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Microdissection of plant chromosomes by argon-ion laser beam

K. Fukui [1], M. Minezawa [2], Y. Kamisugi [3], M. Ishikawa [3,*], N. Ohmido [1], T. Yanagisawa [3,*], M. Fujishita [4], and F. Sakai [3]

[1] Department of Breeding, Hokuriku National Agricultural Experiment Station, Joetsu 943-01, Japan
[2] Department of Animal Science, Hokkaido National Agricultural Experiment Station, Sapporo 004, Japan
[3] Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba 305, Japan
[4] Meridian Instruments Far East K. K., Tokyo 103, Japan

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Summary. Rice and barley chromosomal samples were prepared both on a polyester membrane and on an ordinary glass slide and subjected to microdissection by an argon-ion laser. The intensity and the position of the laser beam were controlled by a microcomputer. The most suitable intensity to obtain chromosomal fragments was determined experimentally. As a result, specific regions of the centromere, satellite, short arm, or long arm, of the barley and rice chromosomes were dissected out from the chromosomal spreads. Chromosomal fragments were also successfully transferred from the sample into an Eppendorf tube.

Key words: Barley chromosomes – Rice chromosomes – Laser dissection – Micromanipulation – Image analysis

Introduction

Microdissection of chromosomes has been attempted for more than ten years. Scalenghe et al. (1981) reported on the microcloning of DNA sequences using chromosomal fragments dissected manually from several polytene chromosomes of *Drosophila* with a fine glass needle. Monajembashi (1986) dissected human chromosomes prepared on a glass slide using a laser beam. Microdissection and subsequent microcloning have not yet become a common technique, although the concept is simple and wide applications of the technique can be anticipated.

One of the obstacles to the wider application of this approach is the skill required for using a micromanipulator. Moreover, even for an expert, it is difficult to dissect and collect numbers of chromosome fragments from the same region. As for the laser-dissection method, control of the focusing positions and regulation of the intensity of the beam are both difficult. Nevertheless, microdissection of human and *Drosophila* chromosomes has been achieved and several genes have already been cloned by manual dissection methods (Röhme et al. 1984; Ludecke et al. 1989; Ponelies et al. 1989).

In the case of plant chromosomes, the situation is even more complex as, with a few exception, there is no objective method, like the G-, R- or Q-banding of vertebrate chromosomes, for identifying each chromosome without affecting its DNA sequence (Kakeda et al. 1990). Until a few years ago, objective and quantitative chromosome maps had not been constructed even for important economic crops such as wheat, barley and rice.

The introduction of image analysis methods for the study of chromosomes has enabled us to achieve considerable progress in this direction (Fukui 1985, 1986, 1988) since the characteristics of the chromosomes analyzed could be described quantitatively by the use of this technique (Fukui and Mukai 1988; Iijima et al. 1991). So-far, quantitative chromosome maps of barley (Fukui and Kakeda 1990), rice (Fukui and Iijima 1991) and *Crepis* (Fukui and Kamisugi, unpublished) have been succcessfully constructed.

The combination of quantitative chromosome maps and micromanipulation methods which have enabled us to dissect specific regions of the chromosomes could also be utilized for the cloning of site-specific DNA sequences from these plants (Fukui et al. 1991).

In this paper, we describe a convenient and efficient method to obtain fragments of plant chromosomes

* Permanent address: Faculty of Agriculture, Meiji University, Kawasaki 214, Japan
** Permanent address: Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.
Correspondence to: K. Fukui

