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Review article

Application of flow cytometry for the study of plant genomes

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Abstract. During the last fifteen years, flow cytometry and sorting greatly contributed to the improvement of our knowledge of plant genome structure and function. This paper reviews the applications of flow cytometry for the analysis of isolated nuclei and chromosomes. Because of its speed, precision and convenience, this method of analysis of the nuclear DNA content finds an enormous number of applications which cover basic research, breeding and production. The results obtained with chromosome analysis and sorting indicate that the technique might greatly simplify the analysis of plant genomes at the molecular level.

Key words: chromosomes, DNA content, flow cytogenetics, flow cytometry, flow karyotype, nuclei, ploidy.

Introduction

Flow cytometry (FCM) was originally developed as a method for rapid counting and analysis of blood cells. With the technical evolution and development of new fluorescent probes it became a useful analytical tool in many areas of biological research. Although the application of FCM for the analysis of plant cells and subcellular organelles has been delayed until the early

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eighties, the number of applications is continuously increasing. The opportunity to sort defined subpopulations of biological particles further increases the number of possible applications. Due to space limitations it is not possible to review all of them, and interested readers are referred to one of general reviews on plant flow cytometry (FOX, GALBRAITH 1990). Other reviews cover various applications of FCM which include cell cycle analysis (BROWN et al. 1991), sorting of protoplasts and somatic hybrids (GALBRAITH 1989, BERGOUNIOUX et al. 1992), and analysis of gene expression (GALBRAITH et al. 1995).

The purpose of this paper is to review the field of flow cytometric analysis of plant cell nuclei and chromosomes. In the first part, basic principles of flow cytometry are reviewed. In the second part, methods for the analysis of nuclear DNA content and the use of FCM for ploidy screening and for genome size estimation, is discussed. In addition to the methodology and its applications, the impact of possible pitfalls is described. The third part concentrates on the use of FCM for the analysis and sorting of plant chromosomes, and on the potential of this technology for gene isolation and mapping.

Basic principles

A flow cytometer is an instrument which analyses optical parameters (light scatter, fluorescence) of particles in flow moving with respect to the point of measurement. This contrasts with other techniques of quantitative analysis of isolated nuclei and chromosomes, such as microspectrophotometry or automatic image analysis, which require the particles to be fixed on a flat surface. The measurement in flow permits analysis at a very high speed, typically 10^2-10^3 per second. Furthermore, the particles to be analysed are selected randomly from the whole population without any bias.

The sample to be analysed is introduced into the centre of the flow chamber filled with a fast moving sheath fluid. The particles contained in the sample are hydrodynamically constrained in the centre of the narrow liquid stream and pass, one after another, through a focus of exciting light. The particles scatter the exciting light and, if they contain a natural fluorochrome, or if they have previously been stained with a fluorescent dye, they will fluoresce. Pulses of scattered light and fluorescence pulses are collected using an optical detector system, separated by optical filters and converted to electric current pulses by optical sensors. The output analog signals are digitized and processed by a computer. Because FCM analyses the particles individually and at a high

speed, large populations of cells and organelles can be measured in a relatively short time and the presence of subpopulations may be detected.

Some instruments (called flow-sorters) can be used to physically separate subpopulations of particles. In a so called "jet-in-air" flow cytometer, sorting is made possible by breaking the liquid stream into droplets by the action of a vibrating transducer attached to the flow chamber. Droplets containing particles of interest are deflected electrostatically by passage through an electric field. In addition to this design, other configurations of flow cytometers have been developed. In one of the "enclosed-stream" designs, excitation and collection of the emitted fluorescence is provided through a single objective. The advantage of this instrument is a high resolution of the measurement. The optical design permits the use of an arc lamp instead of an expensive laser as a source of exciting light without decreasing the sensitivity. Sorting is made possible by switching the flow between two outlets of the flow chamber. The advantage of flow-switching sorters (as compared to droplet sorters) is the negligible physical stress to which sorted particles are exposed. The disadvantage is a considerable dilution of the sorted fraction caused by a continuous flow of about one third of the sheath fluid through the outlet for the sorted fraction.

