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Flow karyotyping and sorting of *Vicia faba* chromosomes

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Summary. Chromosome suspensions were prepared from formaldehyde-fixed, synchronized *Vicia faba* root tips. After staining with the DNA intercalating fluorochrome propidium iodide, the suspensions were analysed with a flow cytometer. The resulting histograms of integral fluorescence intensity contained peaks similar to those of theoretical *V. faba* flow karyotypes. From *V. faba* cv 'Inovec' (2n = 12) only one peak, corresponding to a single chromosome type (metacentric chromosome), could be discriminated. However, it was found that the peak also contained doublets of acrocentric chromosomes. Bivariate analysis of fluorescence pulse area (chromosome DNA content) and fluorescence pulse width (chromosome length) was necessary to distinguish the metacentric chromosome. To achieve a high degree of purity, a two-step sorting protocol was adopted. During a working day, more than 25 000 metacentric chromosomes (corresponding to 0.2 μg DNA) were sorted with a purity of more than 90%. Such chromosomes are suitable for physical gene mapping by in situ hybridization or via the polymerase chain reaction (PCR) and allow the construction of chromosome-specific DNA libraries. While it was only possible to distinguish and sort one chromosome type from *V. faba* cv 'Inovec' with the standard karyotype, it was possible to sort with a high degree of purity five out of six chromosome types of the line EFK of *V. faba*, which has six pairs of morphologically distinct chromosomes. This result confirmed the possibility of using reconstructed karyotypes to overcome existing problems with the discrimination and flow sorting of individual chromosome types in plants.

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Key words: Plant chromosomes – *Vicia faba* – Flow cytometry – Flow karyotype – Chromosome sorting

Introduction

Flow cytogenetics, defined as the use of flow cytometry for the analysis and sorting of metaphase chromosomes (Bartholdi 1990), is developing into a powerful research tool. Flow cytometric analysis of suspensions of isolated chromosomes may be used to classify chromosomes according to their relative DNA content, base content, protein content, and morphological parameters (Gray and Cram 1990). Such histograms are called flow karyotypes. Flow karyotyping has been proven valuable in the monitoring karyotype changes, the detection of aberrant chromosomes, and in mutagenicity testing (Trask et al. 1990; Gray et al. 1988; Dietzel et al. 1990). The sorting of large quantities of chromosomes of a single type has been instrumental in the construction of chromosome-specific DNA libraries (Van Dilla and Deaven 1990) and gene mapping (Cotter et al. 1989).

Flow cytogenetics also has a considerable potential in the plant sciences where it would be of use in the mapping and isolation of agronomically important plant genes. Despite great efforts, however, flow karyotyping has been reported in only a few plant species (De Laat and Blaas 1984; Conia et al. 1987, 1989; Arumuganathan et al. 1991). Furthermore, the sorting of chromosomes of a single type with sufficient purity has been reported so far only in *Haplopappus gracilis* (De Laat and Blaas 1984). The slow progress in applying flow cytometric analysis and sorting of plant chromosomes may be explained by: (1) difficulties

in the preparation of high quality chromosome suspensions, and (2) an inability to resolve single chromosome types on flow karyotypes.

Standard flow cytometric measurements of fluorescent emissions are based on the analysis of the height of the fluorescence pulse. Further information related to particle morphology can be obtained by the analysis of the shape of the fluorescent pulse. If the size of the illuminating laser beam is larger than that of the particle and the fluorescence is collected from the entire illumination spot, three shape descriptors can be obtained: (1) fluorescence pulse height (FPH), which is a measure of the density of the fluorescent molecules when the particle intercepts the laser beam; (2) fluorescence pulse width (FPW), which is a measure of the time during which the particle is emitting light and which is a function of the length of the particle, the width of measuring aperture and the size of the illuminating laser spot; (3) fluorescence pulse area (FPA), which is a measure of the total amount of fluorescence emitted by the particle (Cram et al. 1985).

In this communication, we report for the first time on the successful flow karyotyping and sorting of *Vicia faba* chromosomes. Single chromosome types were unambiguously distinguished using bivariate analysis of fluorescence pulse area versus fluorescence pulse width, and sorted in large quantities and at a high purity. In addition, the sorted chromosomes were shown to be suitable for in situ hybridization.

