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Flow cytometric sorting of maize chromosome 9 from an oat-maize chromosome addition line

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Abstract Large numbers of maize chromosome 9 can be collected with high purity by flow cytometric sorting of chromosomes isolated from a disomic maize chromosome addition line of oat. Metaphase chromosome suspensions were prepared from highly synchronized seedling root-tips of an oat-maize chromosome-9 addition line (OM9) and its parental oat and maize lines. Chromosomes were stained with propidium iodide for flow cytometric analysis and sorting. Flow-karyotypes of the oat-maize addition line showed an extra peak not present in the parental oat line. This peak is due to the presence of a maize chromosome-9 pair within the genome of OM9. Separation of maize chromosome 9 by flow cytometric sorting of a chromosome preparation from a normal maize line was not possible because of its size similarity (DNA content) to maize chromosomes 6, 7 and 8. However, it is possible to separate maize chromosome 9 from oat chromosomes and chromatids. An average of about 6×10^3 chromosomes of maize chromosome 9 can be collected by flow-sorting from chromosomes isolated from 30 root tips (ten seedlings) of the oat-maize addition line. Purity of the maize chromosome 9, sorted from the oat-maize chromosome addition line, was estimated to be more than 90% based on genomic in situ hybridization analysis. Sorting of individual chromosomes provides valuable genomic tools for physical mapping, library construction, and gene isolation.

Keywords Oat-maize chromosome-9 addition line · Maize chromosome-9 sorting · Genomic in situ hybridization · Oat · Maize

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Introduction

Flow cytometric analysis and the sorting of chromosomes have many applications for the study of plant genomes. Since the first report of a plant flow-karyotype in 1984, many such karyotypes have been established. Flow-sorting of chromosomes has been reported for a few plant species including *Haplopappus gracilis* (De Laat and Schel 1986), *Melandrium album* (Veuskens et al. 1995), *Petunia hybrida* (Conia et al. 1987), *Lycopersicon pennellii* (Arumuganathan et al. 1994), *Triticum aestivum* (Schwarzacher et al. 1997; Gill et al. 1999), and *Vicia faba* (Lucretti et al. 1993; Dolezel and Lucretti 1995; Macas et al. 1996).

Maize is an economically important crop. The maize genome has been characterized using molecular markers, cytogenetic stocks, and various molecular genetic tools. The availability of flow-sorted maize chromosomes could greatly enhance genome characterization and cloning studies in this species. We have isolated chromosomes from many maize lines. Flow cytometric analysis of the normal maize lines showed that it is only possible to purify maize chromosome 1 (largest chromosome) from all lines, and maize chromosome 10 from the maize line Seneca 60 (Lee et al. 1996, and unpublished result). It is not possible to purify other chromosomes using any of the other maize lines due to their size similarity (DNA content) to two or more of the other maize chromosomes.

Oat-maize chromosome addition lines have been developed, each containing a single maize chromosome or chromosome pair (Riera-Lizarazu et al. 1996) added to a complete disomic oat genome. These addition lines should be very useful for sorting maize chromosomes because maize chromosomes are significantly smaller than oat chromosomes. Here we test this approach using an oat-maize chromosome-9 addition line and present a first report of the flow-cytometric analysis of this line and the sorting of highly pure maize chromosome 9 based on its flow karyotype.

Materials and methods

Plant material

The oat-maize chromosome-9 addition line used in this study, and herein referred to as OM9, originated from an oat×maize partial hybrid ST 505-5. ST505-5 was produced by crossing the Starter-1 oat line with the Seneca 60 maize line (Riera-Lizaraza et al. 1996). The parental oat (Starter-1) and maize (Seneca 60) lines used to develop the addition lines were also used for chromosome isolation and flow cytometric analysis.

Synchronization of cell cycle and metaphase arrest

Intact metaphase chromosomes were isolated from seedling root-tip meristematic cells of OM9, oat (Starter-1) and maize (Seneca 60). Seedlings with the same-size primary roots (approximately 5 mm length) were placed on a filter paper soaked in 10 ml of Hoagland's solution containing 1.25 mM (for OM9 and oat) or 5 mM (for maize) of hydroxyurea solution. The petri dishes containing the seedlings were sealed with parafilm and incubated at 25°C (for OM9 and oat) or 28°C (for maize) in the dark for 14 h (OM9 and oat) or 18 h (maize). The seedlings were then washed in distilled water three-times and transferred to a filter paper soaked in 10 ml of Hoagland's solution incubated for 1.5 h to release the hydroxyurea block. Then, APM (amiprophos-methyl, Mobay Corporation, Kansas City, Mo.) solution was added to a final concentration of 5 µM and incubated for 5 h in the dark at 25°C (OM9 and oat) or 28°C (for maize).