Analysis of the nuclear DNA content

Estimation of the nuclear DNA content was the first (HELLER 1973) and remains by far the most frequent use of FCM in plant science. Without any doubt, FCM may be considered as the most suitable method for this assay. Biochemical methods are not appropriate because they generate mean values for large numbers of nuclei and cannot detect subpopulations. Absorption microspectrophotometry, cytofluorometry and the more recently introduced image analysis cannot compete with FCM in speed and convenience.

Methodology

FCM analysis of the nuclear DNA content is based on the analysis of the relative fluorescence intensity of nuclei stained with a DNA fluorochrome. The specimen to be analysed by FCM must be in the form of a single particle suspension. Suspensions of protoplasts may be prepared from plant tissues by enzymatic digestion of cell wall. However, autofluorescence and low penetration of DNA stains through the cell wall precludes their use for DNA content estimation. Fixation of plant protoplasts in ethanol : acetic acid fixatives

makes the plasma membrane permeable, elutes endogenous fluorochromes and allows for quantitative DNA staining (PUITE, TEN BROEKE 1983). Nevertheless, fixed protoplasts are not used for DNA analysis due to the decreased resolution of the distribution of DNA content (GALBRAITH 1984, ULRICH et al. 1988).

GALBRAITH et al. (1983) demonstrated that suspensions of intact nuclei may be prepared by chopping a small amount of plant tissue with a razor blade, and that these suspensions are suitable for DNA content analysis. Alternatively, nuclei may be gently released by lysis of protoplasts in a hypotonic buffer (ULRICH, ULRICH 1991). High resolution histograms may be obtained in most species with a more sophisticated nuclei isolation procedure (ULRICH, ULRICH 1991, DOLEŽEL, GÖHDE 1995), originally developed for human cells (OTTO 1990). Some researchers isolated nuclei from fixed cells or tissues (SGORBATI et al. 1986, PFOSSER 1989). This approach is especially attractive for the analysis of cell cycle kinetics (LUCRETTI, DOLEŽEL 1995). Alternatively, nuclei may be fixed after their isolation with ethanol, or ethanol : acetic acid fixatives (HÜLGENHOF et al. 1988). The apparent advantage is a possibility to store

Table 1. Fluorescent dyes most frequently used for flow cytometric estimation of nuclear DNA content*

Fluorochrome	Primary binding mode	Wavelength (nm)	
		excitation	emission
Ethidium bromide**	Intercalation	525	605
Propidium iodide**	Intercalation	535	620
Hoechst 33258	AT-rich regions	345	460
DAPI	AT-rich regions	360	460
Chromomycin A3	GC-rich regions	445	570
Mithramycin	GC-rich regions	445	575

*Actual binding mode as well as spectral properties depend on several factors including DNA/dye ratio, ionic strength and pH of the staining solution

**Binds also to double-stranded RNA

fixed nuclei. The disadvantage is a tendency for nuclei to clump as a result of fixation. Due to changes in chromatin structure, fixed nuclei may not be suitable for some applications. For instance, formaldehyde fixation decreases the resolution of histograms of DNA content obtained after staining with propidium iodide (DOLEŽEL, LUCRETTI 1995).

Various buffers have been used to isolate nuclei from plant cells. Their composition is generally dictated by a requirement to preserve the integrity of nuclei, to protect DNA from degradation, and to provide optimal conditions for DNA staining. The most frequently used buffers include Mg^{2+} cations (GALBRAITH et al. 1983, ARUMUGANATHAN et al. 1994), or polyamines (DOLEŽEL et al. 1989), as stabilizing agents. With some plant species, addition of a reducing agent such as mercaptoethanol, dithiothreitol or polyvinylpyrrolidone, is necessary to inhibit the occurrence of phenolic compounds (BHARATHAN et al. 1994, DOLEŽEL et al. 1994).

During or after the isolation, the nuclei are stained by a fluorescent dye which binds specifically and stoichiometrically to DNA. Table 1 lists six fluorochromes most frequently used for DNA content estimation. Propidium iodide and ethidium bromide quantitatively intercalate into double stranded DNA (LE PECQ, PAOLETTI 1967). Hoechst 33258 and DAPI bind preferentially to AT-rich regions of DNA (PORTUGAL, WARING 1988), while mithramycin and chromomycin A3 bind preferentially to GC-rich regions (van DYKE, DERVAN 1983). The selection of a suitable fluorochrome depends on the type of analysis (DOLEŽEL 1991).