Materials and methods

Intact metaphase chromosomes were isolated from root-tip meristems of broad bean *Vicia faba* cv 'Inovec' (2n = 12, standard karyotype) and from the 'EFK' line of *V. faba* (2n = 12, reconstructed karyotype) in which it is possible to distinguish all six pairs of chromosomes due to the homozygous occurrence of three reciprocal translocations (Schubert et al. 1986). Chromosome suspensions were prepared as described earlier (Doležel et al. 1992) with some modifications.

Synchronization and accumulation of cells in metaphase

V. faba seedlings with about 2-cm-long main roots were incubated for 12 h in a Hoagland solution (Gamborg and Wetter 1975) containing 1.5 m*M* hydroxyurea. The roots were then washed in distilled water and transferred to hydroxyurea-free Hoagland solution. After a 6-h incubation, the roots were treated with 2.5 μ*M* amiprophos-methyl (APM) for 2 h. This treatment routinely resulted in metaphase indices exceeding 50%.

Chromosome isolation and staining

Immediately after the APM treatment, the roots were cut 1 cm from the root tip, rinsed in distilled water and fixed for 30 min at 5 °C in 6% (v/v) formaldehyde made in TRIS buffer (10 m*M* TRIS, 10 m*M* Na_2EDTA, 100 m*M* NaCl, pH 7.5) with 0.1% Triton X-100. After three washes in TRIS buffer, the meristem tips (1.5–2.0 mm) of 30 roots were chopped with a scalpel in a petri dish containing 1 ml LB01 lysis buffer (Doležel et al. 1989) having the following composition: 15 m*M* TRIS, 2 m*M* Na_2EDTA, 80 m*M* KCl, 20 m*M* NaCl, 0.5 m*M* spermine, 15 m*M* mercaptoethanol, 0.1% Triton X-100, pH 7.5. The suspension of released chromosomes and nuclei was filtered through a 50-μm nylon mesh, syringed twice through a 22-G hypodermic needle, and finally filtered through a 15-μm nylon mesh.

To remove interphase nuclei and chromosome clumps, 750 μl of the suspension was layered over 700 μl of 40% (w/v) sucrose in TRIS buffer in a 10-ml centrifuge tube and centrifuged at 200 rpm for 15 min. The supernatant was transferred into a sterile 1.5-ml Eppendorf tube. If the samples were not to be analysed on the same day, hexylene glycol was added to a final concentration of 0.75 *M* to prevent chromosome clumping, and the samples were stored at 4 °C.

Chromosomes were stained with propidium iodide (PI) at a final concentration of 50 μg/ml for at least 30 min before analysis.

Flow cytometric analysis

Chromosome suspensions were analysed with a FACStar^{PLUS} flow cytometer and sorter (Becton Dickinson, San José, USA). Propidium iodide-stained chromosomes and nuclei were optimally excited with a 5-W, argon-ion laser (Coherent INNOVA 90/5 UV) tuned to 514 nm with 200 mW output power. Red signals from stained objects were collected through a combination of a 560-nm-long pass filter and a 630/30-band pass filter. Fluorescence pulses were acquired according to their height, width, and area using logarithmic (4 decades) or linear amplifiers on 1024 channels ADC. The system threshold and the sweep trigger were set on a red fluorescence height signal. A filter-sterilized (0.22 μm) solution made up of 80 m*M* KCl and 20 m*M* NaCl, pH 7.5 was used as a sheath fluid.

Samples were delivered with a motor-driven syringe pump at a flow rate of approximately 400 objects per second. A ceramic nozzle tip of 65 μm was used for analysis and sorting. The instrument was aligned using fixed chicken red blood cell nuclei (CRBC) stained with 50 μg/ml PI. The coefficient of variation (CV) of their fluorescence intensity ranged from 2.0% to 2.3% throughout this study.

Modelling of flow karyotypes

Theoretical monoparametric flow karyotypes were modelled with KARYOSTAR software (Doležel 1991) using a CV of 2.3%. The models were based on relative chromosome lengths (Schubert et al. 1986).

Chromosome sorting

If not mentioned otherwise, sorting gates were set on a bivariate dot-plot of FPA versus FPW. Chromosomes were sorted at a rate of approximately 20 per second and collected in Falcon 24 wells plates containing LB01 buffer. Due to the use of fixed chromosomes, refrigeration of the sheath fluid medium was not necessary. For the analysis of purity of sorted fractions and for in situ hybridization, 1000 chromosomes were sorted on five microscope slides (200 chromosomes each) during each sorting run and air dried.