Preparation of chromosome suspension

At the end of the APM treatment, the root-tips were excised (1.5 mm from the terminal root-tip) and finely chopped with a sharp sterile scalpel blade in a petri dish containing 0.5 ml (per 10 root tips) of ice-cold freshly prepared chromosome isolation buffer. The chromosome isolation buffer was a $MgSO_4$ buffer (10 mM $MgSO_4$, 50 mM KCL, 5 mM hepes , 3 mM dithiothreitol, pH 8.0) (Lee et al. 1996) with propidiom iodide (PI) at a final concentration of 60 µg/ml and Triton-X100 at a final concentration of 0.25%. The resulting chromosome suspension was filtered through a 30-µm nylon mesh.

Flow cytometric analysis and chromosome sorting

Chromosome analysis and sorting was performed using a FACVantage flow cytometer and sorter (Becton Dickinson, San Jose, Calif., USA). $MgSO_4$ buffer without dithiothreitol was autoclaved and employed as the sheath fluid. An argon-ion laser was tuned to 488 nm with 300 mW of power. Red fluorescence signals emitted from PI-stained chromosomes were collected through a 585±21 nm-band pass filter. PI-fluorescence intensities and PI-fluorescence pulse area (FL-A) were measured on a log or linear scale of 1024 channels for chromosomes in the preparations. To ensure the highest purity of sorted chromosomes, sorting windows were set based on PI-fluorescence pulse area (FL-A) versus PI-fluorescence pulse width (FL-W), and chromosomes were sorted into microfuge tubes cooled to 4°C.

Preparation of chromosomes for fluorescence genomic in situ hybridization (GISH)

Chromosomes were also collected by flow-sorting (based on FL-A vs FL-W) directly into 4% (v/v) paraformaldehyde solution (in $MgSO_4$ buffer, pH 8.0) in microfuge tubes. Chromosomes collected in the fixative were re-stained by mixing with 2 vol of the chromosome isolation buffer containing 60 µg/ml of PI and incubated on ice for 30 min before sorting. Chromosomes thus fixed were collected by flow-sorting directly onto a spot on lysine-coated glass slides. A large sorting window was used for sorting, to include all signals on the dot plot of FL-A versus FL-W, and to collect all particles from the first sort. Slides were air-dried at room temperature and stored in a –20°C freezer before being used for GISH.

Metaphase chromosome spreads as controls were made using the protoplast technique as described by Li et al. (1998), with some modifications. Root-tip cell synchronization and metaphase accumulation were done according to the method described earlier. The root tips were fixed in freshly prepared methanol: acetic acid (3:1) fixative solution. The root tips were digested with 1% cellulase (Yakult Honsha Co., Ltd, Tokyo, Japan) and 1% pectolyase (Kikkoman Corporation, Tokyo, Japan) at 28°C for 3 to 4 h. After digestion, the enzyme solution was carefully removed and replaced with distilled water (a hypotonic treatment) for 5 min. Then 2–3 root tips were squashed with forceps in 2–3 drops of fixative on a cold slide and flame-dried. The prepared slides were stored at –20°C or immediately used for GISH.

Digoxigenin labeling of DNA and in situ hybridization

Genomic DNA of maize was labeled with digoxigenin by nick translation using the Dig-Nick Translation Mix (Boehringer Mannheim Corporation, Indianapolis, Ind.). In situ hybridization was performed with the procedure described by Gustafson and Dille (1992) using maize genomic DNA as the probe. The hybridization mixture contained 50% formamide, 10% dextran sulphate, 2×SSC (0.15 M NaCl plus 0.015 M sodium citrate), 1 mg/ml of sheared salmon sperm DNA and 1–2 µg/ml of probe. Slides with the hybridization mixture were placed in a humid chamber and incubated at 37°C for 16 h for hybridization. Fluorescence signals were detected based on the procedure published by Li et al. (1998) with sheep anti-digoxigenin-FITC and Rabbit anti-sheep-FITC. An anti-fade solution containing PI (1 µg/ml) was added to each slide (Molecular Probes, Eugene, Ore., USA), covered with a coverslip and observed. Images were collected using a BioRad HRC 1024ES confocal laser-scanning microscope.

