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High-resolution flow karyotyping and chromosome sorting in *Vicia faba* lines with standard and reconstructed karyotypes

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Abstract Flow cytometric analysis has been performed on chromosomes isolated from formaldehyde-fixed root tips in a *Vicia faba* (2n = 12) line with a standard (wild-type) karyotype and in six *V. faba* translocation lines with reconstructed karyotypes. The resolution of individual chromosome types on histograms of chromosome fluorescence intensity (flow karyotypes) depended on the type of fluorochrome used for chromosome staining. The highest degree of resolution was achieved with 4′,6-diamidino-2-phenylindole (DAPI). The lower resolution obtained after staining with mithramycin A (MIT) and propidium iodide (PI) was probably due to the sensitivity of these stains to changes in chromatin structure induced by formaldehyde fixation. After the staining with DAPI, only 1 chromosome type could be discriminated in the line with a standard karyotype. In the translocation lines, the number of chromosome types resolved on flow karyotypes ranged from 2 in the G and the ACB lines to all (6) chromosome types in the EFK and EF lines. Refined flow karyotyping permitted the sorting of a total of 15 different chromosome types from five of the translocation lines. It is expected that flow sorting of chromosomes from reconstructed karyotypes will become a powerful tool in the study of nuclear genome organisation in *V. faba*.

Key words Chromosome isolation · Chromosome sorting · Flow cytometry · Flow karyotype · *Vicia faba*

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Introduction

Flow cytometric analysis and the sorting of chromosomes has been shown to be an important tool in the study of the mammalian genome. For instance, the quantitative classification of mitotic chromosomes (flow karyotyping) has proven to be invaluable in the detection of numerical and structural aberrations associated with human genetic diseases (Gray et al. 1988; Trask et al. 1990). Chromosomes sorted by flow cytometry have been used for the construction of chromosome-specific DNA libraries and gene mapping in man (Van Dilla and Deaven 1990; McCormick et al. 1993; Carter 1993). With some delay, the methods for chromosome isolation, flow cytometric analysis and sorting have also been developed for several plant species (Conia and Muller 1989; Doležel et al. 1994).

The application of flow karyotyping *per se* in plant genetics will probably be of less importance than chromosome sorting, which may provide large quantities of chromosomes of single type. This potential of plant flow cytogenetics has been confirmed by Wang et al. (1992) who reported the construction of a chromosome-enriched DNA library in *Tritium aestivum*. We have successfully used flow-sorted chromosomes for the physical mapping of several genes and gene families in *Vicia faba* (Macas et al. 1993). In general, the usefulness of a sorted chromosome fraction depends on its purity, i.e. on the contamination with other chromosomes and chromosome fragments. The purity is to a large extent determined by the resolution of the flow karyotype and by the ability to discriminate the chromosome to be sorted from other chromosomes, chromosome fragments and/or clumps (Doležel et al. 1994).

Ideally, the chromosome to be sorted should form a peak in the flow karyotype that contains no other chromosome. We have shown that in *V. faba* only one chromosome type is well-separated on a flow karyotype and amenable for sorting (Lucretti et al. 1993). However, the results of the study indicated that *V. faba* transloca-

tion lines with morphologically distinct chromosome pairs should permit the discrimination and sorting of a larger number of chromosome types. The purpose of this study was to compare the resolution of *V. faba* flow karyotypes obtained after the staining of chromosomes in suspension with three fluorochromes that differ in their binding modes to DNA, and to determine the chromosome types that can be sorted from a standard and six translocation lines of *V. faba*.

Materials and methods

Plant material

Metaphase chromosomes were isolated from meristem root tips of broad bean (*Vicia faba*, 2n = 12) cv 'Inovec' with a standard karyotype and from six lines with reconstrcuted karyotypes (ACB, BKH, EF, EFK, G, JF), kindly provided by Dr. I. Schubert (Gatersleben, Germany). In each of these lines, chromosome morphology has been changed due to one or more homozygous translocations and/or inversions (Schubert et al. 1986).

Cell-cycle synchronisation and metaphase arrest

A slightly modified procedure has been used (Lucretti and Doležel (1995). Briefly, *V. faba* seedlings with about 2-cm- long main roots were incubated for 18.5 h in a Hoagland solution (Gamborg and Wetter 1975) containing 1.25 m*M* hydroxyurea. The roots were then washed in distilled water and transferred to hydroxyurea-free Hoagland solution. After a 4.5-h incubation, the roots were treated with 2.5 μ*M* amiprophos-methyl (APM) for 2 h.