In situ hybridization

In situ hybridization to sorted metacentric satellite chromosomes of the standard karyotype was done according to the method of Pinkel et al. (1986) using a biotinylated rDNA probe VER17

(Yakura et al. 1983) kindly provided by Prof. S. Tanifuji, Saporo. (For details see Schubert 1992).

All experiments were repeated at least 5 times, and mean values were calculated.

Results

Vicia faba with the standard karyotype

Univariate analysis and sorting

Unpurified chromosome suspensions also contained interphase nuclei. To cover a wider range of fluorescence intensities, logarithmic amplification was used. As can be seen from Fig. 1, a theoretical flow karyotype predicted that in addition to two peaks corresponding to interphase nuclei in G_0/G_1 and G_2, two peaks corresponding to chromosomes would be observed. One composite peak would be formed by the five acrocentric chromosomes of similar DNA content and one peak by the metacentric chromosome with approximately twice the DNA content of an average acrocentric. This led to the prediction that from the standard *V. faba* karyotype, only metacentric chromosomes could be sorted. Figure 2 shows a typical histogram obtained after the analysis of the unpurified chromosome suspension. It differed from the theoretical histogram by a peak representing debris background and by a composite peak representing chromatids of acrocentric chromosomes.

Initial attempts to sort metacentric chromosomes were unsuccessful, the sorted fraction was occasionally contaminated even with interphase nuclei. Apparently, the proportion of metacentric chromosomes in unpurified sample was too low. Therefore, in all subsequent experiments, purified chromosome suspensions free of interphase nuclei were used for analysis and sorting.

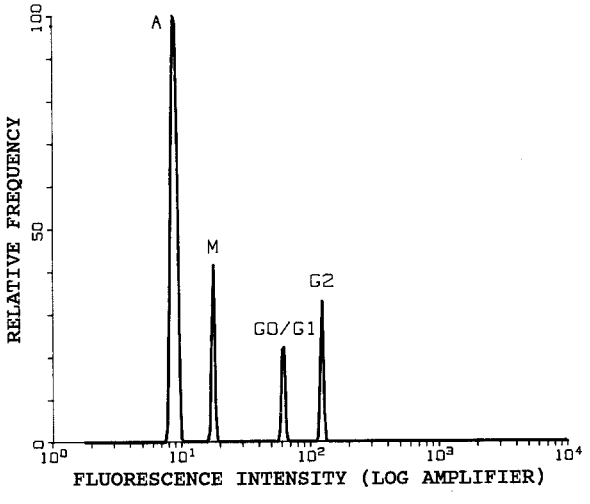

Fig. 1. Theoretical distribution of relative DNA content of chromosomes and interphase nuclei from *V. faba*. The model is based on relative chromosome length (Schubert et al. 1986) and is calculated for CV = 2.3% and logarithmic amplifier. In addition to peaks corresponding to G_0/G_1 and G_2 nuclei, a composite peak corresponding to five pairs of acrocentric chromosomes (*A*) and a peak corresponding to a pair of metacentric chromosomes (*M*) were resolved

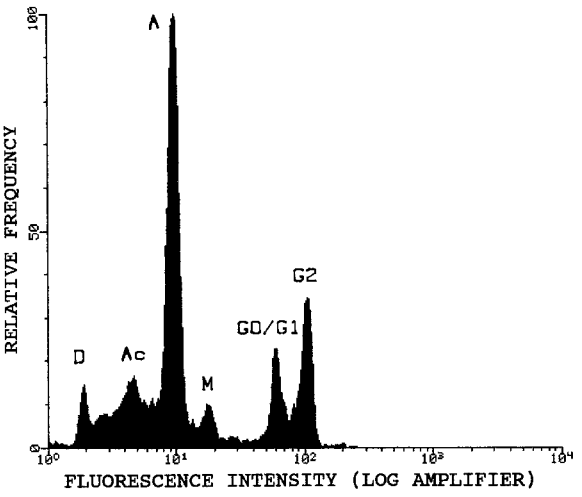

Fig. 2. Histogram of fluorescence intensity obtained from propidium iodide-stained unpurified chromosome suspension prepared from *V. faba* cv 'Inovec'. Logarithmic amplifier was used to cover the whole range of fluorescence intensities. Peaks corresponding to debris (*D*), chromatids of acrocentric chromosomes (*Ac*), acrocentric chromosomes (*A*), metacentric chromosome (*M*), and interphase nuclei in the G_0/G_1 and G_2 phases of the cell cycle were resolved

