

Flow cytometric estimation of nuclear DNA amount in diploid bananas (*Musa acuminata* and *M. balbisiana*)*

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Abstract

Cell nuclei were isolated from leaf tissues of wild banana (*Musa balbisiana*, *M. acuminata* ssp. *banksii* and *M. acuminata* ssp. *errans*) and of the two vegetative clones of diploid cultivar "Pisang Mas". Relative fluorescence intensity was measured on propidium iodide-stained nuclei by flow cytometry. Nuclei isolated from *Glycine max* with known nuclear genome size were used as internal standard to determine nuclear DNA content of *Musa* in absolute units. The results of the study showed that the size of nuclear genome of *Musa* is smaller than previously estimated. In general, it is smaller in comparison with many other angiosperms. Furthermore, it was found that nuclear DNA content of *M. balbisiana* (genome BB) is significantly lower than that of *M. acuminata* subspecies and cultivars (genome AA). This finding should permit estimation of genome composition in triploid *Musa* clones with expected hybrid composition. Flow cytometry is proposed as a useful technique with potential applications in taxonomy, breeding and biotechnology of *Musa*.

Introduction

Musa is a vegetatively propagated crop with a breeding system complicated by widespread sterility and polyploidy among cultivars. Clones with parthenocarpic fruits within *Eumusa* section originate from two species, i.e. *Musa acuminata* and *M. balbisiana* (Simmonds and Shepherd 1955). Among them, it is difficult to distinguish cultivars with different ploidy levels and hybrid constitutions of *A* (*M. acuminata*) and *B* (*M. balbisiana*) genomes. Counting and karyotyping of somatic chromosomes

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is difficult due to very small metaphasic chromosomes ($x = n = 11$) with hardly distinguishable morphology.

Flow cytometry is a rapid and convenient technique that allows accurate determinations of nuclear DNA content (Fox and Galbraith 1990, Doležel 1991). The analysis is based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence intensity emitted by stained nuclei. To determine nuclear DNA content in absolute units, fluorescence intensity of nuclei is compared with the fluorescence intensity of nuclei isolated from a species with known nuclear genome size.

In this communication, we report a modification of the flow cytometry technique for the estimation of nuclear DNA content in *Musa*. The nuclear genome size in two cultivated species of *Musa* has been determined for the first time. This may form a basis for the application of the flow cytometry in *Musa* taxonomy, breeding and biotechnology.

Materials and methods

Plant material: Seeds of *Musa balbisiana* Colla ($2n = 22$, genome BB), *M. acuminata* ssp. *banksii* (F. Muell.) Simmonds and *M. acuminata* ssp. *errans* (Teodoro) Allen (both $2n = 22$, genome AA) were obtained from the *Musa* improvement programme of Fundacion Hondureña de Investigacion Agricola, La Lima, Honduras. Seedlings were grown in the greenhouse to a size of about 50 cm with a minimum of 7 fully developed leaves. Clonally micropropagated plants of sterile diploid cultivars "Pisang Mas" (Syn. "Sucrier", genome AA) were obtained from the Department of Primary Industry, Maroochy Experimental Station, Queensland and from the Malaysian Agricultural Research and Development Institute, Kuala Lumpur. Micropropagated plantlets were acclimatized in the greenhouse and grown to a similar stage as the seedlings.

Isolation and staining of nuclei: To release cell nuclei, approximately 50 mg of *Musa* young leaf tissue was chopped with a sharp scalpel in a glass Petri dish containing 1 ml LB01 lysis buffer of the following composition: 15 mM Tris, 2 mM Na_2EDTA , 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1 % Triton X-100, pH 7.4 (Doležel *et al.* 1989) supplemented with 50 μg propidium iodide and 50 μg RNase. To estimate nuclear DNA content in absolute units, approximately 25 mg of *Glycine* young leaf tissue was simultaneously chopped. The suspensions of released nuclei were passed through a 50 μm nylon mesh and stained in the dark for 30 min. Then the samples were filtered through a 15 μm nylon mesh and analysed.

Flow cytometry: Propidium iodide-stained nuclei were analysed with a *Leitz MPV Compact flow cytometer* (Ernst Leitz Wetzlar GmbH, Wetzlar, Germany). A 100 W high pressure mercury arc lamp was used for excitation. A filterblock N2 was used both for the excitation and for the detection of propidium iodide fluorescence.

Histograms of fluorescence intensity were registered over 512 channels and evaluated using a *Hewlett-Packard HP-86B* microcomputer with a *FLOWSTAR* software (Doležel 1989). At least ten thousand nuclei were analysed in each sample.