Chromosome isolation and staining

Chromosome suspensions were prepared as described earlier (Doležel et al. 1992) with some modifications. Immediately after the APM treatment, the roots were cut 1 cm from the tip, rinsed in distilled water and fixed for 25 min at 5 °C in 4% (v/v) formaldehyde 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 mm) of 30 roots were chopped with a scalpel in a glass petri dish containing 1.25 ml LB01 lysis buffer (Doležel et al. 1989) of 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 and syringed with a 22-G hypodermic needle. The suspension were stored in 2054 Falcon tubes (Becton Dickinson, Heidelberg, Germany) at 4 °C for up to several weeks without any sign of deterioration.

Prior to analysis, the samples were filtered through a 10-μm nylon filter, syringed with a 22-G hypodermic needle and stained with one of the three fluorochromosomes for at least 30 min: propidium iodide (PI) was used at final concentration of 50μg/ml; 4',6-diamidino-2-phenylindole (DAPI) was used at final concentration of 0.5 μg/ml; mithramycin A (MIT) was used at final concentration of 20 μg/ml. The concentrations were selected as being optimal in preliminary experiments and as giving minimal debris background and the lowest coefficients of variation (CV) of chromosome peaks. Because the fluorescence of the DNA-MIT complex is considerably increased in the presence of Mg^{2+} ions (Crissman et al. 1978), $MgSO_4$ was added to the chromosome suspension at final concentration of 10 m*M* prior to staining with MIT.

Flow cytometric analysis

Chromosome suspensions were analysed with a FACStar^{PLUS} flow cytometer and sorter (Becton Dickinson, San José, USA) equipped with an argon ion laser (Coherent INNOVA 90). PI- and MIT-stained chromosomes were excited at 514 nm (PI) and at 457 nm (MIT) with 200-mW output power. DAPI-stained chromosomes were excited at 351–360 nm with a laser adjusted to 100-mW output power. Fluorescence emitted from chromosomes stained with PI, MIT or DAPI was collected through a 630/30-nm band-pass filter, a 490-nm long-pass filter or a 400-nm long-pass filter, respectively.

Fluorescence pulses were acquired according to their height, width and area on 1024 channel ADC. A solution made up of 80 m*M* KCl and 20 m*M* NaCl was used as a sheath fluid. Samples were delivered with a motor-driven syringe pump at flow rate of approximately 200 particles per second. A ceramic nozzle tip of 100 μm was used for analysis and sorting. The instrument was aligned using chicken red blood cell nuclei that had been stained with an appropriate stain. For each karyotype, the instrument gain was adjusted so that the peak of the largest chromosome was positioned approximately at channel 800 (corresponding to channel 200 on 250-channel histograms).

Modelling of flow karyotypes

Theoretical flow karyotypes were modelled with KARYOSTAR software (Doležel 1991) using a CV of 2.5%. The models were based on relative chromosome lengths (Schubert et al. 1986).

Chromosome sorting

In order to confirm the chromosome content of peaks on flow karyotypes, sorting gates were set on histograms of fluorescence pulse area. Chromosomes were sorted at a rate of 5–20 per second directly onto a microscope slide. One thousand chromosomes were sorted from each peak on the flow karyotypes (200 chromosomes per slide) and air-dried.

Fluorescence microscopy

Microscope slides with air-dried chromosome fractions were mounted in LB01 buffer containing corresponding fluorochrome and observed with a Leitz Orthoplan microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany) equipped with a Ploemopak epifluorescent module. Filterblocks N2, H3 and A were used for PI-, MIT- and DAPI-stained samples, respectively.

Results

Idiograms of all *V. faba* karyotypes analysed in this work are shown in Fig. 1. For all of them, theoretical flow karyotypes were modelled on the basis of relative chromosome length and considering CV = 2.5% (Fig. 2). In all models, the peak of the largest chromosome was positioned at channel 200. As can be seen, the number of chromosome types which should be resolvable depends on the type and on the number of translocations involved in the reconstruction of a karyotype.