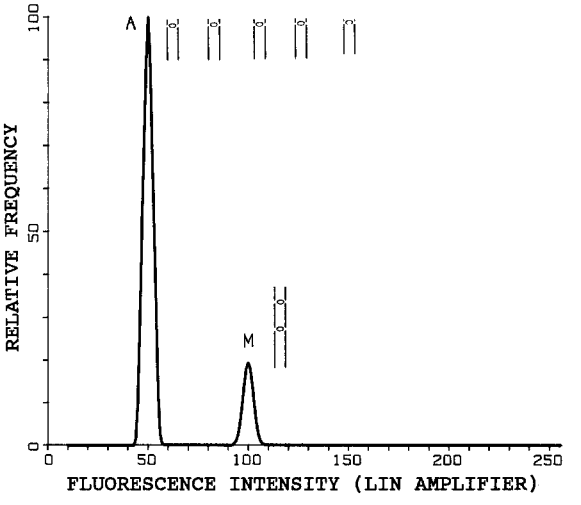

Fig. 3. Theoretical univariate flow karyotype of *V. faba* modelled for CV = 2.3%. A composite peak corresponding to five pairs of acrocentric chromosomes (*A*) and a peak corresponding to a pair of metacentric chromosomes (*M*) are indicated. The model is based on relative chromosome length (Schubert et al. 1986)

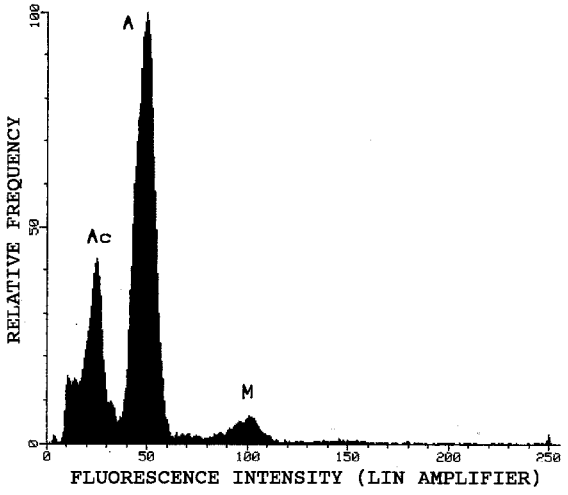

Fig. 4. Univariate flow karyotype obtained after the analysis of propidium iodide-stained purified chromosomes suspension prepared from *V. faba* cv 'Inovec'. Peaks corresponding to chromatids of acrocentric chromosomes (*Ac*), five pairs of acrocentric chromosomes (*A*), and a pair of metacentric chromosomes (*M*) are well resolved

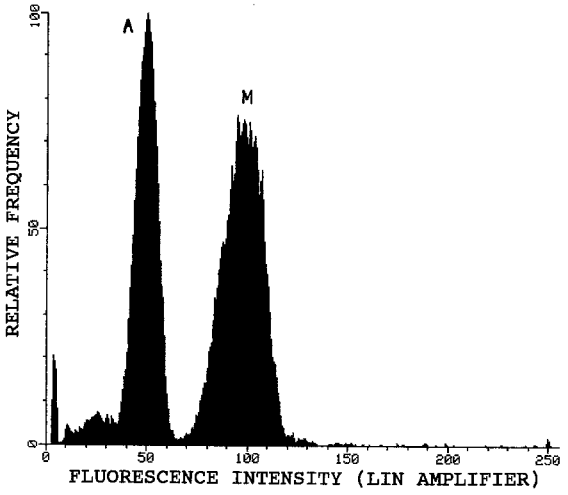

Fig. 6. Univariate flow karyotype obtained after the analysis of a propidium iodide-stained *V. faba* cv 'Inovec' chromosome suspension enriched for the metacentric chromosome. Note the marked increase in the frequency of metacentric chromosomes (*M*) with respect to acrocentric chromosomes (*A*)