Estimation of nuclear genome size: Symbol C is used for DNA content of haploid set of chromosomes. Determinations of genome size in *Musa* were done by using *Glycine max* cv. "Polanka" as an internal standard. Nuclear DNA content of *G. max* was estimated in preliminary experiments in relation to an assigned value of $2C = 7.00$ pg in fresh human male leukocytes (Tiersch *et al.* 1989). Nuclear DNA content of *G. max* was found to be $2C = 2.50 \pm 0.04$ pg (mean \pm SD, $n = 11$). This value is similar to that of 2.35 pg reported by Hammatt *et al.* (1991) for a different cultivar of *G. max*.

Data analysis: The ratio of means of the $G_{0/1}$ peaks corresponding to the reference standard and sample nuclei (fluorescence ratio) was calculated. The ratio was corrected for zero offset error using the DNA ratio between single and double chicken red blood cell nuclei clumps (Givan *et al.* 1988). Nuclear genome size of *Musa* was then calculated according to a formula:

$$2C \text{ nuclear DNA content [pg]} = \frac{2.50}{\text{fluorescence ratio}}$$

Evaluation of the single phases of the cell cycle was performed according to a non-parametric curve fitting method (Dean and Jett 1974). Each sample was analysed five times and the whole experiment was repeated six times. Statistical analysis was performed using a *Hewlett-Packard* software.

Results

Chopping of young *Musa* leaf tissue with a scalpel lead to the release of high numbers of intact nuclei into the LB01 buffer. On average 4×10^5 nuclei were isolated from 50 mg of leaf tissue. Most of the nuclei form a single peak at channel 64 corresponding to $G_{0/1}$ phase of the cell cycle (Fig. 1A). The coefficient of variation (CV) for $G_{0/1}$ peak varied between 2.5 % and 4.5 % throughout this study. The analysis of the distribution of nuclei in various stages of the cell cycle showed that more than 90 % of nuclei were in $G_{0/1}$ phase. The cell cycle distribution was similar in all *Musa* genotypes studied. When analysed at the same instrument setting, the distribution of fluorescence intensity of *Glycine* nuclei stained with propidium iodide was similar to that of *Musa*, however, the distribution was shifted more to the right with the $G_{0/1}$ peak at approximately channel 130.

When *Musa* and *Glycine* nuclei were isolated, stained and analysed simultaneously, the histograms of fluorescence distribution contained two large peaks corresponding to $G_{0/1}$ nuclei of both species (Fig. 1B). The ratio of $G_{0/1}$ peak means was taken as a basis for calculation of 2C nuclear DNA content of *Musa*.

Nuclear DNA content of all *M. acuminata* genotypes was found to be higher than

that of *M. balbisiana*. This difference was statistically highly significant ($P = 0.001$).

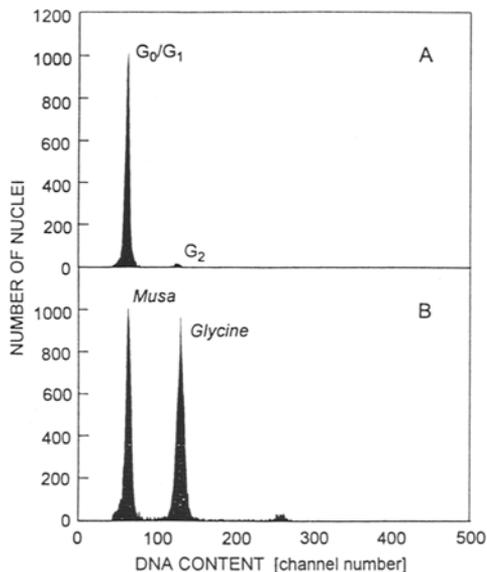


Fig. 1. Histogram of relative nuclear DNA content. A - The nuclei isolated from young leaf of *M. acuminata* ssp. *errans*. Most of nuclei are in $G_{0/1}$ phase of the cell cycle and form a large peak at channel 64. B - The nuclei isolated from *M. acuminata* ssp. *errans* and *G. max* cv. "Polanka". The nuclei were isolated, stained and analysed simultaneously. The first peak on the left represents $G_{0/1}$ nuclei of *Musa* while the second peak represents $G_{0/1}$ nuclei of *Glycine*.

However, statistically highly significant difference ($P = 0.001$) was found also between 2C nuclear DNA content of *M. acuminata* subsp. *banksii* and the other three genotypes of *M. acuminata*. These genotypes (*M. acuminata* subsp. *errans*, diploid cultivars "Pisang Mas" from Australia and "Pisang Mas" from Malaysia) had very similar 2C DNA content and the differences between them were not significant at $P = 0.05$ level (Table 1).

