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Flow-Sorted chromosomes: a fine material for plant gene physical mapping

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Abstract — Fluorescence *in situ* hybridization (FISH) on flow-sorted plant chromosomes is described. The protocol involves flow cytometric sorting of metaphase chromosomes, then fixing them with 4% paraformaldehyde solution, and re-sorting these chromosomes directly onto a spot on poly-lysine coated slides after stained. FISH mapping experiments were performed on the sorted chromosomes from oat and barley using 45S and 5S ribosomal DNAs as probes. The chromosome morphology was very well preserved and there were nearly no chromosomes lost after procedures of hybridization and fluorescent staining. Sorted chromosomes are advantageous over metaphase chromosomes as targets for FISH mapping studies because a large number of target chromosomes with better chromosome morphology on a small area on the slide are easy to gain by flow-sorting, background is very clear and hybridization sensitivity is enhanced. The successful and reliable location of rDNA genes in these two plant genomes, combining with the data published before in maize proves that flow-sorted chromosomes are fine materials for gene physical mapping in plants.

Key words: flow-sorted chromosomes, fluorescence *in situ* hybridization, gene mapping, rDNA.

INTRODUCTION

Fluorescence *in situ* hybridization (FISH) is a powerful tool for understanding of plant genome organization and evolution (Bentivegna *et al.* 2001). The targets for FISH are always metaphase and pachytene chromosomes and DNA fibers. Preparation of well-dispersed metaphase chromosomes with high division index is always laborious and it is difficult to get a good slide suitable for FISH mapping analysis from a lot of preparations in some plants.

Chromosome flow-sorting allows identification and isolation of individual chromosome types. Flow-sorted chromosomes have been effectively exploited for human chromosomes (Gray and Langlois 1986). Flow-sorting of chromosomes could be done from various plant species including *Haplopappus gracilis* (De Laat and Schel 1986), *Petunia hybrida* (Conia *et al.* 1987), *Lycopersicon pennellii* (Arumuganathan *et al.* 1994), *Vicia faba* (Macas *et al.* 1996), *Hordeum vulgare* (Lysak *et al.* 1999; Lee *et al.* 2000), *Melandrium album* (Kejnovsky *et al.* 2001), *Triticum aestivum* (Gill *et al.* 1999; Kubalakova *et al.* 2002), *Pisum sativum* (Neumann *et al.* 2002), *Secale cereale* (Kubalakova *et al.* 2003) and *Zea may* (Li *et al.* 2004).

Flow cytometers can evaluate and sort chromosomes of interest at an extremely rapid rate (up to 20,000 events per second). Thus, a number of chromosomes may be gained rapidly by flow-sorting (Huang *et al.* 2004). Flow-sorted chromosomes have been used to construct chromosome-specific libraries in plants (Arumuganathan *et al.* 1994; Macas *et al.* 1996; Li *et al.* 2004). However, localization of genes on flow-sorted chromosomes has only been accomplished in field bean using chromosome-specific PCR (Macas *et al.* 1993; Neumann *et al.* 2002) except for our report on FISH mapping genes on maize sorted chromosomes (Li and Arumuganathan 2001). Fixation of sorted chromosomes is a prerequisite for application of *in situ* hybridization or storage for later use. But chromosome suspension prepared from fixed plant materials (especially cereals) is not suitable for chromosome sorting (Lee *et al.* 1996). To overcome this problem and widely apply FISH to sorted chromosomes for gene mapping, in this study, we further developed and proved a method

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for FISH directly on sorted chromosomes based on data published before in maize (LI and ARUMUGANATHAN 2001). Chromosomes are first sorted into a fixative (4% paraformaldehyde solution), then stained and re-sorted directly onto a spot on poly-lysine coated slides. FISH experiments were performed on the sorted chromosomes from oat and barley using 45S and 5S ribosomal DNAs as probes. Thus the ability to detect clear hybridization signals on these sorted chromosomes allows us to consider sorted chromosomes as a fine material for physical mapping of genes in plants.

MATERIALS AND METHODS

Plant material - Oat and barley were used for chromosome isolation and flow sorting.

Synchronization of cell cycle, metaphase arrest and preparation of chromosome suspensions - These experiments were performed according to the methods described by LI et al. (2004).