Ploidy analysis

Analysis of the relative fluorescence intensity of nuclei isolated from young leaf tissue yields a histogram showing a dominant peak corresponding to nuclei in the G_1 phase of the cell cycle and a minor peak corresponding to G_2 nuclei. To estimate ploidy level, the position of the G_1 peak on a histogram is compared to that of a reference plant with known ploidy. The comparison can be made between individual histograms obtained under identical conditions (Fig. 1). This procedure is called external standardization. A more reliable approach involves a comparison within a single histogram, comparing G_1 peak positions of two samples (reference + specimen), processed in one tube. This approach, called internal standardization, eliminates errors due to machine instability and variability in sample preparation.

FCM assay has some important advantages over chromosome counting. It is convenient (sample preparation is easy), rapid (several hundreds of samples can be analysed in one working day), it does not require dividing cells, it is non-destructive (one sample can be prepared, e.g., from a few milligrams of leaf tissue), and can detect mixoploidy. Therefore the method is used in different areas ranging from basic research to plant breeding and production.

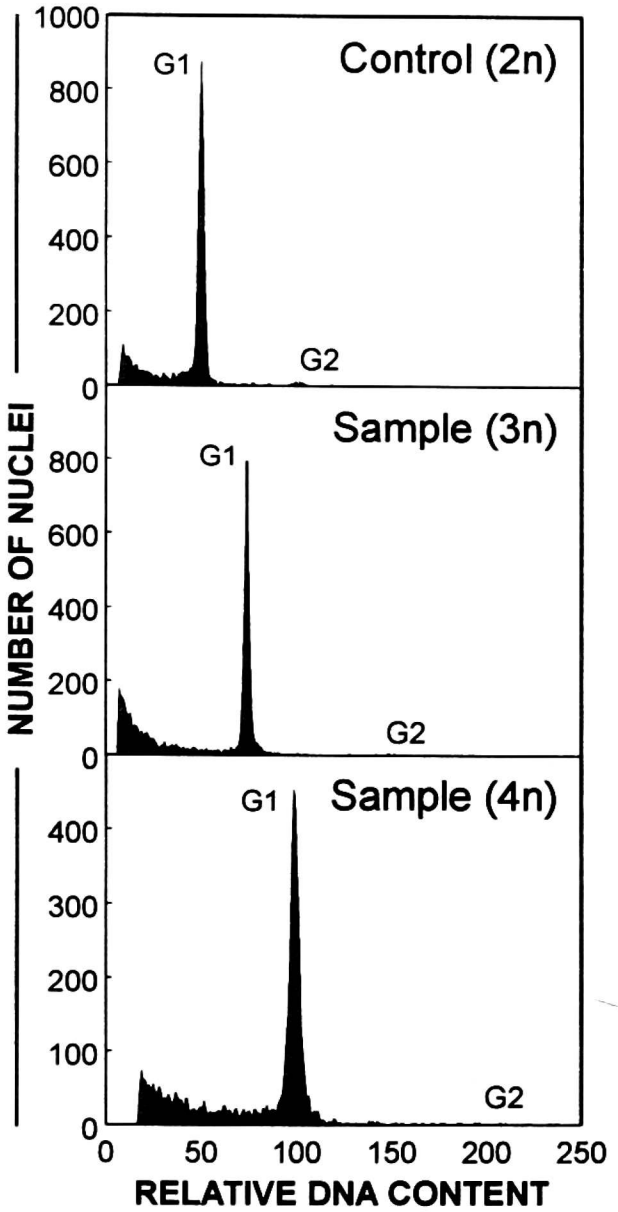

Fig. 1. Ploidy analysis in banana (*Musa* spp.). Histograms of relative nuclear DNA content obtained after analysis of nuclei isolated from leaf tissues. Gain of the instrument was adjusted so that the G_1 peak of nuclei isolated from a control diploid plant was at channel 50. This setting was kept constant during the analysis of samples prepared from plants of unknown ploidy. Peaks representing G_1 nuclei appeared on channels 75 and 100, corresponding to the triploid and tetraploid level, respectively. N.B., the distribution of DNA content is characterized by dominant G_1 peaks, which makes ploidy estimation easy.