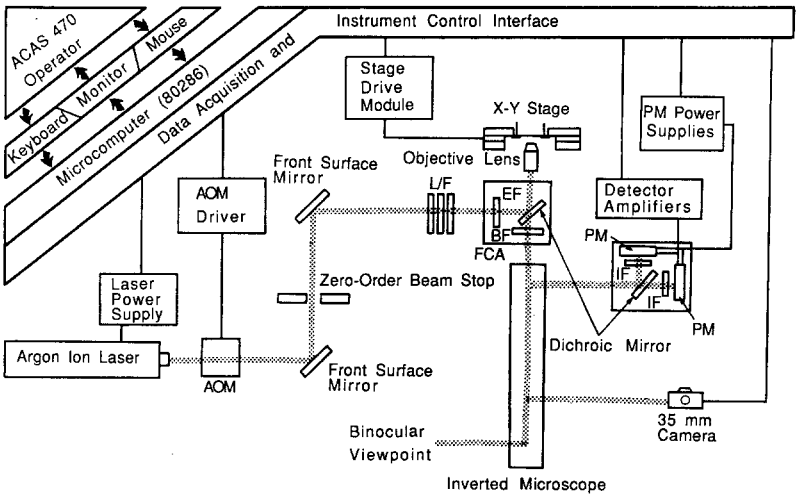

Fig. 1. A Block diagram of a cell work-station, ACAS 470. *AOM*, Acousto-optic modulator; *BF*, Barrier filter; *EF*, Excitation filter; *FCA*, Fluorescence cube assembly; *IF*, Isolation filter; *L/F*, Lens filter holders; PM, Photomultiplier

targeted by applying a computerized laser-dissection method.

Materials and methods

Plant materials and cytology

Seeds of the barley variety, "New Golden" (2n = 14) and a rice variety, "Nipponbare" (2n = 24) were sown in Petri dishes and were germinated at 28 °C. Root tips 1–2 cm long were excised and, in the case of barley root tips, pretreated with distilled water at 0 °C for 18 h. Then they were fixed in methanol and acetic acid (3:1). Root tips of rice 1–2 cm long were fixed in ethanol and acetic acid (4:1) without any pretreatment.

After fixation, the root tips were washed thoroughly and subjected to enzymatic maceration both on glass slides and on a polyester membrane fixed at the bottom of a 35 mm ∅ plastic Petri dish. The enzymatic cocktail, which consisted of 2% Cellulase Onozuka RS (Yakult Honsha Co. Ltd., Tokyo), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Tokyo) and 1.5% Macerozyme R200 (Yakult Honsha Co. Ltd., Tokyo), was adjusted to pH 4.2 (Kamisugi and Fukui 1990). The barley and rice root rips were macerated at 37 °C for 30 min and 60 min, respectively.

The cocktail was removed by washing and the macerated root tips were tapped with the tip of a fine forceps into small, almost invisible, fragments by the addition of a few drops of fresh fixative (3:1 methanol:acetic acid) and then air-dried. After complete drying, several drops of a 2% Giemsa solution were added to the plastic Petri dish until the surface of the membrane was completely covered. The chromosomes were stained for 30 min. In the case of glass slides, they were dipped into the Giemsa solution for 30 min. Then they were washed and air dried.

System and implementation

A cell work-station (ACAS 470, Meridian Instruments Inc., Okemos, Mich., USA) was used for the microdissection of the plant chromosomes. This system consists of an argon-ion laser tube, an acousto-optic modulator (AOM), an inverted microscope, an automatic X-Y stage, a photo-multiplier, and a controlling microcomputer (CPU 80286) equipped with a keyboard, monitors, and a mouse (Schindler et al. 1985). The argon-ion laser tube emits laser beams with wave lengths ranging between 350 and 528.7 nm. A single laser beam with a wave length of 488 nm was used for microdissection of the chromosomes. The intensity of the laser output was controlled by the regulation of the electric power supply (unit = mW) and by the acousto-optic modulator (unit = %). The laser beam was introduced into the center of the light axis of the inverted microscope through slits and several mirrors. The beam was focussed to 1 μm by a ×40 objective lens and irradiated to the targeted region of the chromosomes according to the speck of the system. A ×100 objective lens was used throughout the study to obtain a beam less than 1 μm in diameter to focus onto the chromosomes.

The automatic stage controlled by the host computer with a pitch of 0.25 μm was operated both manually, by using a mouse, and automatically by computer programing. The laser focusing site was directly monitored by a TV display. Figure 1 illustrates the block diagram of ACAS 470 after slight modification from the operation manual.