Analysis of the relative fluorescence intensity of chromosomes stained either with PI, MIT or DAPI showed that flow karyotypes obtained after staining with MIT consistently gave the lowest degree of resolution. Significantly better resolution was observed after PI staining. However, the chromosome peaks on flow karyotypes were still rather broad, with the CV ranging from 3.8% to 5.8%. Flow karyotypes with the highest degree of resolution in all *V. faba* lines were obtained after DAPI staining. The CVs of the chromosome peaks ranged from 2.1% to 4.1%. Examples of flow karyotypes obtained with all three stains in the EF translocation line are shown in Fig. 3. Based on this observation, we made

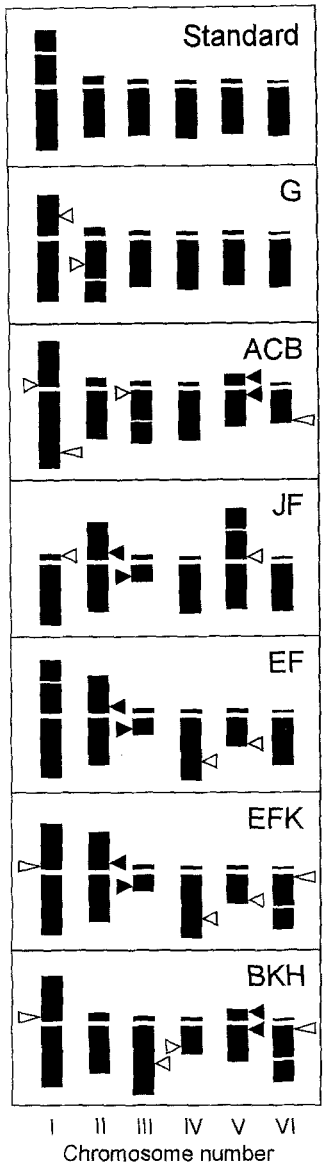


Fig. 1 Idiograms of *V. faba* (2 = 12) lines with standard (wildtype) and reconstructed karyotypes used in this study. *Arrows* point to presumable translocation and inversion breakpoints. Relative chromosome lengths were taken from Schubert et al. (1986)

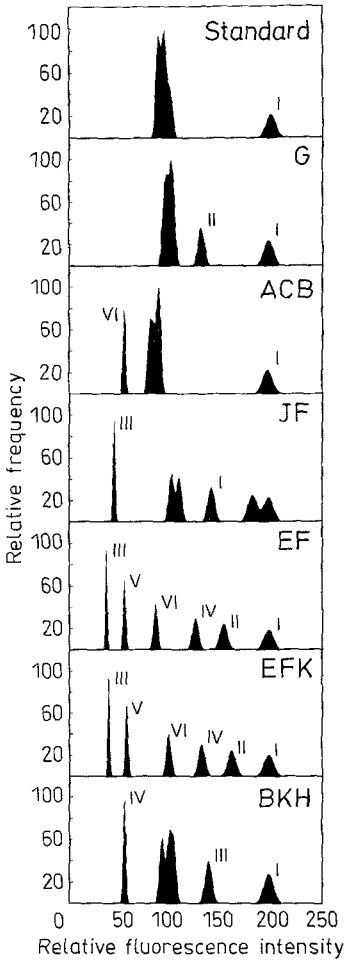


Fig. 2 Theoretical flow karyotypes modelled for all *V. faba* lines analysed in this work. All theoretically distinguishable chromosomes are labelled by their respective *numbers*

no attempt to estimate the number of chromosome types that could be discriminated after MIT or PI staining, and more detailed analysis was performed on flow karyotypes obtained after DAPI staining. Representative flow karyotypes of the remaining lines studied in this work are given in Fig. 4.

In the standard karyotype, only one peak corresponding to a pair of metacentric chromosomes I approximately at channel 200 could be discriminated. All other chromosomes (II–VI) formed a composite channel 100, with a closely attached peak at channel 90. In the flow karyotype of the ACB translocation line, 2 chromosome types could be easily discriminated. Chromosome I formed a peak at channel 203, while the peak of chromosome VI was located at channel 57. Remaining chromosomes formed partially overlapping peaks at channels 104, 91 and 83. Two chromosome types could also be discriminated in karyotype *G*. Namely, chromosome I, forming a peak at channel 197, and chromosome II, at channel 123. The remaining four acrocentric chromosomes formed 2 overlapping peaks with their means at channels 97 and 89.

On the flow karyotype obtained after the analysis of DAPI-stained chromosomes isolated from the BKH line, 3 chromosome types could be discriminated: chromosome I, with a peak at channel 203; chromosome III, on channel 148; and chromosome IV, at channel 51. Chromosomes II, V and VI formed closely attached peaks at channels 92 and 106. Four peaks corresponding to chromosome V (at channel 202), chromosome II (at channel 176), chromosome I (at channel 135) and chromosome III (at channel 46) could be discriminated on the JF flow karyotype. The remaining 2 chromosome types (IV and VI) formed a composite peak approximately at channel 100.