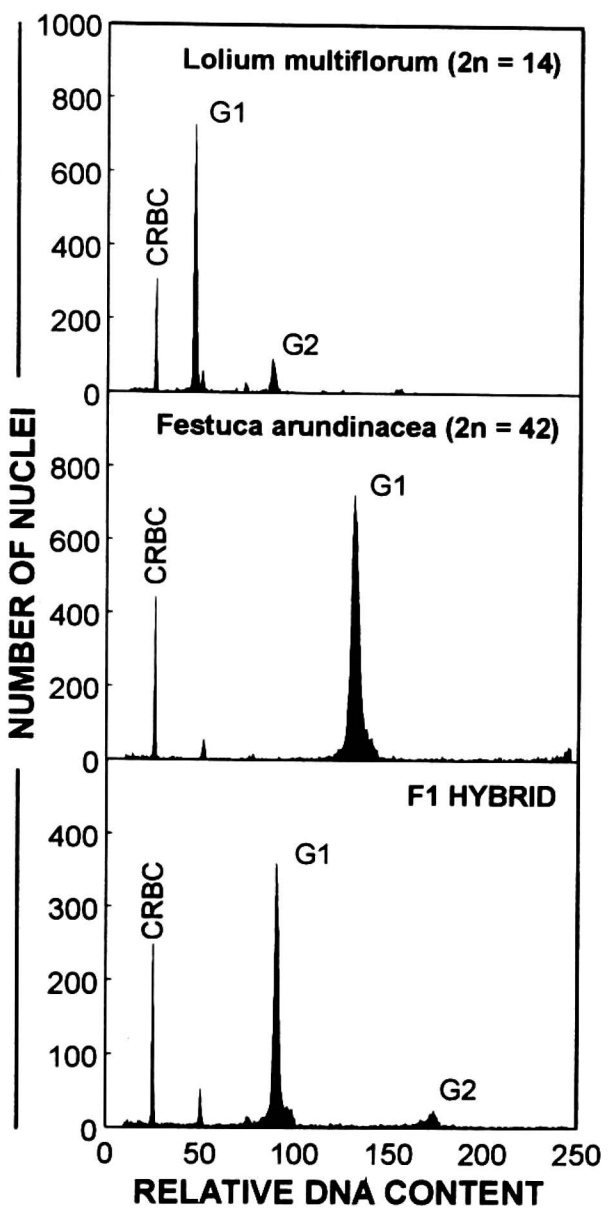

Fig. 2. Identification of hybrid plants obtained after crossing *Lolium multiflorum* with *Festuca arundinacea*. Histograms of relative DNA content were obtained after analysis of nuclei isolated from leaf tissues. Chicken red blood cell nuclei were used as an internal standard. N.B., this type of analysis cannot discriminate F_1 hybrids from rarely occurring tetraploid *L. multiflorum* plants which have the same DNA content.

In some crops, commercially used cultivars are triploid (e.g., sugar beet, hops, banana), and their breeding is based on different crossing strategies to obtain the desired ploidy level. In other crops (e.g., some grasses), tetraploid cultivars may outperform diploid ones. Tetraploids are usually produced after treatment with a polyploidizing agent. In some species, detection of 2n-gamete producers is of great importance. Van TUYL et al. (1989) reported on successful FCM screening for 2n-pollen producers in *Lilium* using FCM. The method is also extensively used for rapid assessment of quality ("ploidy purity") of seed lots. De LAAT et al. (1987) demonstrated that high numbers of individual plants can be pooled in one sample to speed up the analysis. Nevertheless, precise estimation of a rare contamination by seeds or plants of undesirable ploidy requires individual plant analyses (ŚLIWIŃSKA, STEEN 1995).

In vitro cultures are usually characterized by a low frequency of mitotic cells. Many cells differentiate, do not divide, and their ploidy cannot be estimated by chromosome counting. FCM was found invaluable for estimating the ploidy stability of callus, cell or protoplast cultures, including the effects of ageing and culture conditions (BINAROVÁ, DOLEŽEL 1988, KUBALÁKOVÁ et al. 1996). The applications also include assessment of ploidy stability after in vitro multiplication (JACQ et al. 1992, WYMAN et al. 1992). In some species, including cereals, plants can be regenerated in large numbers from anther, microspore or ovary cultures. Establishment of ploidy is important because haploid regenerants must be treated with a polyploidizing agent to produce fertile homozygous diploids, while spontaneously occurring dihaploids can be left untreated. FCM is the only method which can cope with high numbers of regenerants (MARTÍNEZ et al. 1994, BOHANEC et al. 1995). In vitro cultures can be used for mass production of polyploid plants. Complete systems for production of solid tetraploids based on rapid screening of regenerants using FCM were developed and successfully used in cassava and banana (AWOLEYE et al. 1994, van DUREN et al. 1996).