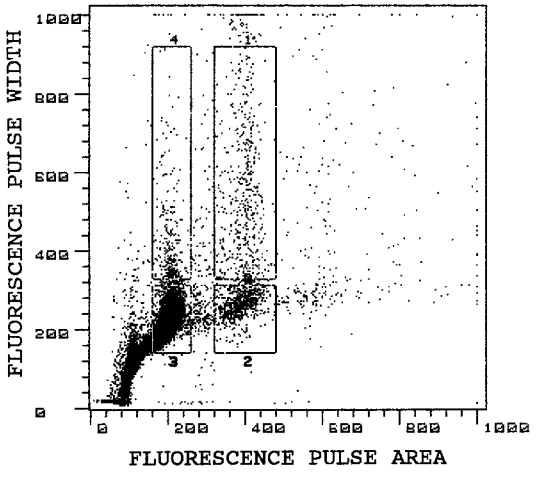

Fig. 5. Bivariate flow karyotype of *V. faba* chromosomes. Fluorescence pulse area and fluorescence pulse width were used simultaneously as parameters. Groups corresponding to five pairs of acrocentrics (*window 3*), chromatids of metacentric chromosome (*window 4*), doublets of acrocentrics (*window 2*), and metacentric chromosome (*window 1*) were resolved

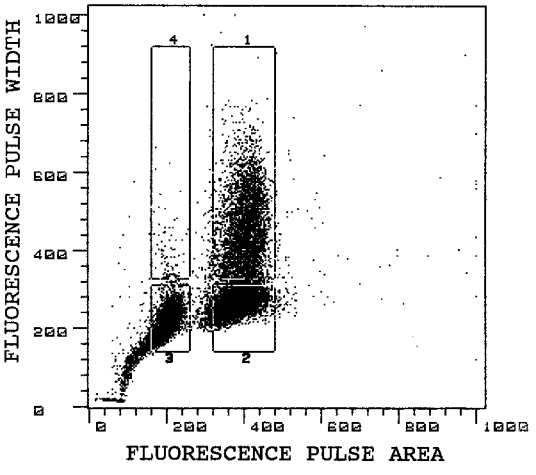

Fig. 7. Bivariate flow karyotype obtained after the analysis of a *V. faba* cv 'Inovec' chromosome suspension enriched for the metacentric chromosome. Note the high frequency of metacentric chromosomes (*window 1*).

A theoretical flow karyotype modelled for a chromosome suspension free of interphase nuclei and for linear amplification is shown in Fig. 3; a typical histogram obtained after analysis is shown in Fig. 4. Again, the only marked difference was the presence of debris background and chromatids of acrocentric chromosomes.

Sorting of the peak corresponding to the metacentric chromosome (channels 80 to 120), which represented 5.9% of the total number of analysed objects, resulted in 18.3% purity. The sorted fraction was heavily contaminated with doublets of acrocentric chromosomes sticking to each other (51.5%) and lightly contaminated by single acrocentric chromosomes (30.2%). Because a doublet of acrocentrics has a DNA content similar to that of a metacentric chromosome, single parametric DNA analysis could not be used to discriminate between them.