In the karyotype EFK, peaks corresponding to all chromosome types (I–VI) with peaks at channels 203, 162, 44, 134, 60 and 107, respectively, could be discriminated on a histogram of DAPI fluorescence intensity (Figs. 4, 5). Similarly, all 6 chromosome types could be easily discriminated on a flow karyotype obtained in the EF translocation line (Fig. 3). Peak means were on channels 198, 146, 117, 84, 54 and 39 for chromosomes I, II, IV, VI, V, and II, respectively.

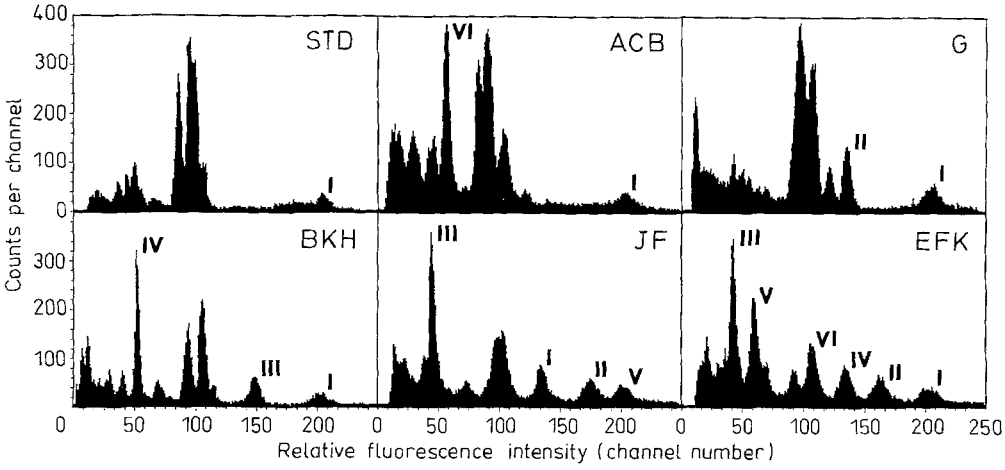


Fig. 3a–c Distributions of relative fluorescence of chromosomes isolated from the *V. faba* EF line and stained by **a** propidium iodide, **b** mithramycin A and **c** DAPI. Note the differences in the degree of resolution of individual chromosome peaks. The peaks on DAPI-derived flow karyotype which represent single chromosome types are labelled by their respective *numbers*