Interspecific crossing is used to transfer desired characters from one species into another. When parental species differ enough in their nuclear DNA content, FCM can detect interspecific hybrids according to their intermediate DNA values (KELLER et al. 1996). We have used FCM routinely to detect interspecific hybrids between *Lolium multiflorum* and *Festuca arundinacea* (Fig. 2). Similarly, genome composition of allopolyploids may be determined by this method (AFZA et al. 1992, SABHARWAL, DOLEŽEL 1993). While the detection of changes involving whole chromosome sets is a simple task, detection of aneuploidy, especially if only one chromosome is involved, is technically more demanding. Nevertheless, BASHIR et al. (1993) showed that FCM is

sensitive enough to detect the presence of a pair of rye chromosomes in wheat-rye addition lines. More recently, PFOSSER et al. (1995) demonstrated that this method may detect aneuploids in triticale and the presence of a pair of chromosome arms of rye added to the wheat genome.

An important application of FCM ploidy screening involves plant taxonomy. Because chromosome counting is laborious, only a limited number of plants can be analysed and phenotypically similar populations which differ in ploidy level may be overlooked (KEELER et al. 1987, MISSET, GOURRET 1995, LYSÁK et al. 1997). FCM has an enormous potential to study the structure and dynamics of plant populations belonging to species with anomalous types of genetic systems (e.g., agamospermy). Such taxa are characterized by large numbers of microspecies differing in ploidy (RENNO et al. 1995). HUFF and BARA (1993) found that FCM ploidy analysis may be used to determine the genetic origins of aberrant progeny from the facultatively apomictic *Poa pratensis*.

Even if FCM ploidy analysis is relatively simple, some precautions should be taken during the analysis and data interpretation. Ploidy is defined as the number of copies of the chromosome complement. Because FCM analyses DNA content, a term "DNA ploidy" should be used to distinguish the results obtained by chromosome counting from those obtained by FCM (HIDDEMAN et al. 1984). Differentiated plant tissues should not be used to establish the ploidy status of a plant because of a higher proportion of cells in G_2 phase (4C DNA content) or having undergone endoreduplication. Simple FCM analysis cannot distinguish these cells from polyploid cells. On the other hand, because endoreduplication plays a major role in the development of seed endosperm, FCM may be employed to study this process, for instance with the aim to increase the grain yield in cereals (SCHWEIZER et al. 1995).

Genome size estimation

The knowledge of genome size is important in many areas of research. It is useful in studies aiming to resolve phylogenetic relationships, to analyse the correlation between the genome size and physiological or agronomical characters, and to estimate the effect of environmental factors on genome size. Molecular biologists mapping a genome need to know its complexity. As pointed out by BENNETT and LEITCH (1995), nuclear genome size is still not known in about 99% of angiosperm species. It is a general opinion, because of its speed and precision, that FCM is the preferred method to handle this task.