Table 1. Laser beam intensity and width of cut bands

Laser beam intensity		Band width (μm ± SD)	Number of bands measured
mW	%		
50	15	0.50 ± 0.11	32
50	20	0.78 ± 0.15	21
50	40	1.01 ± 0.13	23
50	60	1.19 ± 0.23	35
50	80	1.39 ± 0.13	25
50	100	1.43 ± 0.14	36
100	15	0.56 ± 0.13	36
100	20	0.69 ± 0.12	30
100	40	1.10 ± 0.13	24
100	60	1.47 ± 0.15	51

Results and discussion

Table 1 shows the relationship between the laser beam intensity and the width of the ablated bands. The intensity of the laser beam depends both on the supply of elec-

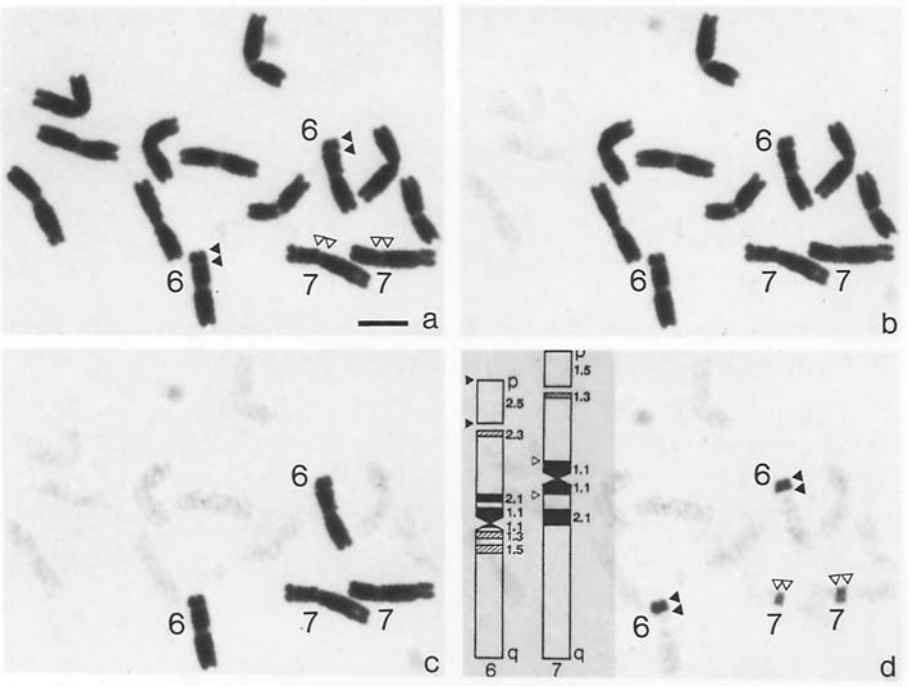

Fig. 2a–d. Dissection of the satellite regions (6p2.5) and centromeric regions (7p1.1 and 7q1.1) respectively of barley chromosomes no. 6 and no. 7. The nomenclature of the chromosome regions was slightly modified from the barley chromosome map of Fukui and Kakeda (1990). Bar shows 5 μm

tric power and the AOM regulation. Ten combinations were tested for barley chromosome samples in order to determine the optimal laser beam intensity to dissect the chromosomes. In general, the stronger the laser beam intensity, the wider the band cut.

It was thus concluded that a band width of 0.5 μm, which was attained by the laser beam under a 15% AOM regulation with a 50 mW electricity output, was optimal for very fine manipulation of the chromosomes. It was found useful to remove unnecessary chromosomal regions or nuclei in the vicinity using a laser beam 1 μm in width by increasing the intensity of the laser beam through an increase of the AOM regulation of up to 40%.

The remaining part of the chromosomal DNA was obtained after laser ablation under a 40% AOM regulation with an electricity output of 50 mW. Chromosome elimination throughout the study was monitored by staining the membrane with the DNA-binding fluorescent dyes, DAPI (1 μg/ml) and PI (10 μg/ml), using a solution with a concentration ten times that of the standard used for chromosome staining. No DNA-specific fluorescence was observed after overnight staining with either dye. Thus it was concluded that the chromosomal DNAs had been removed in the laser-irradiated regions.