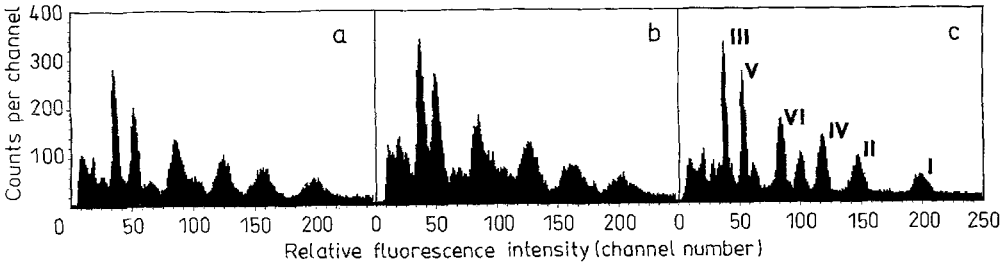
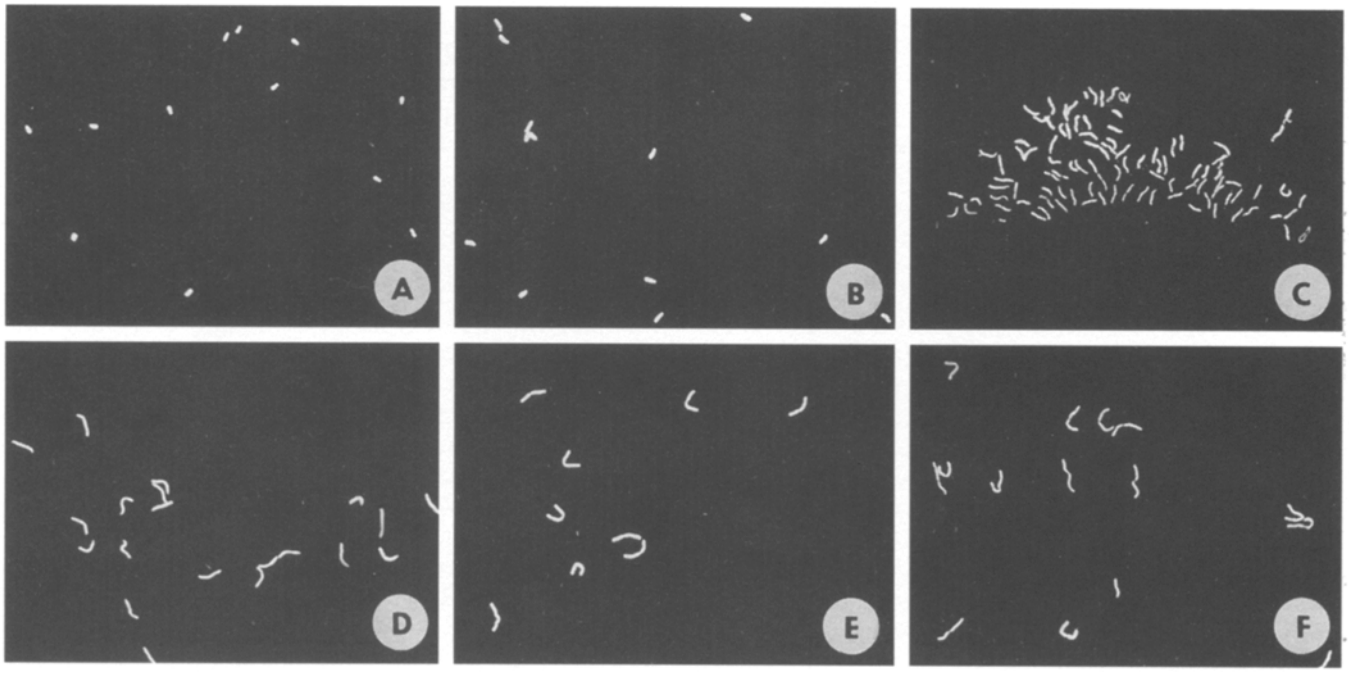


Fig. 4 Distributions of relative fluorescence intensities of chromo- somes isolated from *V. faba* lines with standard and reconstructed karyotypes. In all cases, the chromosome suspensions were stained by DAPI. The peaks which can be clearly discriminated and correspond to single chromosome types are labelled by their respective *numbers*

In most *V. faba* lines, the number of chromosome types which could be discriminated, as well as positions of peaks on experimental flow karyotypes corresponding to these chromosomes, agreed well with theoretical

Fig. 5A–F Chromosomes sorted from *V. faba* karyotype EFK. **A** Chromosomes III, **B** chromosomes V, **C** chromosomes VI, **D** chromosomes IV, **E** chromosomes II, **F** chromosomes I



flow karyotypes. However, in the ACB line, chromosome III, which was not resolved on the theoretical karyotype, formed a peak at channel 104 that only partially overlapped with the two peaks at channels 91 and 83 corresponding to chromosomes II, IV and V. It is interesting to point out that chromosome III was not resolved on the MIT or PI flow karyotypes. Another difference between the theoretical and experimental flow karyotype was observed in the JF line, where the peak mean of chromosome II (channel 176) was lower than predicted. This permitted the discrimination of chromosome II from the chromosome V with its peak on channel 202. As in the case of the ACB line, the two chromosomes could not be resolved on the MIT or PI karyotypes.

In all of the histograms obtained after DAPI staining, a continuous debris background was present over which chromosome peaks were positioned. As can be seen in Figs. 3 and 4, the proportion of these particles was rather low in all *V. faba* lines, and thus their presence did not hamper the discrimination and sorting of individual chromosome types. In addition to peaks representing single chromosome types, some experimental flow karyotypes (e.g. G or BKH) contained other well-resolved peaks which, however, corresponded to a mixture of chromosomes with single chromosome arms or chromatids all having very similar DNA content. The third category of peaks detected on experimental flow karyotypes consisted of peaks representing only chromatids or chromosome fragments (single arms, satellites).

Discussion

The purpose of this work was twofold: (1) to select an optimal staining procedure to achieve the highest resolution of individual chromosome types on *Vicia faba* flow karyotypes and (2) to determine the chromosome types that can be clearly discriminated in *V. faba* lines with standard and reconstructed karyotypes.