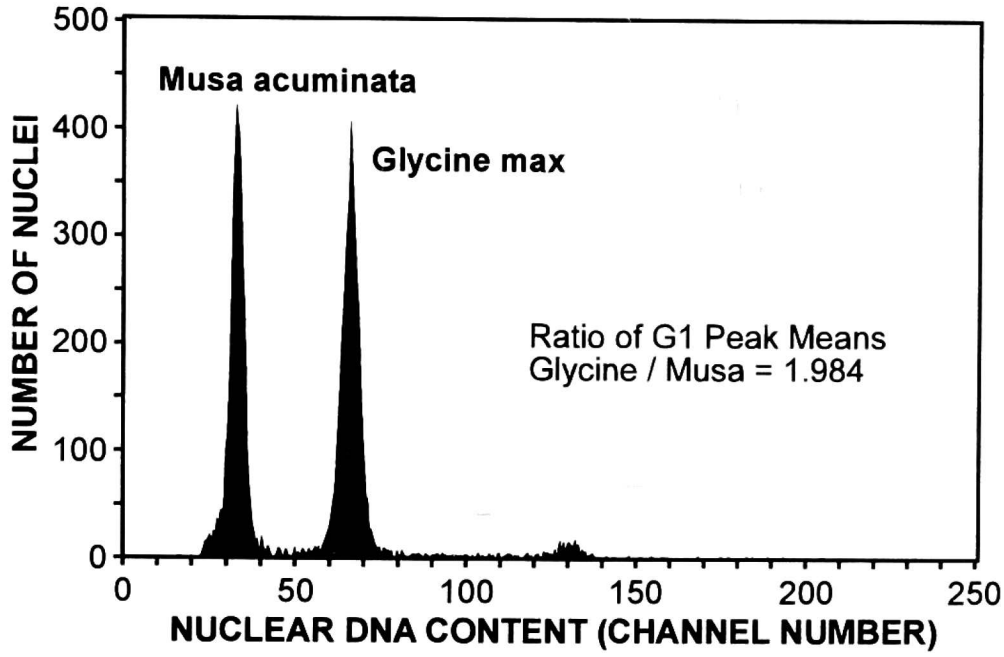

Fig. 3. Estimation of genome size in diploid banana (*Musa acuminata errans*). Soybean (*Glycine max*, 2C = 2.50 pg DNA) was used as an internal reference standard. Nuclei of banana and soybean were simultaneously isolated from leaf tissues, stained by propidium iodide and analysed. The 2C DNA content of *M. acuminata errans* can be calculated using the ratio of G_1 peak means: 2.50/1.984 = 1.26 pg DNA.

Flow cytometric estimation of nuclear genome size in pg DNA or Mbp can be achieved by simultaneous measurement of specimen and reference standard nuclei. The genome size of the specimen is then calculated from the ratio of G_1 peak positions specimen/standard (Fig. 3). Recently, FCM has been used extensively to estimate the genome size in cultivated and wild species. BENNETT and LEITCH (1995) included results obtained by flow cytometry in their extensive list of DNA amounts. The technique was successfully used in various cytotaxonomic and evolutionary studies (CROS et al. 1994, CERBAH et al. 1995). The precision of FCM analysis is very high and detection of statistically significant differences in genome size as low as 0.02 pg has been reported (DOLEŽEL et al. 1994).

The precision and reproducibility of analysis is critical especially in studies of intraspecific genome size variation. There are several reports showing considerable variation of genome size in many species. These results, based mainly on Feulgen microspectrophotometry, were interpreted in terms of nucleotype theory (BENNETT 1985). However, in the light of new results obtained by FCM, it seems that some of these results might be technical artefacts. For instance BARANYI and GREILHUBER (1995, 1996) re-analysed genome size in *Pisum sativum*. In contrast to previous reports, all accessions were practically constant in genome size. On the other hand, it was found that some

of the accessions classified as subspecies of *P. sativum* differed significantly in genome size from this species. The authors suggested that these accessions should be treated as separate taxa.

In some species, a relationship was found between genome size and agronomical parameters. According to BIRADAR et al. (1994), all growth and yield parameters in *Zea mays* are negatively correlated with nuclear DNA amount. The authors found negative correlations of nuclear DNA amount and important yield parameters, such as seed weight and seed number per plant. The results of BIRADAR and RAYBURN (1993) demonstrated an association between heterotic response and nuclear DNA content inheritance in F_1 hybrids of *Z. mays*. In *Glycine max*, a highly significant correlation between maturity and the genome size of twenty cultivars was found (GRAHAM et al. 1994). However, the existence of intraspecific variation of genome size in *G. max* has not been confirmed (GREILHUBER, OBERMAYER 1997). Clearly further studies will be needed to confirm these results and to prove the usefulness of DNA content estimation for prediction of agronomically-important characters.