Figure 2 shows the process of laser dissection of specific chromosomal regions. In the case of barley, chromosomes no. 6 and no. 7 were both identified by their morphological characteristics as indicated by the solid and open arrow heads in Fig. 2a. Satellites and centromeric regions of chromosomes no. 6 and no. 7, respectively, were targeted for dissection by laser-beam irradiation. Unnecessary chromosomes were removed one by one by a stronger laser beam (Fig. 2b) until only four chromosomes remained (Fig. 2c). Unnecessary neighboring regions of the targeted chromosomes were carefully ablated with a very fine laser beam at first and the cut ditch was subsequently enlarged by repeated application of the laser beam. Then the rest of the targeted chromosomal regions were irradiated by the stronger laser beam. The targeted regions were dissected out after removal of the remaining barley chromosomes. As a result, only chromosomal fragments of the 6p2.5 region of chromosome no. 6 and regions 7p1.1 and 7q1.1 of chromosome 7 remained. The barley chromosomal addresses were determined according to the slightly modified barley chromosome map of Fukui and Kakeda (1990).

Figure 3 illustrates the laser dissection of the rice chromosomes. Rice chromosomes differ from those of barley by their size, which, as shown in Fig. 3a, is only one third to one fifth that of barley. Thus a more careful orientation of the laser beam was required for a precise dissection of the rice chromosomes. However, all of them can be readily identified by their morphological characteristics under the microscope. Fukui and Iijima (1991) and Iijima et al. (1991) identified 117 characteristic fea-

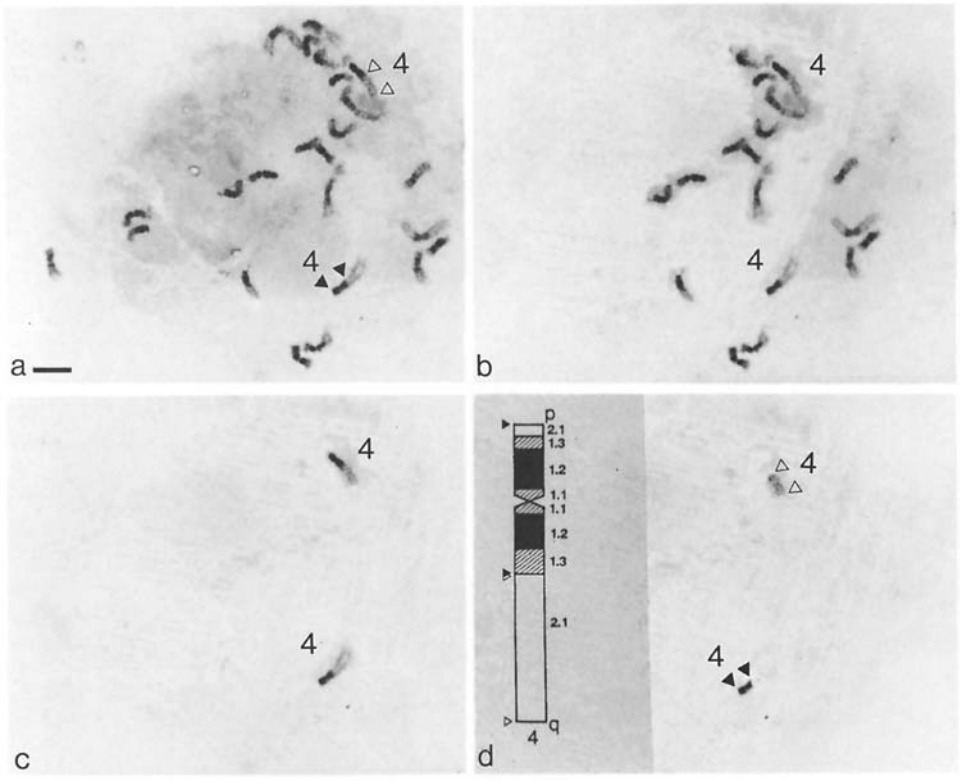

Fig. 3. a–d. Dissection of the condensed region (4p1 and 4q1) and dispersed region (4q2.1) of rice chromosome no. 4 by laser-beam irradiation. The nomenclature of the chromosome regions was based on the rice chromosome map of Fukui and Iijima (1991). Bar shows 5 μm

