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Plant Genome Size Measurement with DNA Image Cytometry

BARBARA VILHAR*†, JOHANN GREILHUBER‡, JASNA DOLENC KOCE†, EVA MARIA TEMSCH‡ and MARINA DERMASTIA†

†*University of Ljubljana, Biotechnical Faculty, Department of Biology, Večna pot 111, 1000 Ljubljana, Slovenia and* ‡*Institute of Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria*

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To test the reliability of DNA image cytometry for the measurement of nuclear DNA content in plant material, we conducted independent experiments in two laboratories using different image analysis instruments for densitometric measurement of nuclear DNA amount in Feulgen-stained squash preparations of root tips. The 2C nuclear DNA content of the nine species studied spanned a 100-fold range (approx. 0·3–33 pg). The estimates of nuclear DNA content measured with image cytometry methods were comparable to values obtained previously using both photometric cytometry and flow cytometry. Image cytometry methods showed little variation among repeated experiments within each laboratory or among different operators using the same instrument. Furthermore, the interphase-peak method (measurement of several hundred interphase nuclei per slide) was comparable to the classical prophase/telophase approach (measurement of ten early prophase and ten late telophase nuclei per slide). Hence, DNA image cytometry gives accurate and reproducible results and may be used as an alternative to photometric cytometry in plant nuclear DNA content measurements. In the present study, we propose that two standards for quality control of nuclear DNA content measurement are used in plant DNA image cytometry: (1) the coefficient of variation of the peak should be lower than 6 %, and (2) the 4C/2C ratio should be between 1·9 and 2·1.

Key words: DNA image cytometry, nuclear DNA content, genome size, quality control standards for slides, prophase/telophase method, interphase-peak method.

INTRODUCTION

The most widely used cytometric methods for nuclear DNA content measurement rely on quantitative staining of DNA molecules *in situ*. The methods used for such measurements can be classified into two groups, fluorometry and densitometry. Fluorometry relies on staining of DNA with fluorochromes, such as propidium iodide, and the amount of DNA is estimated from the amount of emitted light measured with a microscope photometer or, more frequently, with a flow cytometer (FC; Doležel, 1997; Doležel *et al*., 1998; Johnston *et al*., 1999). Densitometry, on the other hand, comprises methods which deduce DNA quantity in the nucleus from the optical density of the stained regions. In this case, the most common and reliable procedure for DNA staining is the Feulgen reaction (Feulgen and Rossenbeck, 1924). The classical densitometric method, which has been used in botany for over 50 years (Swift, 1950), involves measurement of optical density with instruments that combine a microscope with a photometer and have been variously referred to as microdensitometers, cytophotometers and microspectrophotometers. The photometer can now be replaced with an image analysis system which grabs images from the microscope via a video or digital camera, and calculates optical density from the grey values of pixels in the nucleus. Given that this new technology may soon become widely accepted in plant science, we propose that the classical method is referred to as photometric cytometry (PC), in contrast to the computer-based image cytometry (IC).

Whereas FC and PC are both well established methods for plant nuclear DNA content measurement (Bennett and Leitch, 1995; Doležel *et al*., 1998), IC has received less attention and has only rarely been used in botany (Temsch *et al*., 1998; Dimitrova *et al*., 1999). In particular, the published data give very fragmentary evidence that IC gives results comparable to FC and PC, and only for a few plant species with a restricted range of C-values.

In contrast, DNA IC has gained importance in medical science for assistance in diagnosis and grading of malignant tumors, and stringent international standards have been proposed and set for quality control of instrumentation (Böcking *et al*., 1995; Puech and Giroud, 1999) as well as quality control of DNA content measurement in individual microscope slides (Kindermann and Hilgers, 1994; Böcking *et al*., 1995). Presently, no internationally recognized botanical standards exist that can be consulted in these matters. However, from the standards for accuracy of medical DNA IC, several criteria can be adopted directly for the measurement of C-values in general, and should be considered when measuring plant material.