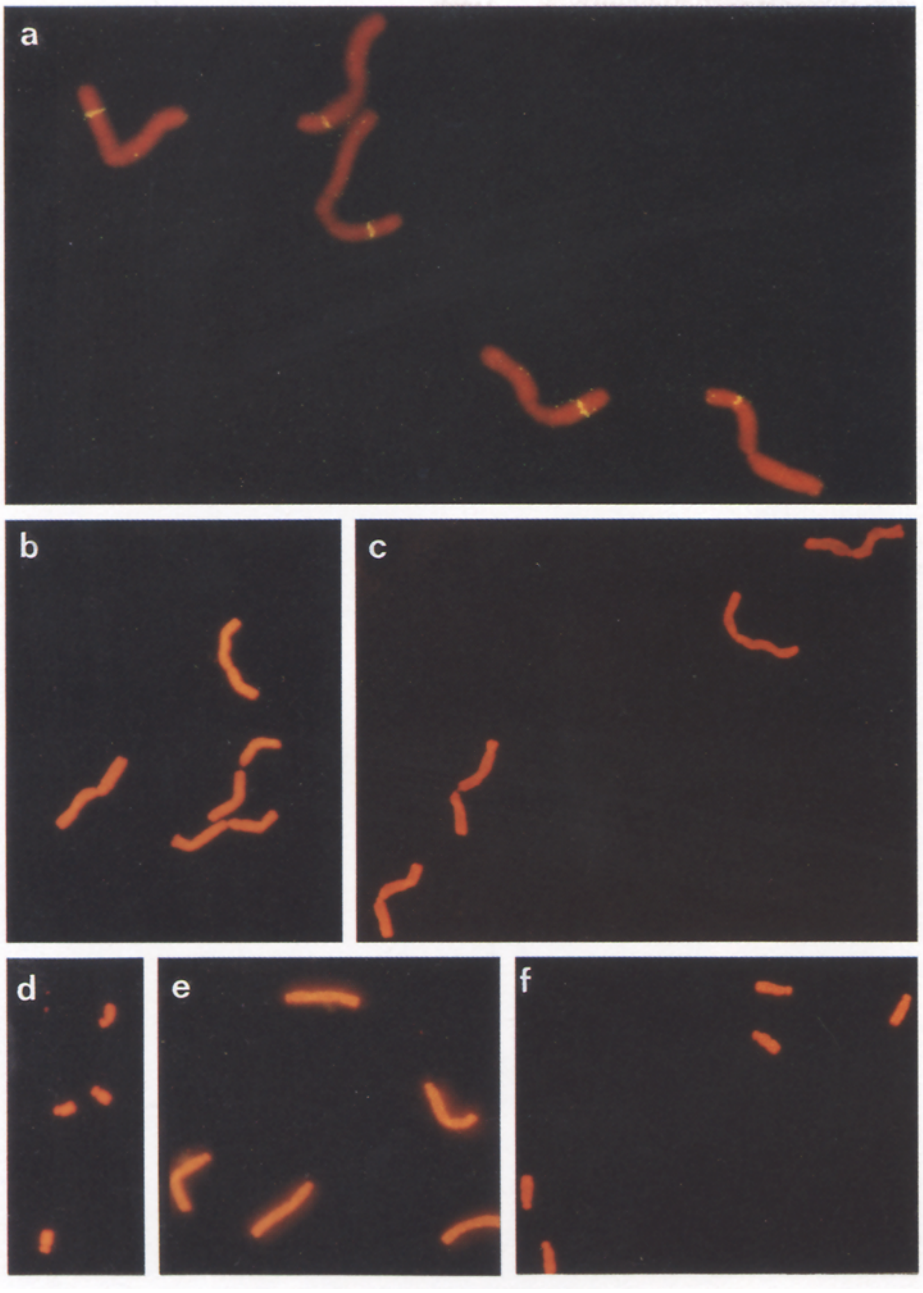

Fig. 8a–f. Sorted *V. faba* chromosomes, **a** Metacentric chromosomes of the standard karyotype after in situ hybridization with biotinylated rDNA probe. **b–f** Chromosomes of karyotype EFK; the enumeration of peaks corresponds to Fig. 10: **b** chromosomes I (peak 6), **c** chromosomes II (peak 5), **d** chromosomes III (peak 1), **e** chromosomes IV (peak 4), **f** chromosomes V (peak 2)

Bivariate analysis and sorting

When acrocentric and metacentric chromosomes crossed the 20-μm elliptical laser spot at 12.9 m/s the FPW ranged from 0.7 to 2 μs and from 1.5 to 3.1 μs, respectively. A typical result of a bivariate analysis of FPA versus FPW displayed in dot-plot mode is shown in Fig. 5. A peak corresponding to acrocentric chromosomes (FPA: channels 160–260, FPW: channels 140–920) was resolved into two groups. The bottom group (97.8% of the total peak count) represented acrocentrics, and the top group represented chromatids of the metacentric chromosome. Similarly, the peak corresponding to metacentric chromosomes (FPA: channels 320–480, FPW: channels 140–920) was resolved into a group representing doublets of acrocentrics plus metacentric chromosomes with their arms lying side by side, and a group (41.2% of the total peak count) representing only metacentric chromosomes.

To sort metacentric chromosomes, sort window no. 1 was set up in a bivariate flow karyotype as shown in Fig. 5. The average purity of the sorted fraction,

contaminated mostly with acrocentrics, was 52.1%. The window represented only 2.3% of the total number of objects analysed. The low proportion of metacentrics was considered to be the main reason for this result.

Two-step sorting procedure

To improve the purity, a two-step sorting procedure was adopted. During the first sorting run, the sample was 'enriched' for metaphase chromosomes. The sort window no. 1 was set up as indicated in Fig. 5; however, the FACS was set to ENRICH sort mode. In this mode, abort circuitry is disabled, and recovery is preferred to purity. From 1 ml of chromosome suspension, 34 000 objects were sorted into 200 µl of LB01 buffer. The analysis of enriched samples showed that they comprised on average 46.7% metacentric chromosomes, the main contamination being acrocentrics.