tures from the 12 rice chromosomes by the application of image-analysis methods. Using these features as key characters to discriminate individual rice chromosomes most of the prometaphase chromosomes could be easily identified even under the light microscope. Chromosome no. 4 is characterized by the presence of a phantom-like figure as indicated by the solid and open arrow heads in Fig. 3a. Uneven condensation along the chromosomes was clearly observed and served as a marker for the idiogram. Chromosomal addresses were assigned based on the quantitative rice chromosome map constructed by Fukui and Iijima (1991). Scattered cytoplasmic debris was eliminated at first and unnecessary chromosomes were also removed (Fig. 3b). Then all the rice chromosomes within the spread except for chromosome 4 were ablated (Fig. 3c). A fine laser beam made it possible to eliminate the 4p1.1, 4p1.2, 4p1.3, 4p2.1, 4q1.1, 4q1.2, 4q1.3 condensed regions so that only 4q2.1 remained (indicated by the open arrow head). The portion of chromosome no. 4 indicated by the solid arrow head was also dissected subsequently and in this case the chromosomal regions 4p1.1, 4p1.2, 4p1.3, 4p2.1, 4q1.1, 4q1.2, 4q1.3 remained (Fig. 3d).

The time from the initial identification of the target barley chromosomal spread (Fig. 2a) until the final stage of the dissection (Fig. 2d) was about 10 min and the manipulation of rice chromosomes took almost the same time. The small size of the rice chromosomes did not prevent laser dissection since the laser beam could be narrowed down sufficiently and fine positioning of the beam could be attained by computer control. Therefore, it is concluded that the method described here is very efficient for the manipulation of plant chromosomes regardless of their size.

Transfer of the chromosomal fragments into, for example, an Eppendorf tube had hitherto been difficult. The use of microglass pipettes controlled by micromanipulators has been successfully tried (Röhme et al. 1984; Bates et al. 1986). Scalenghe et al. (1981) succeeded in the mechanical dissection and collection of the fragments of specific regions of *Drosophila* polytene chromosomes on a glass slide. It is, however, rather difficult to recover somatic chromosomal fragments manually, although laser ablation of human somatic chromosome has already been achieved (Monajembashi et al. 1986). Moreover, the production of suitable glass pipettes and the manual control of the micromanipulator require a great deal of experience.

Figure 4 shows the dissection by laser-beam irradiation of the tinted polyester membrane on which chromo-

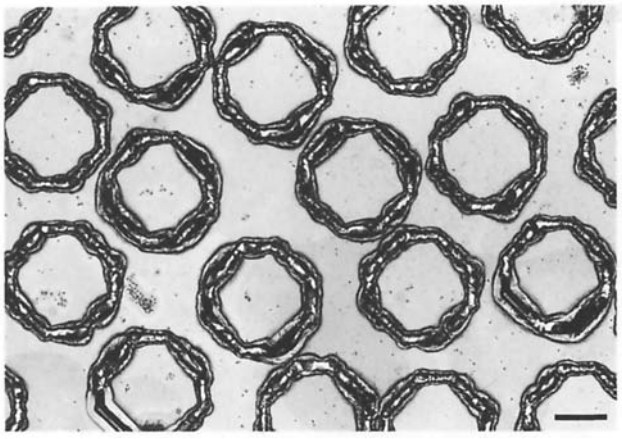

Fig. 4. Dissection of the tinted polyester membrane where the chromosomal samples were overlaid. Bar shows 500 μm.

somal samples were overlaid. The stronger laser beam automatically cut the membrane into an octagonal shape with a diameter ranging from 10 μm to 2 mm. Octagonal pieces of the membranes, 1 mm in diameter, were cut and picked up with a fine forceps under a stereomicroscope. They were put into a 500 μl Eppendorf tube and then stored at −20 °C for further use.

The advantages of the method described here are as follows: (1) Control of the intensity of the laser beam, and orientation to the irradiation site, could be simply achieved without much experience. (2) Fine processing of the plant chromosomes could be easily performed by controlling the laser-beam intensity, and (3) Recovery of the dissected chromosome fragments could also be easily attained.

Along with progress in genome analysis, information on the position of the genes on the chromosomes is becoming increasingly important. Genetic maps based on RFLP analysis have been reported for several crops (Tanksley et al. 1988; Weber and Helentjaris 1989; Wang and Tanksley 1989). The construction of a sufficiently informative RFLP map with a large number of genetic markers is, however, laborious. The laser dissection method on the other hand enables one to obtain genetic information directly from specific chromosomal regions. Therefore, it is anticipated that chromosome dissection may provide an alternative method for the analysis of the genome of various organisms since chromosomal samples are readily available.

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