The important steps in nuclear DNA content measurement in plants are standardized procedures for DNA staining and measurement, as well as the choice of the standard species. Ideally, the standard species should give reliable results with different methods for nuclear DNA

* For correspondence. Fax: +386 1 257 33 90, e-mail barbara.vilhar@uni-lj.si

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content measurement. The attributes of a good standard are negligible variation among cultivars or populations, low variation among measurements with different methods and/or in different laboratories, low variation among samples or individuals in one experiment, low variation of nuclear DNA content readings for nuclei falling within the same C-value class in one sample, and low proportionality error (4C/2C ratio in one sample close to 2·00). Additional criteria that a standard should fulfil are non-problematic staining with fluorochromes and the Feulgen reaction, ease of sample preparation, fairly homogeneous chromatin, ease of growing and availability of seed material (Bennett and Smith, 1976; Johnston *et al*., 1999). Finally, one standard species will have to be chosen to act as the calibration standard upon which all other standards are calibrated. This species needs to fulfil an additional criterion of intermediate C-value. Presently, the most likely candidate to be generally recognized as the calibration standard is *Pisum sativum* (Doležel *et al*., 1998; Johnston *et al*., 1999). In the past, *Allium cepa* has frequently served as the calibration standard (Van't Hof, 1965; Bennett and Smith, 1976). Given that most plant species have a much lower C-value (Bennett *et al*., 1998), this taxon appears to us less optimal for that purpose than *P. sativum*. However, the absolute DNA amount of the *Pisum sativum* genome has not yet been determined by chemical methods, while that of *A. cepa* has been (Van't Hof, 1965) and fits well to chemically determined human and animal C-values when compared by cytophotometry (Greilhuber *et al*., 1983).

To test the reliability of IC for measurement of nuclear DNA content in a wide range of plant species, we conducted independent experiments in two laboratories using different IC instruments, and results were compared to those obtained with both FC and PC (Doležel *et al*., 1998). The 2C nuclear DNA content of the measured species spanned a 100-fold range (approx. 0·3–33 pg DNA), and several of the species included in the study are among the candidates for the standard species in C-value measurement.

Moreover, a different general approach to the measurement of nuclear DNA content was used in each laboratory. In one laboratory, ten early prophase and ten late telophase nuclei were measured per slide; this is an accepted method in PC and has also been used in IC (Temsch *et al*., 1998; Dimitrova *et al*., 1999). In the other laboratory, several hundred interphase nuclei were measured per slide, revealing integrated optical density (IOD) peaks similar to those obtained with FC, and the 2C and 4C nuclear DNA content was calculated from the position of the two peaks. Although similar approaches have occasionally been used to measure nuclear DNA content in problematic plant material (e.g. the epidermal peeling technique by Price *et al*., 1980; Hörandl *et al*., 2000), this is the first attempt to demonstrate systematically that the interphase-peak method can be used in Feulgen densitometry as an alternative to the prophase/telophase method and that it gives results comparable to other methods.

To our knowledge, the present study is the first inter-laboratory comparison of plant DNA densitometry.

MATERIALS AND METHODS

Plant material

Nuclear DNA content was measured in eight plant species: *Allium cepa* L. 'Alice', *Vicia faba* L. 'Inovec', *Secale cereale* L. 'Dankovske', *Hordeum vulgare* L. 'Ditta', *Zea mays* L. line CE-777, *Glycine max* (L.) Merr. 'Polanka', *Raphanus sativus* L. 'Saxa' and *Arabidopsis thaliana* (L.) Heynh. 'Columbia', using the ninth species *Pisum sativum* L. 'Ctirad' as the calibration standard. All seeds were the gift of Dr J. Doležel (Olomouc) and were received as certified seed lots from breeders responsible for maintenance breeding of respective cultivars, with the exception of *A. thaliana*, which was donated by Dr E. Chytilová (Masaryk University, Brno). Seeds were from the same cultivars and came from the same source as those used in Doležel *et al*. (1998). Seeds were germinated at room temperature on plastic germinating plates and young seedling roots were harvested and fixed.

Experimental design

Two laboratories participated in the project. Both laboratories measured nuclear DNA content in eight plant species with DNA image cytometry, using *P. sativum* as the calibration standard against which the 2C and the 4C ratios for the other species were estimated. Each laboratory used established methods for nuclear DNA content measurement specific to the laboratory (Table 1). Quantitative DNA staining was conducted by treating test species strictly in parallel in the same test tube. The 2C and 4C nuclear DNA contents were measured for each species.