Table 1. 2C nuclear DNA content (mean \pm S.D.) and genome size of *Musa* genotypes estimated by flow cytometry*.

Genotype	Genome	2C DNA content [pg]	CV[%]	Genome size [Mbp]**
<i>M balbisiana</i>	BB	1.14 \pm 0.03	2.57	552
<i>M. acuminata</i> ssp. <i>banksii</i>	AA	1.23 \pm 0.02	1.48	593
<i>M. acuminata</i> cv. "Pisang Mas" (Australia)	AA	1.25 \pm 0.02	1.62	605
<i>M. acuminata</i> ssp. <i>errans</i>	AA	1.26 \pm 0.02	1.72	606
<i>M. acuminata</i> cv. "Pisang Mas" (Malaysia)	AA	1.26 \pm 0.01	1.14	607

**Glycine max* cv. "Polanka" (2C DNA content = 2.50 pg) was used as an internal reference standard

**1 pg DNA = 965 Mbp (Bennett and Smith 1976).

Discussion

The current investigation proved that flow cytometry may be used for rapid and convenient analysis of nuclear DNA content in *Musa*. The chopping technique described by Galbraith *et al.* (1983) was used successfully for isolation of intact *Musa* cell nuclei. However, optimum results (low CV of G_{0/1} peaks and low background) were obtained only when using young leaf tissues. Furthermore, the use of the LB01 buffer that contains mercaptoethanol as a reducing agent (Doležel *et al.* 1989) was found to be important to prevent browning of suspensions of nuclei due to phenolic compounds.

As the nuclear DNA content of G_{0/1} cells reflects the ploidy status of a plant, the technique may be used for rapid estimation of ploidy level. Considering the difficulties encountered in application of classical karyological techniques in *Musa*, flow cytometry may be a powerful alternative. The validity of this approach was proved *e.g.* by De Laat *et al.* (1987) who used flow cytometry for determination of ploidy and breeding of sugar beet.

Propidium iodide was used as a DNA stain throughout this study because it quantitatively intercalates DNA without base specificity (Crissman and Steinkamp 1990), which could otherwise bias the results when comparing DNA content of genotypes with unknown AT/GC ratios. Propidium iodide can be used as a DNA-specific stain following pretreatment of nuclei with RNase and was shown to be suitable for flow cytometric determinations of absolute DNA amounts both in animals (Tiersch *et al.* 1989) as well as plants (Michaelson *et al.* 1991, Dickson *et al.* 1992, Doležel *et al.* 1992).

To estimate nuclear DNA content in absolute units by flow cytometry, an internal reference standard is used (Doležel 1991). After a preliminary screening, we have chosen *G. max*. Its genome size is not very different from that of *Musa* which is important to minimise the risk of the zero level error (Vindeløv *et al.* 1983), on the other hand, it is high enough to allow the analysis of DNA content also in economically important triploid *Musa* cultivars (Afza *et al.* 1992).

The analysis of DNA content revealed that *Musa* belongs to genera at the low end of the range of known plant genome sizes (Bennett and Smith 1976, 1991, Bennett *et al.* 1982). Our estimation of haploid genome size in several *Musa* genotypes (range 552 - 607 Mbp) is significantly lower than the value of 873 Mbp estimated previously (Arumuganathan and Earle 1991). However, as the 2C DNA content of 1.81 pg DNA estimated by these authors is about 50 % higher than our estimations we believe that the discrepancy could be explained by the fact the authors did not analyse diploid but a triploid plant (most of cultivated clones are triploid). The fact that the A and the B genomes of *Musa* differ in size should permit estimation of nuclear genome composition in triploid *Musa* clones with expected hybrid composition. We have used successfully flow cytometry to estimate genome composition in *Brassica* interspecific hybrids (Sabharwal and Doležel 1993). Smaller differences in genome sizes were found also among subspecies of *Musa acuminata* (genome A). These differences might reflect evolutionary changes of the A genome

(e.g. amplification of repetitive DNA) since their separation from a common ancestor (Moore *et al.* 1993).

To conclude, flow cytometry has been shown to be a useful tool for rapid and accurate estimation of nuclear DNA content in *Musa*. The small amount of leaf tissue needed for the analysis will allow to estimate ploidy and/or nuclear genome size even in small plantlets without destroying them. The method should find application in various fields ranging from *Musa* taxonomy to *Musa* breeding and biotechnology.