Univariate and bivariate flow cytometric analysis of a typical enriched sample is shown in Figs. 6 and 7. As can be seen in Fig. 6, the area of the peak corresponding to the metacentric chromosome (channels 80–120) increased significantly to 53.3% of the total number of objects analysed. Similarly, the number of objects in the group representing the metacentric chromosome (window no. 1) increased to 20.1% (Fig. 7). During the second sorting run ('resorting'), metaphase chromosomes were sorted from enriched suspensions. This time, the FACS was set to COUNTER mode, at which mode abort circuitry is established and purity is preferred to recovery. The sorted fraction then contained on average 92.8% of metacentric chromosomes.

With this procedure it was possible to sort approximately 25 000 metacentric chromosomes during a working day. This was equivalent to 0.2 µg DNA. The figures concerning the purity of the sorted fractions were based on visual evaluation of the number of sorted objects. However, as many applications of sorted chromosomes are based on the quantity of DNA, it is useful to express purity in terms of the amount of contaminating DNA. Because the sorted fractions were contaminated exclusively with acrocentric chromosomes having approximately half of the DNA content of the metacentric, the resulting purity was about 96% in terms of DNA amount.

In situ hybridization to sorted chromosomes

Chromosomes sorted on glass slides proved suitable for in situ hybridization even after the dried slides were stored for several weeks at 4 °C. Hybridization of a biotinylated rDNA probe to sorted metacentric satellite chromosomes of the standard karyotype revealed after detection by FITC-conjugated streptavidine a strong fluorescence signal at the nucleolus-organizing secondary constriction (Fig. 8a).

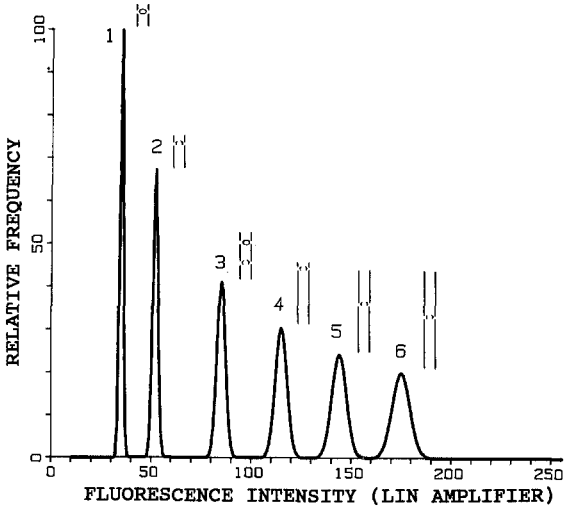

Fig. 9. Theoretical univariate flow karyotype of *V. faba* EFK line with reconstructed karyotype modelled for CV = 2.3%. The model is based on relative chromosome lengths (Schubert et al. 1986). All six pairs of chromosomes are well resolved

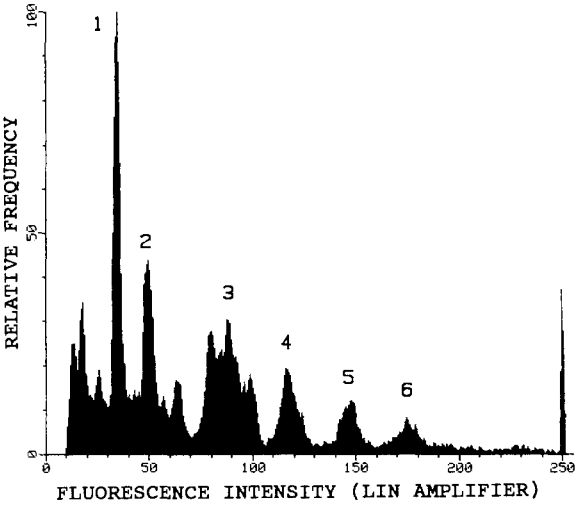

Fig. 10. Univariate flow karyotype obtained after the analysis of a propidium iodide-stained chromosome suspension prepared from the EFK line of *V. faba* (2n = 12, reconstructed karyotype). With the exception of chromosome VI, that formed peak no. 3, all of the chromosome types can be discriminated

Vicia faba with reconstructed karyotype EFK

Only the metacentric chromosome could be sorted from *V. faba* with the standard karyotype. Therefore, line EFK, which possesses a reconstructed karyotype with chromosomes differing sufficiently in size, was used.