DNA image cytometry with the CIRES software package

Root tips were fixed in methanol : acetic acid (3 : 1; MAA fixative) or 4 % buffered formaldehyde (1·5 h, then rinsed in MAA) and stored in ethanol at −20 °C. The procedures described in Greilhuber and Ebert (1994) were followed for Feulgen staining. Root tips were hydrolysed in 5 M HCl at 20·0 °C for 60 min (MAA fixative) or 75 min (formaldehyde), and stained in Schiff's reagent for about 24 h in a refrigerator. After rinsing the material for 45 min at room temperature in SO_2 water, the meristems were squashed onto glass slides, the coverslips removed over a cold plate, and the slides air dried.

The microscope was a Zeiss Axioskop with a stabilized light source. A neutral density filter and a green interference filter were used. Measurements were done under a 63 × 1·25 oil iris immersion objective. The green channel of the camera (Sony 3CCD colour camera, model DXC-930P with adaptor CMA-D2, Tokyo, Japan) was used to grab images (frame grabber KONTRON Smart DFC 500). The CIRES software package (release 3·1, KONTRON, Munich, Germany) was used to process the images with local background determination, i.e. the nucleus was segmented and light intensity of the reference background was automatically determined from a narrow blank zone surrounding the nucleus. The images were corrected for uneven illumination of the field of view (shading

correction). Integrated optical density (IOD) was measured for ten early prophase and ten late telophase nuclei per slide.

In each experiment, three slides per species were measured against three slides of *P. sativum*. For each of the fixatives, the experiment was repeated four times by one researcher, and twice by another.

DNA image cytometry with the KS 400 software package

Root tips were fixed in ethanol : acetic acid (3 : 1) for 24 h at 4 °C and then stored in 96 % ethanol at −20 °C. The procedures described in Greilhuber and Ebert (1994) were followed for Feulgen staining (hydrolysis in 5 M HCl, 60 min, 20·0 °C) and preparation of squash microscope slides. After removal of the cover slips, the slides were briefly washed in ethanol, air-dried for 1 h and mounted in DPX (Fisons, Ipswich, UK).

The image analysis system consisted of a light microscope (Axioskop MOT, Carl Zeiss, Jena, Germany) connected to a personal computer via a colour CCD camera (Sony DXC-950P). A green filter was inserted in the light path (band pass interference filter, 540 nm, width at half-peak transmission 90 nm) and Köhler illumination was used, with objective magnification 40× (Plan-Neofluar, Carl Zeiss). Grey images were grabbed using the green camera channel with the frame grabber Matrox Meteor (image size 760 × 560 pixels) and processed using a macro based on the software package KS 400 version 3·0 (Carl Zeiss Vision), which has been developed in the laboratory at the University of Ljubljana, Slovenia. The system was densitometrically calibrated prior to each measurement session with a set of neutral density filters. The images of nuclei were averaged (32 sequential images), and corrected for uneven background illumination and glare. Integrated optical density (IOD) was measured in several hundred interphase nuclei per slide (excluding the dense mitotic figures from late prophase to early telophase). The 2C and 4C values as well as an estimate of the coefficient of variation of the peak (CV_p) were calculated from IOD cumulative frequency plots.

For one experiment, slides of all species were measured in one measurement session. The experiment was repeated twice, with up to five slides measured per species.

Measurement units for nuclear DNA content

With both software packages, 2C and 4C nuclear DNA contents were measured in arbitrary IOD units. The nuclear DNA content ratios against *P. sativum* (the calibration standard) were expressed as a percentage of the 2C nuclear DNA content of *P. sativum*. Where estimates of nuclear DNA content are given in pg DNA, *P. sativum* was used as the calibration standard at 2C = 8·84 pg DNA (Greilhuber and Ebert, 1994).

Quality control standards for slides

Due to lack of international botanical quality control standards, two medical standards for DNA image cytometry were adopted and tested in the present study. The first criterion requires that the coefficient of variation for the nuclei on each slide which fall into the 2C or the 4C group must not exceed 6 % (Böcking *et al.*, 1995). The second criterion involves the deviation of the ratio between the 4C and 2C value from the ideal value 2·0; this proportionality error should be lower than 5 %, i.e. the 4C/2C ratio should be between 1·9 and 2·1 (Kindermann and Hilgers, 1994).