Results and discussion

Large numbers of mitotic metaphase chromosomes were successfully isolated from synchronized root tips of an oat-maize chromosome-9 addition line (OM9) and its parental oat (Starter-1) and maize (Seneca 60) lines. Chromosome suspensions prepared from Seneca 60 were analyzed on the flow-cytometer. Then, chromosomes isolated from oat and the oat-maize addition line were analyzed without changing the flow-cytometer settings. Figure 1a shows the Seneca 60 flow-karyotype on a log scale. Four clear peaks were seen for the ten maize chro-

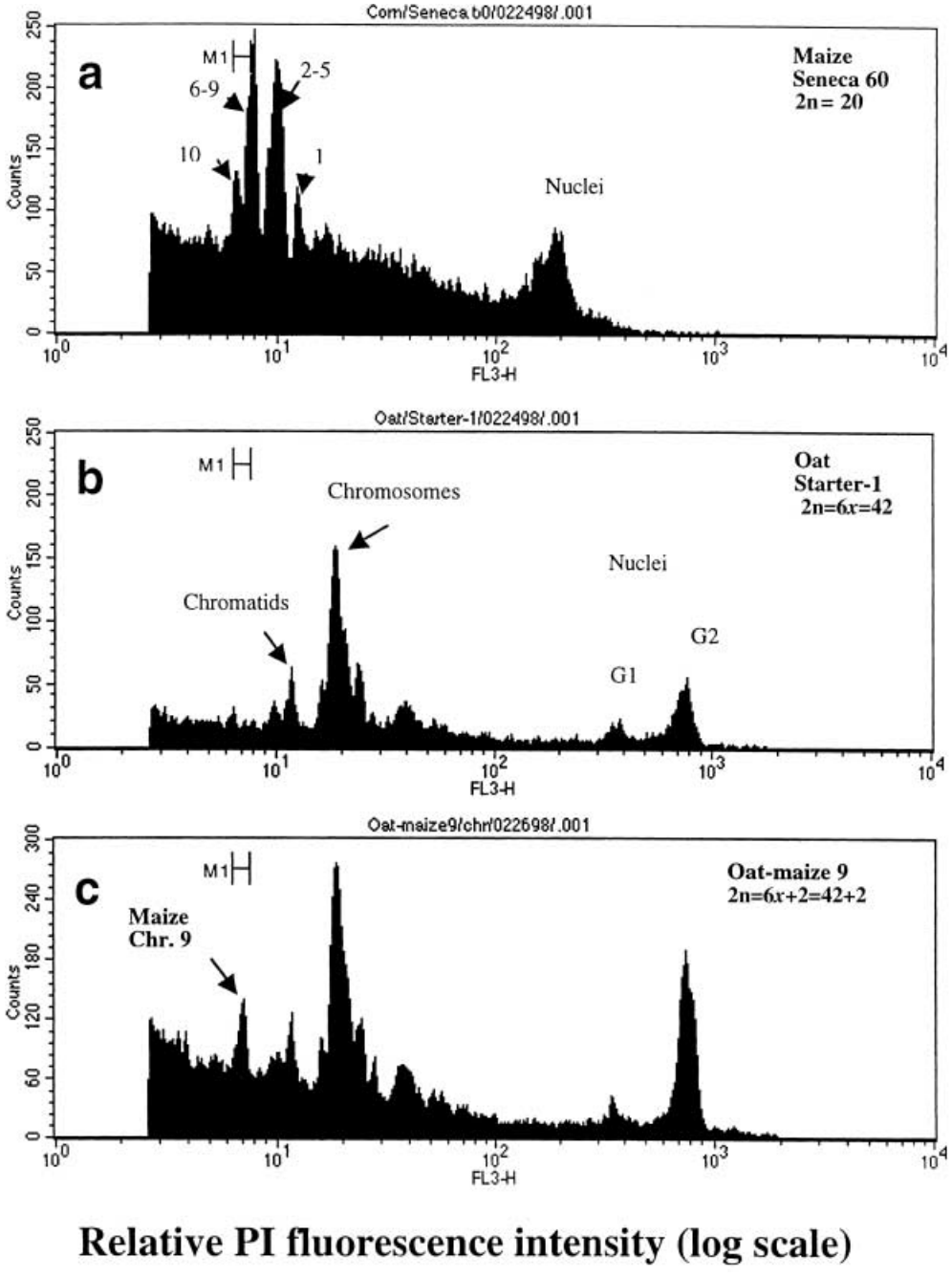

Fig. 1 Univariate flow-karyotypes on a log scale derived from the analysis of mitotic metaphase chromosomes isolated from synchronized seedling root tips of maize (Seneca 60) (**a**), oat (Starter-1) (**b**), and an oat-maize chromosome-9 addition line (**c**). Chromosomes were stained with propidium iodide. Note that the peak for maize chromosome 9 is not separable in Seneca 60. Maize chromosome 9 can be separated from oat chromosomes (and chromatids) in the oat-maize addition line and purified by flow-cytometric sorting

mosomes on the flow-karyotype. This figure shows that chromosome-9 cannot be separated from other similar-sized maize chromosomes by flow-sorting based on this normal maize karyotype. The flow-karyotype of oat (Fig. 1b) shows the peaks for oat chromosomes and