Flow-sorting of chromosomes for fluorescent in situ hybridization (FISH).- Chromosome sorting was performed using a FACVantage flow cytrometer and sorter (Becton Dickinson, San Jose, USA). $MgSO_4$ buffer without dithiothreitol was autoclaved and employed as sheath fluid. An argon ion laser tuned to 488 nm with 300 mW out power. Chromosomes were collected by flow sorting directly into 4% (v/v) paraformaldehyde solution in microfuge tubes cooled to 4°C. Chromosomes collected into the fixative were restained by mixing with two volumes of the chromosome isolation buffer with 60μg/mL propidium iodide and incubated on ice for 30 mins before used for sorting. Chromosomes thus fixed were collected directly onto a spot on lysine coated glass slides by flow sorting. Slides were air-dried at room temperature and stored in –20°C freezer or immediately used for FISH.

Digoxigenin labeling DNA and in situ hybridization - Plasmid 45S and 5S rDNAs were digoxigenin-labeled by a nick translation method using Dig-Nick Translation Mix provided by Boehringer Mannheim Corporation (Indianapolis, IN). *In situ* hybridization was performed using the procedure described by LI and ARUMUGANATHAN (2001). Hybridization mixture contained 50% formamide, 10% dextran sulphate, 2xSSC, 1mg/mL of sheared salmon sperm DNA and 1-2μg/mL probes.

Detection - The detection reagents offered by Boehringer Mannheim Corporation. The procedure for detection of fluorescent signals includes following steps: 1) after hybridization, the coverslip was removed by dipping the slides in a 2xSSC solution and the slides were washed in 2xSSC at room temperature (RT) for 10 min, 2xSSC at 37°C for 10 min, 2xSSC at RT for 5 min and 1xPBS at RT for 5 min; 2) each slide was added 100μl of sheep anti-digoxigenin-FITC, covered with a coverslip, incubated in a humid chamber at 37°C for 30 min and the slides were washed 3 times, each for 5 min in PBS at RT; 3) the slides were added 100μl of rabbit anti-sheep-FITC for 30 min at 37°C in a humid chamber, and then were washed as above; 4) the slides were added 10μl (1μg/ml) propidium iodide in an anti- fade solution (Molecular Probes, Eugene, Oregon USA), covered with a coverslip, and observed. Images were collected using a Bio-Rad HRC 1024ES confocal laser scanning microscopy.

RESULTS

Large numbers of mitotic metaphase chromosome suspensions could be successfully isolated from synchronized root tips of barley and oat. 100 chromosomes were sorted at rates of 5-20 per second. FISH experiments were performed on sorted chromosomes from oat and barley using 45S and 5S ribosomal DNAs as probes. Another FISH experiment was performed on oat metaphase chromosomes as a control. The FISH results showed that the chromosomes were red, while the signals were green (Fig.1 and Fig. 2). The clear hybridization signals were seen on sorted chromosomes after procedures of hybridization and fluorescent staining. Fig. 1b and 2a are a low magnification to show some sorted oat chromosomes with yellow-green fluorescent signals in the field.

FISH, with a probe for 45S rDNA on oat chromosomes, resulted in strong and wide bands in distal and subterminal regions of the short arms on two pairs of chromosomes, and sharp signal bands in the short arms of another pair of chromosomes (Fig. 1). Fig. 2 shows the results of fluorescent *in situ* hybridization (FISH) with 5S DNA as probes. FISH with a 5S rDNA on oat chromosome 2 and 4, resulted in stronger signals in interstitial region of the short arms and minor signals at proximal of the long arms, respectively, and these signals were identified on each chromatin (Fig. 2). The mapping results on sorted chromosomes are in agreement with those on metaphase