Statistical analysis

Standard statistical procedures were used as indicated in the Results and Discussion.

RESULTS AND DISCUSSION

The prophase/telophase method and the interphase-peak method

The difference in the general approach to measurement of nuclear DNA content in the two laboratories is illustrated in Fig. 1 and Table 1. With the prophase/telophase method, ten prophase and ten telophase nuclei were selected and measured per slide, and the mean of the telophase and the prophase classes of nuclei was calculated as the 2C and 4C nuclear DNA content, respectively. With the interphase-peak method, several hundred nuclei were measured per slide, and the 2C and 4C nuclear DNA content was calculated from the position of the steepest slope of the cumulative frequency plots. The medical guidelines applied

TABLE 1. *Experimental design*

Laboratory	Abbreviation	Fixative	Software	Measured nuclei on a slide	Operator
1	CIRES/MAA	Methanol/acetic acid (3 : 1)	CIRES	10 early prophase 10 late telophase	A, B
1	CIRES/formaldehyde	4 % buffered formaldehyde	CIRES	10 early prophase 10 late telophase	A, B
2	KS 400	Ethanol/acetic acid (3 : 1)	KS 400	Several hundred interphase	C

For each of the two participating laboratories, the type of fixative, the type of software and the method for selection of measured nuclei are shown. Operator: in laboratory 1, operators A and B prepared and measured the slides independently; in laboratory 2, one operator C prepared and measured the slides.

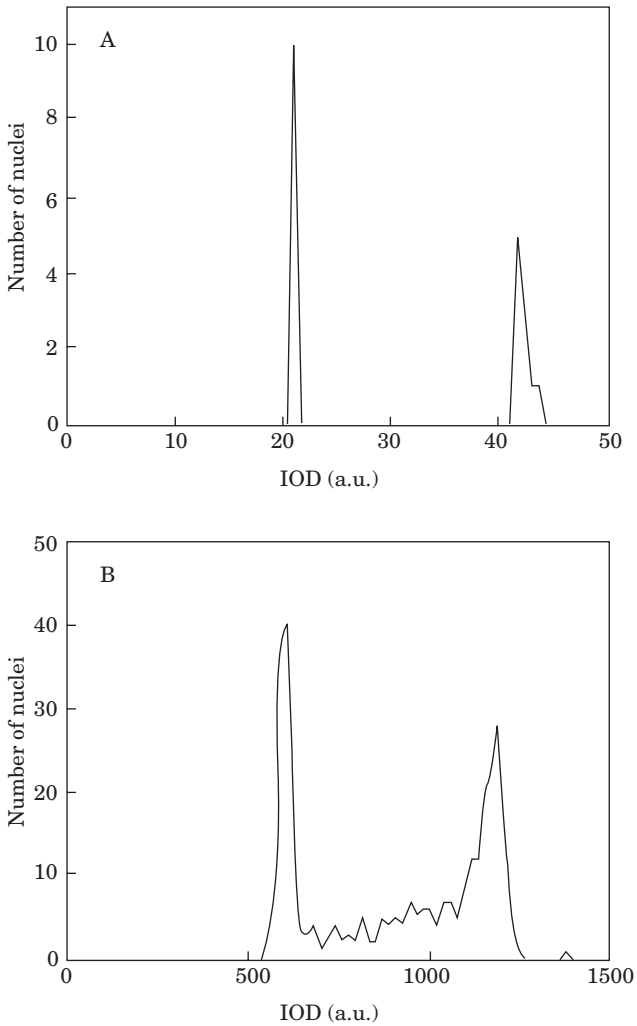


FIG. 1. The prophase/telophase method and the interphase-peak method. A microscope slide of a *Pisum sativum* root tip was prepared and measured in each of the two laboratories. The graph shows IOD frequency polygons obtained with the prophase/telophase method (ten early prophase and ten late telophase nuclei; CIRES/formaldehyde; A) and with the interphase-peak method (323 nuclei; KS 400; B).

in this study correspond methodologically to the interphase-peak method (see below).