chromatids. Peak positions for the chromosomes of oat and maize indicate that all the maize chromosomes are smaller than all the oat chromosomes. Figure 1c shows the flow-karyotype obtained by analyzing chromosome suspensions prepared from OM9. As expected, an extra peak, well-separated from the peaks for oat chromosomes and chromatids, was observed in the flow-karyotype of OM9.

The OM9 is well-characterized and is known to have a single pair of maize chromosome-9 present as additions to the oat parent chromosomes (Riera-Lizarazu et al. 1996). The location of the extra peak corresponded well with that of the size of chromosomes 6–9 in the flow-karyotype of the parental maize from which chromosome-9 was derived. Figure 2 is a flow-karyotype of OM9 on a linear scale. Maize chromosome 9 is about 2.4-times smaller than the smallest oat chromosomes (based on the relative peak positions in Fig. 2). Maize chromosome-9 is also distinguishable in size from oat chromosomes by a standard light microscope. Our cytological observation of sorted chromosomes from the ex-

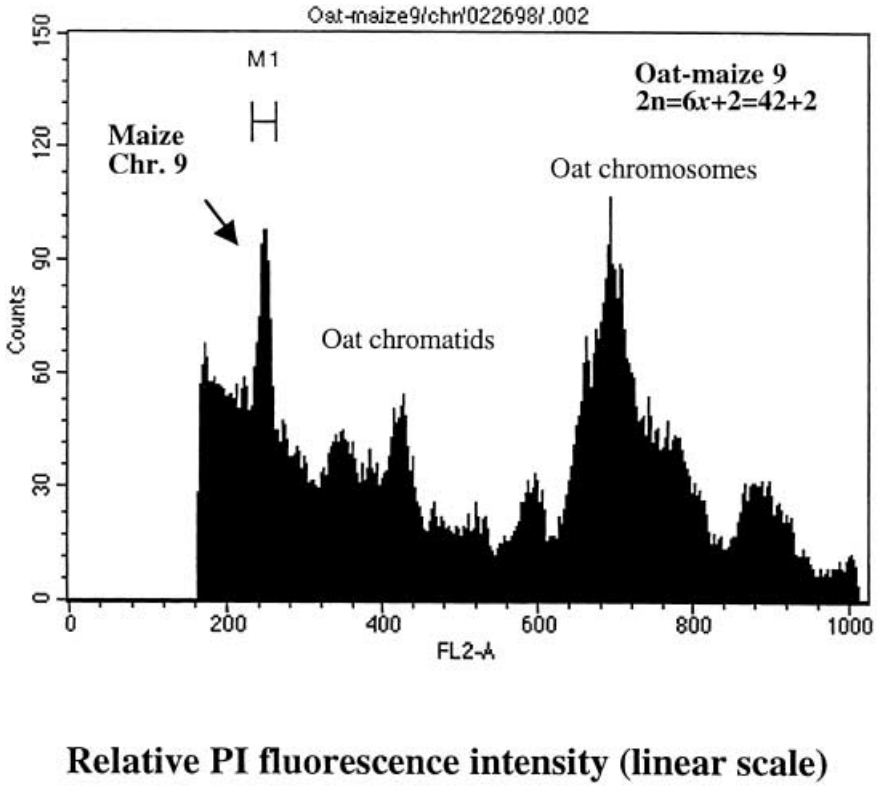

Fig. 2 Univariate flow-karyotypes of the oat-maize chromosome-9 addition line on a linear scale. Note that maize chromosome 9 is 2.4-times smaller than the smallest oat chromosome (*small arrow*)

tra peak verified the presence and apparent purity of the maize chromosomes (Fig. 3a, b).