The selection of a proper standard is crucial for correct estimation of nuclear genome size. Some authors used chicken red blood cell nuclei and a value of 2.33 pg DNA is usually considered (GALBRAITH et al. 1983), although other values (e.g., 2.50 pg) were also reported (RASCH 1985). When using chicken erythrocytes, one should also keep in mind that sex-specific differences in DNA content exist, and that inbred strains of chicken differ in DNA content (NAKAMURA et al. 1990). Because the genome size of the standard should not differ significantly from that of the specimen (VINDELØV et al. 1983), a range of standards is needed to cover the range of genome size variation observed in plants. DOLEŽEL et al. (1992) calibrated genome size of selected cultivars of *Raphanus sativus* (2C = 1.11 pg), *Lycopersicon esculentum* (2C = 1.96 pg), *Z. mays* (2C = 5.72 pg), *P. sativum* (2C = 9.07 pg), *Vicia faba* (2C = 26.90 pg) and *Allium cepa* (2C = 34.76 pg) against human leukocytes (2C = 7.0 pg DNA, TIERSCH et al. 1989). These species are routinely used as internal standards. Other plant species frequently used for DNA standardization include *Petunia hybrida* (2C = 2.85 pg; MARIE, BROWN 1993), *G. max* (2C = 2.50 pg; DOLEŽEL et al. 1994) and *Hordeum vulgare* (2C = 11.12 pg; OBRIEN et al. 1996). Unfortunately, no general agreement on DNA standards for plant FCM has been achieved so far. This makes comparison of data obtained in different laboratories difficult, if not impossible.

An attractive application of FCM is the analysis of the effect of environment and genotoxic compounds on the nuclear DNA amount. SELVAN and THOMAS (1995) reported on FCM detection of DNA content changes in *A. cepa* which

were induced by irradiation. Similarly, BIRADAR et al. (1994) reported on nuclear DNA changes in *Z. mays* induced by a fungicide Triticonazole and MC MURPHY and RAYBURN (1993) observed changes in the nuclear DNA content of *Z. mays* seedlings grown in media including coal fly ash.

As with ploidy analysis, some precautions are warranted in this application of FCM. As mentioned above, some DNA fluorochromes bind preferentially to AT- or GC-rich regions of DNA. Because the AT/GC ratio is not constant in plants, these dyes should not be used for genome size estimation in absolute units (DOLEŽEL et al. 1992). On the other hand, such dyes may be useful to estimate the DNA base content (GODELLE et al. 1993, BLONDON et al. 1994). An important issue is the possibility to detect differences in DNA content between various accessions. Flow cytometric analysis is usually characterized by a high degree of resolution with coefficient of variation of DNA peaks ranging from 1 to 3%. However, this precision is not related to the reproducibility of DNA content estimation. The experiment should include replicate measurements to allow for statistical analysis of variability within and between individual accessions.

Chromosome analysis and sorting

Genome analysis and mapping in most cultivated crops is hampered by the large size of the genome. Its complexity may be reduced by purification of isolated chromosomes by flow cytometry and sorting (DOLEŽEL et al. 1994). In this approach, suspensions of intact chromosomes are prepared from synchronized cells, chromosomes are stained by a DNA specific fluorochrome and their fluorescence is measured and used to quantitatively classify each chromosome (LUCRETTI, DOLEŽEL 1995). Resulting fluorescence distribution (flow karyotype) usually consists of several peaks representing individual chromosomes or chromosome groups (Fig. 4). De LAAT and BLAAS (1984) were the first to demonstrate the possibility of obtaining purified fractions of plant chromosomes. Since that time, chromosome sorting was described in several species (see DOLEŽEL et al. 1995 for recent review).

Chromosomes which can be unambiguously discriminated on a flow karyotype may be sorted in high numbers and used for subsequent molecular analysis. The applications envisaged include localization of DNA sequences, isolation of complex probes for chromosome painting, construction of chromosome-specific DNA libraries, and isolation of chromosome-specific molecular markers. WANG et al. (1992) reported construction of chromosome-