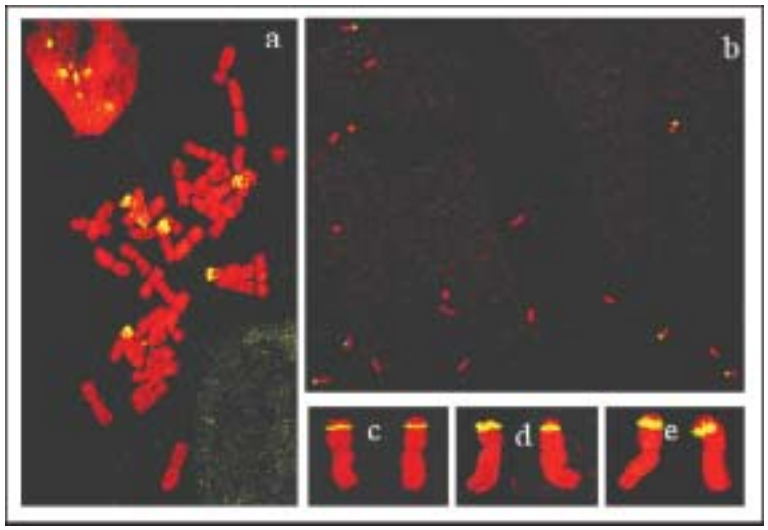

Fig. 1 — FISH of 45S rDNA on a metaphase chromosome spread of oat (a) and sorted oat chromosomes (b-e).

chromosomes (Fig. 1a and Fig. 2b) (Roser *et al.* 2001; Linares *et al.* 1996). The distribution of FISH signals was comparable both on sorted and metaphase chromosomes (Fig. 1 and 2). However, the signals on sorted chromosomes were stronger. The strength of FISH signals on the sorted chromosomes increased about 2 fold for 5S rDNA (Fig. 2) although the difference was not obvious for 45S rDNA (Fig. 1). Due to lower copy number of 5S rDNA than that of 45S rDNA in plant genomes, it is often a little more difficult to obtain clearer and stronger signals using 5S rDNA as probes on metaphase chromosomes in some plants. Fig. 2f shows that 5S rDNA probes were hybridized to the sorted barley chromosomes and the hybridization signals were identified on the

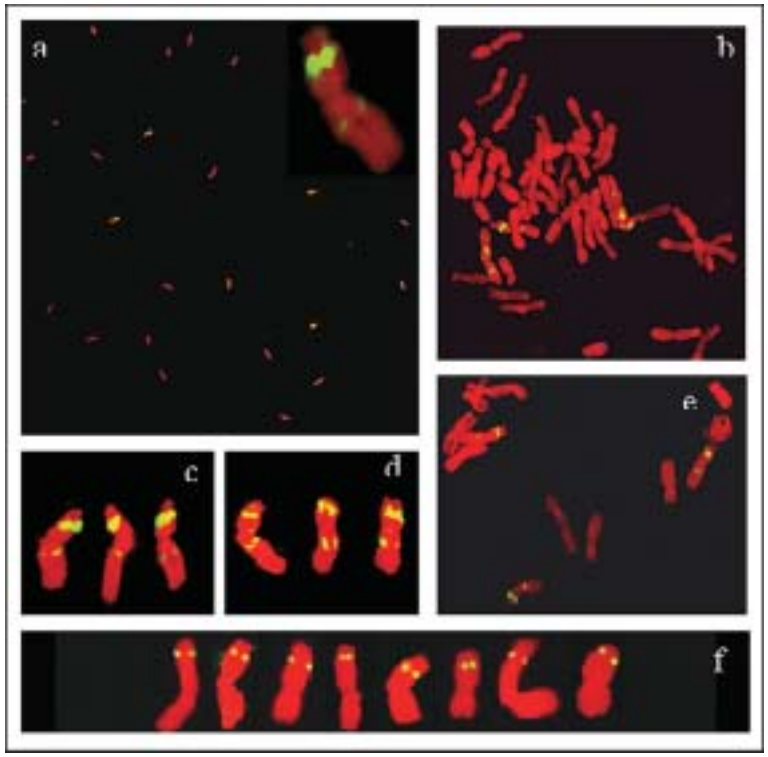

Fig. 2 — FISH of 5S rDNA on sorted chromosomes (a,c,d,e) and a metaphase chromosome spread of oat (b), and sorted chromosomes of barley (f).

long arm of chromosome 2 with a percentage of 73, which were consistent with the hybridization sites gained on the metaphase chromosome preparations (TAKETA *et al.* 1999). Large number of target chromosomes can be easily gained on a small area on the slide by flow-sorting compared to metaphase. The chromosome morphology was very well preserved and there were nearly no chromosomes lost after procedures of hybridization and fluorescent staining.