We wanted to confirm, by fluorescence in situ hybridization, whether we could collect chromosome 9 with high purity. The contents of the putative chromosome-9 peak were sorted into microfuge tubes containing paraformaldehyde. After fixing for a minimum of 30 mins in paraformaldehyde, all the particles from the first sort were again sorted onto a spot on a lysine-coated glass slide. Genomic in situ hybridization (GISH) was conducted using digoxigenin-labeled maize genomic DNA as a probe on the metaphase chromosome spread and on sorted chromosomes (Fig. 3c–f). GISH effectively identified maize chromosome 9 in OM9 (Riera-Lizarazu et al. 1996). Our genomic in situ hybridization showed a pair of uniformly yellow-green fluorescein-labeled maize chromosomes and oat chromosomes that are not fluorescein labelled (stained red with PI) (Fig. 3c). The blocking DNA was omitted in our GISH. The major part of the maize genome consists of repetitive nucleotide sequences that do not cross-hybridize to oat genomic sequences under standard hybridization conditions (Ananiev et al. 1997). The success of identifying maize chromosomes by hybridization of maize total genomic DNA to OM9 chromosome spreads (Fig. 3c), with no blocking DNA, suggests that this procedure can be applied for identifying sorted maize chromosomes from oat chromosomes. This method was used to determine the chromosome origin of the peaks in a hamster-human cell-flow karyotype (Van Dilla et al. 1986). Hybridization of labeled maize total genomic DNA to sorted chromosomes revealed dense yellow-green fluorescein labeling of maize chromosomes (Fig. 3d–g), while no labeling was detectable on sorted oat chromosomes. Figure 3f–g is a photograph taken at low magnification that shows many sorted maize chromosomes with yellow-green fluorescein label. A few sorted particles from the extra peak observed were not yellow-green labeled (Fig. 3e–g). These red particles are suspected to be broken oat chromosome fragments and/or debris. In cytometric sorts from OM9, the labeled chromosomes were over 90% (Fig. 3e–g), and the unlabeled red particles similar to broken chromosomes were less than 10%, of the particles detected (Fig. 3d–g). The purity of maize chromosome-9 sorted from an oat-maize line thus was estimated to be more than 90% based on GISH analysis. Sorts from some hamster-human hybrids contained about 15% hamster material (Van Dilla et al. 1986). Our sorting purity of 90% should be suitable for many applications.

We estimated that an average of about of 6×10^3 chromosomes of maize chromosome-9 can be collected by flow-sorting from chromosomes isolated from 30 root tips (ten seedlings) of the oat-maize addition line. The flow-sorting of maize chromosome-9 from OM9 provides unique starting material to enable the physical mapping of maize chromosome-9. Flow-sorted chromosomes have been effectively exploited in human genome analyses (Gray and Langlois 1986; Van Dilla and Deaven 1990; McCormick et al. 1993; Kim et al. 1995). The sorted specific chromosomes from plants have also been used to construct chromosome-specific libraries