We have found that the resolution of *V. faba* flow karyotypes can be determined by the type of fluorochrome used to stain the chromosomes. The lowest resolution was observed after staining with MIT, which binds to G-C base regions of DNA by a non-intercalating mechanism (Zimmer and Wähnert 1986). Although the reason for such low resolution is not clear, it might be explained by a negative influence of formaldehyde fixation on MIT binding to DNA. Crissman et al. (1978) showed that formaldehyde fixation of mammalian cells quenched the fluorescence of MIT-stained nuclei and decreased the resolution of DNA content histograms. Thus, the rather poor resolution of MIT flow karyotypes in *V. faba* could be due to formaldehyde fixation, which is an integral part of our chromosome isolation procedure (Doležel et al. 1992). Considerably better resolution was observed after staining with PI, a fluorochrome that intercalates into double-stranded DNA and RNA without base specificity (Crissman and Steinkamp 1990). However, it is highly probable that also in this case formaldehyde fixation negatively influenced the binding of PI to DNA. For instance, Becker and Mikel (1990) showed that the fixation decreased PI fluorescence of mammalian nuclei. Recently, we have found that the resolution of DNA content histograms of formaldehyde-fixed plant nuclei can be significantly improved by acid hydrolysis, which increases the accessibility of DNA for PI (Moretti, Lucretti, Doležel, unpublished results).

In contrast to MIT or PI, high-resolution flow karyotypes were obtained after staining with DAPI, a fluorochrome that binds specifically to A-T base pairs in the minor groove of DNA (Portugal and Waring 1988). This fact indicated that changes in chromatin structure caused by formaldehyde fixation does not impede the binding of DAPI to chromosomal DNA. The work of Sgorbati et al. (1986) supports this hypothesis. The authors did not find any difference in resolution between DNA content histograms obtained after the analysis of non-fixed and formaldehyde-fixed plant nuclei.

The good agreement between theoretical and experimental flow karyotypes observed in this study confirmed the usefulness of theoretical models in the planning and execution of flow cytometric analysis and sorting of chromosomes (Conia et al. 1989; Doležel 1991). The reason for the discrepancies between theoretical and experimental flow karyotypes in the peak positions of chromosome III in the ACB line and chromosome II in the JF line is not clear. Considering the preference of DAPI for A-T-rich DNA regions (Portugal and Waring 1988), these observations indicate a higher A-T content of the short arm of chromosome I of the standard karyotype.

The assignment of peaks on flow karyotypes to individual chromosome types was based on the analysis of sorted chromosome fractions (Fig. 5). Although the fractions were occasionally contaminated with chromosome fragments, chromatids or chromosome doublets, their purity was high enough (75–90%) to avoid any misclassification. Considering the purpose of this work, no attempt was made to sort high purity fractions. In our previous work (Lucretti et al. 1993), we demonstrated that such fractions can be obtained using enriched suspensions and bivariate analysis.

The sorting of fractions of chromosomes of a single type from *V. faba* translocation lines provides unique material that enables the physical mapping of DNA sequences at the chromosomal and subchromosomal level by the polymerase chain reaction (PCR) (Macas et al. 1993). This approach is especially attractive for the localisation of genes that cannot be mapped genetically. In this work, we have shown that refined flow karyotyping enabled us to discriminate and sort 15 different chromosome types from six translocation lines. Although other reconstructed karyotypes are available in *V. faba* (Schubert et al. 1986) from which 1 or more chromosome types could be discriminated, from a prac-

tical point of view it is reasonable to reduce the number of karyotypes needed for chromosome isolation and sorting. Based on the results of this study a suitable set is as follows: EF line (all 6 chromosome types), BKH line (chromosomes I, III and IV), G line (chromosomes I and II), and JF line (chromosomes I and V). Karyotype EFK may be omitted as chromosomes II, III, IV and V can be sorted from the EF line and chromosomes I and VI can be obtained from the BKH line. The sorting of chromosomes I and VI from karyotype ACB would be warranted only to confirm and/or further specify sequence location on standard (wildtype) chromosomes I, II, and VI. The same holds true for other karyotypes not analysed in this work.

In conclusion we have demonstrated that high-resolution flow cytometric analysis and the sorting of chromosomes can also be performed in plants. Work is in progress to study the *V. faba* genome organisation using the approach described here and to modify the technique for other economically important crops.

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