DISCUSSION

Recent progress in plant flow cytogenetics enabled chromosome sorting in a far larger range of plant species than before (DOLEZEL *et al.* 2004). Now flow sorting of chromosomes could be done from various cereal plants including rye, maize, oat, barley and wheat (GILL *et al.* 1999; LYSAK *et al.* 1999; KUBALAKOVA *et al.* 2002; KUBALAKOVA *et al.* 2003; LI *et al.* 2004). Sorted chromosomes have many important applications for plant genome research, one of which is for gene mapping in plants. Localization of genes on flow-sorted chromosomes has been accomplished by PCR in field bean (MACAS *et al.* 1993; NEUMANN *et al.* 2002) and we ever reported mapping genes on maize sorted chromosomes by FISH (LI and ARUMUGANATHAN 2001). In this study, we further developed and proved a technique for mapping genes on the sorted chromosomes by *in situ* hybridization. Many chromosome isolation methods for chromosome sorting are not compatible to fixed chromosomes (LEE *et al.* 1996). But fixation of sorted chromosomes is a prerequisite for application to in situ hybridization location or storage for later use, so in this method, specific chromosomes or a group of chromosomes in large quantities were first sorted into a fixative (4% paraformaldehyde solution) from a suspension of chromosomes prepared from actively growing synchronised root tip meristems by a flow cytometer, then stained and re-sorted directly onto a spot on poly-lysine coated slides.

FISH showed that sorted chromosomes were suitable for localization of DNA sequences in these two species used in the present study. The success of carrying out FISH on sorted chromosome would provide a unique starting material that enables physical mapping genome. Flow-sorted chromosomes have been effectively exploited in human chromosomes (GRAY and LANGLOIS 1986). The sorted chromosomes of a single type from plants have also been used to construct chromosome-specific library and physically map DNA sequences at the chromosomal level (ARUMUGANATHAN *et al.* 1994; MACAS *et al.* 1996; LI *et al.* 2004).

The precision of the chromosomal position of a hybridization site depends on the number of measurements, the extension of the hybridization signal, and its position relative to the stable cytological markers (PEDERSEN and LINDE-LAURSEN 1994). Sorted chromosomes are advantageous over metaphase chromosomes as targets for FISH mapping studies because a large number of target chromosomes with better chromosome morphology on a small area on the slide are easy to gain by flow-sorting and background is very clear. However metaphase chromosome slides always contains cellular debris and nuclei, which hinder hybridization of probes to target chromosomes. Large numbers of target chromosomes on a small area on the slide will facilitate more precise mapping genes, because there are more chromosomes with signals for the calculation of the percentage distances.

Using this method, we have performed in *situ* hybridization experiments on sorted chromosomes from maize (LI and ARUMUGANATHAN 2001), barley and oat (present study). The success of location of rDNA on chromosomes sorted from these few species by hybridization of rDNA to the sorted chromosomes suggests that this procedure can be widely applied to mapping genes on sorted chromosomes in plants. The ability to detect clearly hybridization signals on sorted chromosomes allows us to consider sorted chromosomes as a fine material for physical mapping of genes in plants.

The positions of FISH signals obtained with rDNA probes accorded with the results of FISH on metaphase chromosomes. However, the signals on sorted chromosomes covered much larger areas, indicating that increased sensitivity was obtained with sorted chromosomes. This could be a result of higher efficiency of hybridization on sorted chromosome, enhanced penetration and hybridization of the probes to sorted chromosomes and smaller debris steric inhibition of probes and detection system molecules as compared with metaphase chromosome preparations.

Recently a novel method for high-resolution FISH, using super-stretched mitotic chromosomes was presented, which provided a unique system for controlling stretching degree of mitotic chromosomes and high-resolution bar-code FISH (VALARIK *et al.* 2004). Furthermore, a possibility to sort a specific chromosome may be an important benefit for direct mapping genes or DNA sequences onto specific chromosomes. FISH on

sorted chromosomes will be useful for the detailed analysis of chromosomal structure evolution and function.

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