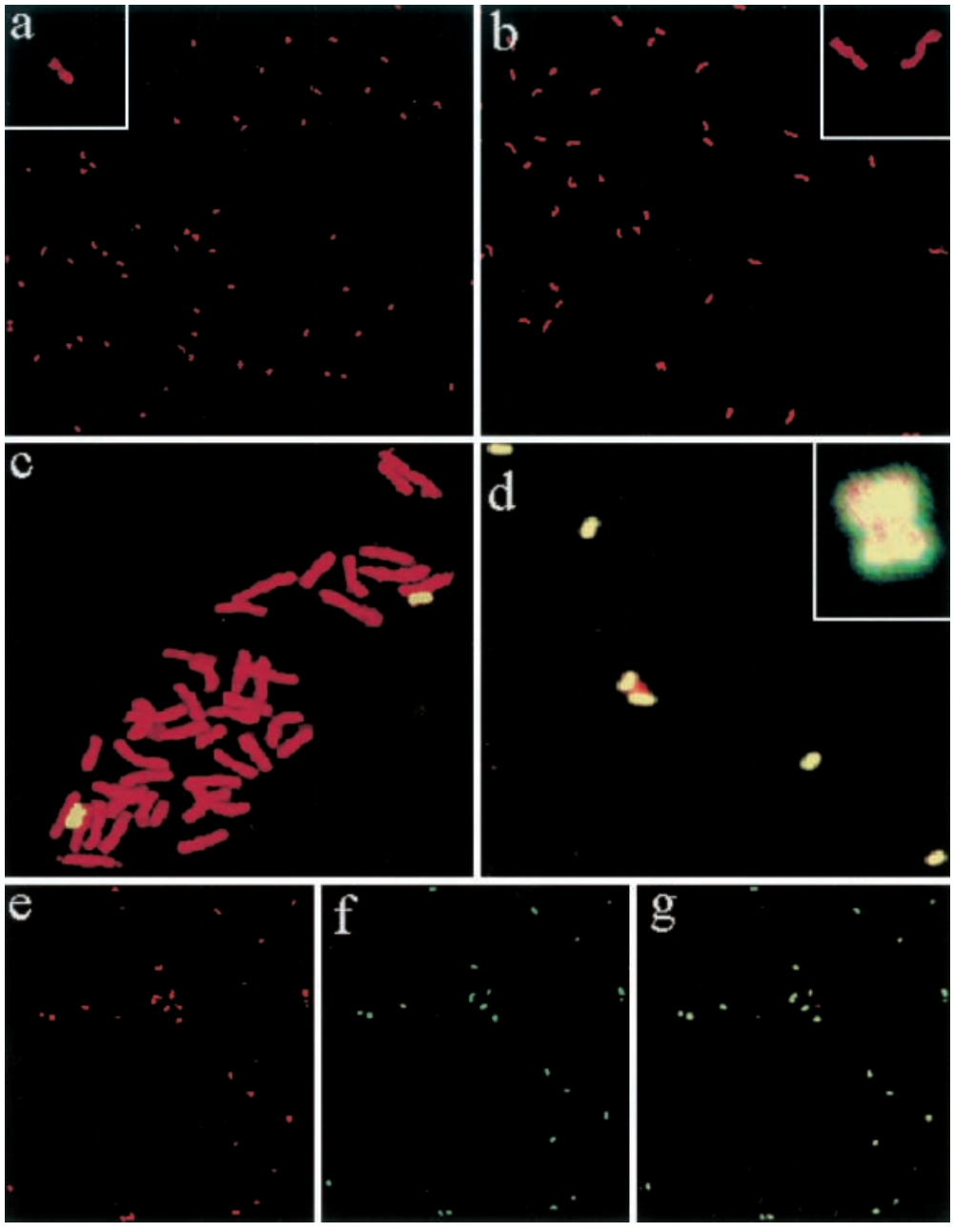

Fig. 3a–g. a–b. Propidium iodide-stained (*red*) maize chromosome 9 (**a**) and oat chromosomes (**b**) collected by flow sorting. **c–g** Show fluorescence genomic in situ hybridization (GISH) with labeled total maize DNA. **c** A pair of yellow-green labeled maize chromosomes 9 within the metaphase spread of the oat-maize addition line. **d** Yellow-green labeled flow-sorted maize chromosomes. **e, f and g** A color image (*red and green*) was digitally separated into red and green layers: **e** the red layer shows PI-stained chromosomes; **f** the green layer shows green fluorescein-labeled chromosomes; **g** a color image from merging **e** and **f** shows the high purity of sorted maize chromosome 9 with a yellow-green fluorescein label

and to physically map DNA sequences at the chromosomal level (Wang et al. 1992; Arumuganathan et al. 1994; Macas et al. 1996). We are presently sorting and collecting maize chromosome 9 in an attempt to construct a maize chromosome 9-specific DNA partial digestion library, and to map genes on the sorted chromosome-9.