A theoretical flow karyotype (Fig. 9) predicted six well-separated peaks corresponding to the six chromosomes of the EFK karyotype. Figure 10 shows a typical result of univariate analysis. As can be seen, five chromosomes out of six could be well resolved.

The assignment of individual peaks was confirmed by microscopic evaluation of the sorted fractions (Fig. 8b–f). With the exception of peak number 3, the purity of the sorted fractions ranged from 74.2% to 88.9%. To obtain samples with a better purity a two-step sorting procedure would be necessary.

Discussion

The feasibility of using flow-sorted chromosomes for the construction of chromosome-specific DNA libraries and for gene mapping in humans has been established: DNA libraries have been constructed for each of the 24 human chromosome types (Van Dilla and Deaven 1990; Cotter et al. 1989). Despite great efforts to modify this methodology for application to plant species, the results obtained so far are rather scarce. While the preparation of chromosome suspensions and subsequent flow karyotyping has been described in some species (De Laat and Blaas 1984; Conia et al. 1987, 1989; Arumuganathan et al. 1991), the sorting of chromosomes of a single type has been described only in *Haplopappus gracilis* (De Laat and Blaas 1984).

The preparation of high quality chromosome suspensions is a prerequisite for successful flow karyotyping and chromosome sorting. Several factors can be responsible for low quality plant chromosome suspensions: the splitting of metaphase chromosomes into chromatids, chromosome breakage, chromosome clumping, and the presence of cellular and chromosomal debris (Conia et al. 1987; Arumuganathan et al. 1991). In agreement with other authors (Fantes and Green 1989; Van Dilla and Deaven 1990) we have found that the presence of interphase nuclei negatively influences the purity of sorted chromosome fractions.

Conia et al. (1987) and Arumuganathan et al. (1991) noted that long treatments with mitotic spindle inhibitors lead to chromosomes splitting into chromatids, chromosome decondensation, and micronuclei formation. Our chromosome isolation procedure (Doležel et al. 1992) employs only a 2-h treatment with a spindle poison, thus avoiding chromosome decondensation and micronuclei formation and the separation of chromosomes into single chromatids.

The lower than expected frequency of metacentric chromosomes (2.3% versus 16.7%) could be explained by the breakage of metacentrics during the isolation procedure. Further loss was apparently caused by negligence during the analysis and sorting of those metacentrics that had both arms lying side by side. In this arrangement they could not be distinguished from doublets of acrocentrics and were aborted.

Several factors are of importance for achieving a high purity of sorted fractions. For the first time in plant flow karyotyping, we have used fluorescence pulse shape analysis. The bivariate analysis of FPA versus FPW enabled us to discriminate metacentric chromosomes from the doublets of acrocentrics. Our instrument was adjusted so that the size of the illuminating laser beam at the measuring point was similar to that of the chromosomes, and emitted fluorescence was collected from the entire illumination slit. Under these conditions, only low spatial resolution measurements (zero resolution system) can be performed (Cram et al. 1985). A more detailed morphological analysis of the chromosomes can be obtained by reducing the size of the measuring slit and the illuminating spot, thus enabling the analysis of the pulse profile (slit-scan flow cytometry). However, at present the use of slit-scan flow cytometry is limited by its technical complexity (Lucas et al. 1991).

Since fixed chromosomes are resistant to shearing forces during the sorting procedure a two-step sorting is possible. In addition to achieving a higher purity of the sorted fraction, this procedure made the results of the sorting experiments less sensitive to variations in the quality of the original chromosome suspension (data not shown). A two-step sorting procedure has also been shown to be useful for the sorting of minor subpopulations of cells (McCoy et al. 1991) and nuclei (Knopf and Bromova 1989).

During a working day, 25000 of metacentric chromosomes could be sorted with a purity higher than 90%. This result is comparable to the data reported for the sorting of human chromosomes (Bartholdi 1990). The corresponding quantity of DNA (0.2 μg) is sufficient for the direct construction of DNA libraries (Van Dilla et al. 1990). However, the recent development of methods for enzymatic DNA amplification (PCR) has made possible to reduce the number of chromosomes required for the preparation of chromosome-specific DNA to only several hundred (Cotter et al. 1989). Such quantities of chromosomes can be sorted within a very short time. The application of suitable karyotypes enables each chromosome type to be sorted separately and the use of pure chromosome fractions for physical sequence mapping by in situ or filter hybridization of via PCR.

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