References

- Afza, R., Kamer, D., Doležel, J., Konig, J., Van Duren, M., Kahl, G., Novák, F.J.: The potential of nuclear DNA flow cytometry and DNA fingerprinting for *Musa* improvement programmes. - In: Wills, B. (ed.): Proc. Int. Symp. Breeding Banana and Plantain for Resistance to Diseases and Pests. Pp. 65-75. CIRAD, Montpellier 1992.
- Arumuganathan, K., Earle, E.D.: Nuclear DNA content of some important plant species. - Plant mol. Biol. Rep. 9: 208-218, 1991.
- Bennett, M.D., Smith, J.B.: Nuclear DNA amounts in angiosperms. - Phil. Trans. roy. Soc. London B 274: 227-274, 1976.
- Bennett, M.D., Smith, J.B.: Nuclear DNA amounts in angiosperms. - Phil. Trans. roy. Soc. London B 334: 309-345, 1991.
- Bennett, M.D., Smith, J.B., Heslop-Harrison, J.S.: Nuclear DNA amounts in angiosperms. - Proc. roy. Soc. London 216: 179-199, 1982.
- Crissman, H.A., Steinkamp, J.: Cytochemical techniques for multivariate analysis of DNA and other cellular constituents. - In: Melamed, M.R., Lindmo, T., Mendelsohn, M.L. (ed.): Flow Cytometry and Sorting. 2nd Edition. Pp. 227-247. Wiley-Liss, New York 1990.
- Dean, P.N., Jett, J.H.: Mathematical analysis of DNA distributions derived from flow microfluorometry. - J. Cell Biol. 60: 523-527, 1974.
- De Laat, A.M.M., Gohde, W., Vogelzang, M.J.D.C.: Determination of ploidy of single plants and plant populations by flow cytometry. - Plant Breed. 99: 303-307, 1987.
- Dickson, E.E., Arumuganathan, K., Kresovich, S., Doyle, J.J.: Nuclear DNA content variation within the *Rosaceae*. - Amer. J. Bot. 79: 1081-1086, 1992.
- Doležel, J.: FLOWSTAR: A microcomputer program for flow cytometric data manipulation and analysis. - Biológia (Bratislava) 44: 287-291, 1989.
- Doležel, J.: Flow cytometric analysis of nuclear DNA content in higher plants. - Phytochem. Anal. 2: 143-154, 1991.
- Doležel, J., Binarova, P., Lucretti, S.: Analysis of nuclear DNA content in plant cells by flow cytometry. - Biol. Plant. 31: 113-120, 1989.
- Doležel, J., Sgorbati, S., Lucretti, S.: Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. - Physiol. Plant. 85: 625-631, 1992.
- Fox, M.H., Galbraith, D.W.: Application of flow cytometry and sorting to higher plant systems. - In: Melamed M.R., Lindmo T., Mendelsohn, M.L. (ed.): Flow Cytometry and Sorting. 2nd Edition. Pp. 633-650. Wiley-Liss, New York 1990.
- Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E.: Rapid flow cytometric analysis of the cell cycle in intact plant tissues. - Science 220: 1049-1051, 1983.
- Givan, A.L., Shenton, B.K., Carr, T.W.: A correction required for calculation of DNA ratios in flow cytometric analysis of ploidy. - Cytometry 9: 271-274, 1988.
- Hammatt, N., Blackhall, N.W., Davey, M.R.: Variation in the DNA content of *Glycine*. - J. exp. Bot. 42: 659-665, 1991.

- Michaelson, M.J., Price, H.J., Ellison, J.R., Johnston, J.S.: Comparison of plant DNA contents determined by Feulgen microspectrophotometry and laser flow cytometry. - *Amer. J. Bot.* **78**: 183-188, 1991.
- Moore, G., Gale, M.D., Kurata, N., Flavell, R.B.: Molecular analysis of small grain cereal genomes: current status and prospects. - *Bio/Technology* **11**: 584-589, 1993.
- Sabharwal, P.S., Doležel, J.: Interspecific hybridization in *Brassica*: Application of flow cytometry for analysis of ploidy and genome composition in hybrid plants. - *Biol. Plant.* **35**: 169-177, 1993.
- Simmonds, N.W., Shepherd, K.: The taxonomy and origins of the cultivated bananas. - *J. Linnean Soc. Bot.* **55**: 302-312, 1955.
- Tiersch, T.R., Chandler, R.W., Wachtel, S.S.M., Elias, S.: Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. - *Cytometry* **10**: 706-710, 1989.
- Vindeløv, L.L., Christensen, I.J., Nissen, N.I.: Standardisation of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. - *Cytometry* **3**: 328-331, 1983.