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References

Ananiev EV, Riera-Lizarazu O, Rines HW, Phillips RL (1997) Oat-maize chromosome addition lines: a new system for mapping the maize genome. Proc Natl Acad Sci USA 94: 3524–3529

Arumuganathan K, Martin GB, Telenius H, Tanksley SD, Earle ED (1994) Chromosome 2-specific DNA clones from flow-sorted chromosomes of tomato. Mol Gen Genet 242:551–558

Conia J, Bergounioux C, Perennes C, Muller P, Brown S, Gadal P (1987) Flow cytometric analysis and sorting of plant chromosomes from *Petunia hybrida* protoplasts. Cytrometry 8:500–508

De Laat AMM, Schel JHN (1986) The integrity of metaphase chromosomes of *Haplopappus gracilis* (Nutt) Gray isolated by flow cytometry. Plant Sci 47:145–151

Dolezel J, Lucretti S (1995) High-resolution flow karyotyping and chromosome sorting in a *Vicia faba* line with standard and reconstructed karyotypes. Theor Appl Genet 90:797–802

Gill KS, Arumuganathan K, Lee J-H (1999) Isolating individual wheat (*Triticum aestivum*) chromosome arms by flow cytometric analysis of ditelosomic lines. Theor Appl Genet 98: 1248–1252

Gray JW, Langlois RG (1986) Chromosome classification and purification using flow cytometry and sorting. Annu Rev Biophys Chem 15:195–235

Gustafson JP, Dille JE (1992) The chromosome location of *Oryza sativa* recombination linkage groups. Proc Natl Acad Sci USA 89:9646–8650

Kim UJ, Shizuya H, Chen XN, Deaven L, Speicher S, Solomon J, Korenberg J, Simon MI (1995) Characterization of a human chromosome 22-enriched bacterial artificial chromosome sublibrary. Genetic Anal 12:73–79

Lee J-H, Arumuganathan K, Kaeppler SM, Kaeppler HF, Papa CM (1996) Cell synchronization and isolation of metaphase chromosomes from maize (*Zea mays* L.) root tips for flow cytometric analysis and sorting. Genome 39:697–703

Li LJ, Song YC, Yan HM, Wang L, Liu LH (1998) The physical location of the gene *ht1* (*Helminthosporium turcicum* resistance1) in maize (*Zea mays* L.). Hereditas 92:131–138

Lucretti S, Dolezel J, Schubert I, Fuchs J (1993) Flow karyotyping and sorting of *Vicia faba* chromosomes. Theor Appl Genet 85:665–672

Macas J, Gualberti G, Nouzova M, Samec P, Lucretti S, Dolezel J (1996) Construction of chromosome-specific DNA libraries covering the whole genome of field bean (*Vicia faba* L.). Chrom Res 4:531–539

McCormick MK, Campbell E, Deaven L, Moyzis R (1993) Low-frequency chimeric yeast artificial chromosome libraries from flow-sorted human chromosomes 16 and 21. Proc Natl Acad Sci USA 90:1063–1067

Riera-Lizarazu O, Rines HW, Phillips RL (1996) Cytological and molecular characterization of oat x maize partial hybrids. Theor Appl Genet 93:123–135

Schwarzacher T, Wang ML, Leitch AR, Miller N, Moore G, Heslop-Harrison JS (1997) Flow cytometric analysis of the chromosomes and stability of a wheat cell-culture line. Theor Appl Genet 94:91–97

Van Dilla MA, Deaven LL (1990) Construction of gene libraries for each human chromosome. Cytometry 11:208–218

Van Dilla MA, Deaven LL, Albright KL, Allem NA, Aubuchon M, Bartholdi M, Brown N, Campbell E, Carrano A, Clark L, Cram L, Crawford B, Fuscoe J, Gray J, Hildebrand E, Jackson P, Jett J, Longmire J, Lozes C, Luedemann M, Martin J, Mcninch J, Meincke L, Mendelsonhn M, Meyne J, Moyzis R, Munk A, Perlman J, Peters D, Silvama, Trask B (1986) Human chromosome-specific DNA libraries:construction and availability. Biotechnology 4:537–552

Veuskens J, Marie D, Brown SC, Jacobs M, Negrutiu I (1995) Flow sorting of the Y sex chromosome in the dioecious plant *Melandrium album*. Cytometry 21:363–373

Wang ML, Leitch AR, Schwarzacher T, Heslop-Harrison JS, Moore G (1992) Construction of a chromosome- enriched *Hpa*II library from flow-sorted wheat chromosomes. Nucleic Acids Res 20:1897–1901