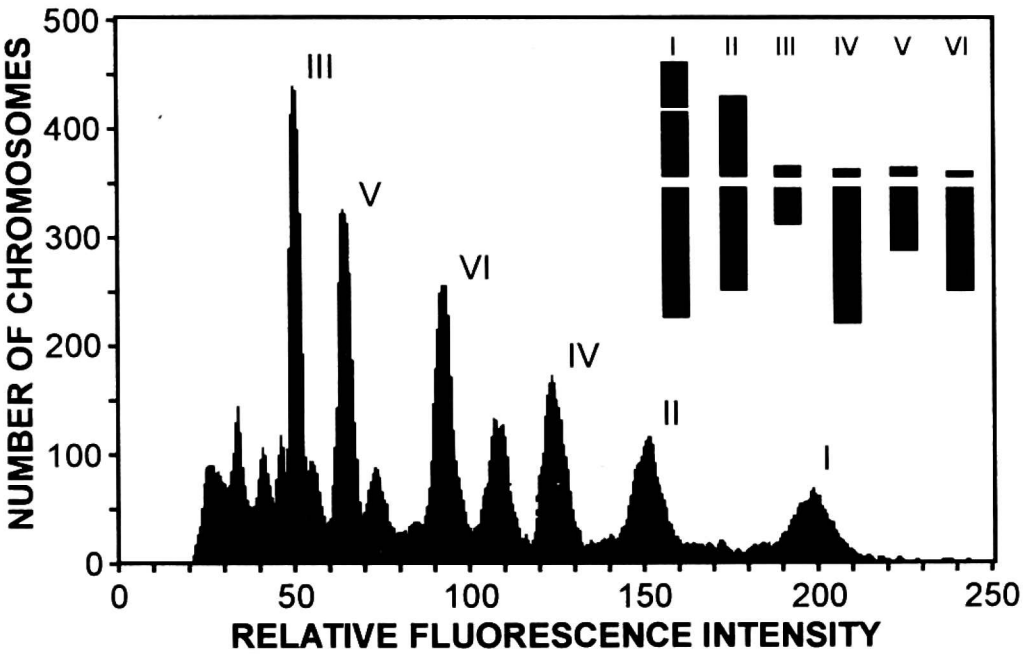

Fig. 4. Analysis of relative fluorescence intensity of *Vicia faba* (2n = 12) chromosomes after staining with DAPI. Chromosomes were isolated from the EF line with a reconstructed karyotype (the idiogram is shown inside the frame). Peaks corresponding to all chromosomes are well separated. Good discrimination permits sorting of all chromosome types within the karyotype. In addition to chromosome peaks, the histogram (flow karyotype) contains peaks corresponding to chromosome fragments, chromatids and chromosome debris.

-enriched DNA library from sorted *Triticum aestivum* chromosome 4A. In *V. faba*, sorted chromosomes were used for PCR with sequence-specific primers to localize seed-specific protein genes and pseudogenes to specific chromosome regions (MACAS et al. 1993). ARUMUGANATHAN et al. (1994) reported the generation of a DNA library specific to chromosome 2 in *Lycopersicon pennellii*. The authors used the library to isolate eleven chromosome 2-specific RFLP markers and placed them on the genetic linkage map of *L. esculentum*. Recently, we have constructed a set of chromosome-specific libraries in *V. faba* (MACAS et al. 1996). This is the first example in plants of a complete set of chromosome-specific libraries covering the whole genome.

The progress in plant flow cytogenetics has been hampered by difficulties in discrimination of individual chromosome types. We have shown that the use of chromosome translocation lines with morphologically distinct chromosomes may greatly aid in chromosome discrimination (DOLEŽEL, LUCRETTI 1995). Furthermore, the use of reconstructed karyotypes permitted subchromosomal gene localization. In those species where translocation lines are not available, chromosome discrimination may be improved by fluorescent labelling using chromosome-specific probes. We have developed a protocol for primed in situ DNA labelling en suspension (PRINSES) (MACAS et al. 1995) which may be used to sort otherwise indistinguishable chromosomes (PICH et al. 1995).

One of the applications of sorted mammalian chromosomes is chromosome painting, a powerful technique for the visualization of defined chromosomes during mitosis or interphase. Unfortunately, the attempts to use sorted plant chromosomes for chromosome painting have so far not been successful, the reasons being a large proportion of dispersed repetitive sequences and insufficient signal intensity of short unique sequences (FUCHS et al. 1996). An important advantage of flow cytogenetics is the potential to provide a high (microgram) quantity of chromosome DNA which is required for construction of large-insert libraries. The availability of chromosome-specific BAC or YAC libraries would greatly simplify gene isolation and mapping.

Conclusions

Flow cytometry has become a powerful tool for the study of plant genomes with applications ranging from basic research to industrial uses. It may be expected that the number of practical applications will increase and flow cytometry will be even more extensively used by plant breeders and seed companies. Numerous applications of the technique are foreseen in the field of plant taxonomy and in evolutionary studies. Monitoring of mutagen-induced DNA damage remains an unexplored possibility. Considering the recent results, it may be expected that flow cytometric sorting of chromosomes will play an increasingly important role in the analysis of plant genome structure and function.

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