Quality control standards for slides

For all methods used to measure nuclear DNA content, including IC used in the present study, it is essential that all the steps of the experiment are optimized, from fixation of the plant material to DNA staining and the measurement procedure itself. Given that no botanical guidelines are available for evaluation of DNA IC measurement, we relied on medical quality control standards (Kindermann and Hilgers, 1994; Böcking *et al*., 1995). Hence, the width of the 2C peak expressed as the coefficient of variation of the peak (CVp; Fig. 2) and the proportionality error (discrepancy in 4C/2C ratio; Fig. 3) were examined in all slides.

For measurements using the CIRES software (the prophase/telophase method), the 2C CVp corresponded

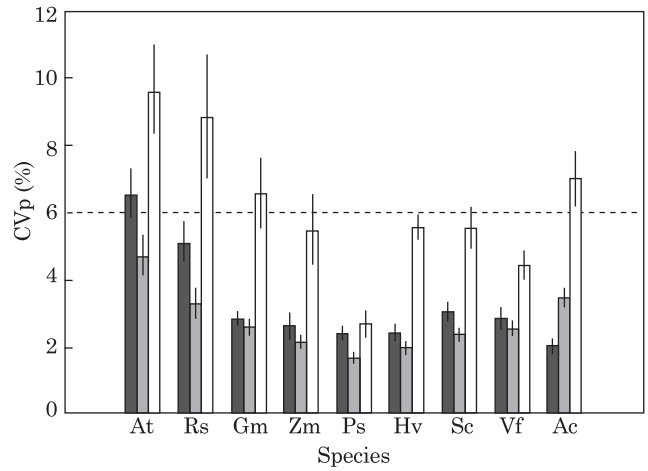


FIG. 2. Variation of 2C nuclear DNA content measurement within the sample, expressed as the coefficient of variation of the 2C peak (2C CVp). Results for the three IC methods [CIRES/MAA (■), CIRES/formaldehyde (▒) and KS 400 (□)] are shown for the nine plant species (mean CVp ± SEM). CIRES/MAA and CIRES/formaldehyde, 12 slides per bar ($N = 12$); KS 400, all slides of experiments 1 and 2 (N as indicated in Table 3). At, *Arabidopsis thaliana*; Rs, *Raphanus sativus*; Gm, *Glycine max*; Zm, *Zea mays*; Ps, *Pisum sativum*, Hv, *Hordeum vulgare*; Sc, *Secale cereale*, Vf, *Vicia faba*; Ac, *Allium cepa*. The species are ordered from the smallest 2C nuclear DNA content (left) to the largest 2C nuclear DNA content (right).

to the CV of ten telophase nuclei (Fig. 2). For the KS 400 software (the interphase-peak method), the CVp was calculated from the IOD cumulative frequency plot using standard statistical procedures (Fig. 2).

The general pattern of the CVp for different species was similar regardless of the measurement software or the fixative used. Thus, low CVp values were recorded in the middle C-value range, from *G. max* to *V. faba*, while higher CVp values were typical of species with small nuclear DNA contents (*R. sativus*, *A. thaliana*) as well as for *A. cepa* with the largest C-value (Fig. 2).

For most of the species studied, the 2C CVp was close to or less than 6 % (Fig. 2), which corresponds to the CVp limit recommended by medical standards (Böcking *et al*., 1995). The criterion was not met when *A. thaliana* was fixed in MAA and measured with the CIRES software, and when *A. thaliana* and *R. sativus* were measured with the KS 400 software. With this software package, CVp for *G. max* and *A. cepa* was marginally higher than the standard 6 % limit.

The 4C/2C ratio did not deviate from the ideal 2·00 ratio by more than 5 % in any species (Fig. 3), which is the recommended limit for medical applications (Kindermann and Hilgers, 1994). The only exception to this was a marginally higher 4C/2C ratio for *A. thaliana* measured with the KS 400 software.

The lower 2C CVp values measured with the CIRES software compared to the KS 400 software are probably due to methodological differences in the selection of measured nuclei on a slide. In the case of the CIRES software, only late telophase nuclei were selected, and care was taken to avoid optically defective nuclei. On the other hand, the 2C peak of the KS 400 measurements consists of interphase G1 nuclei and late telophase nuclei, hence the

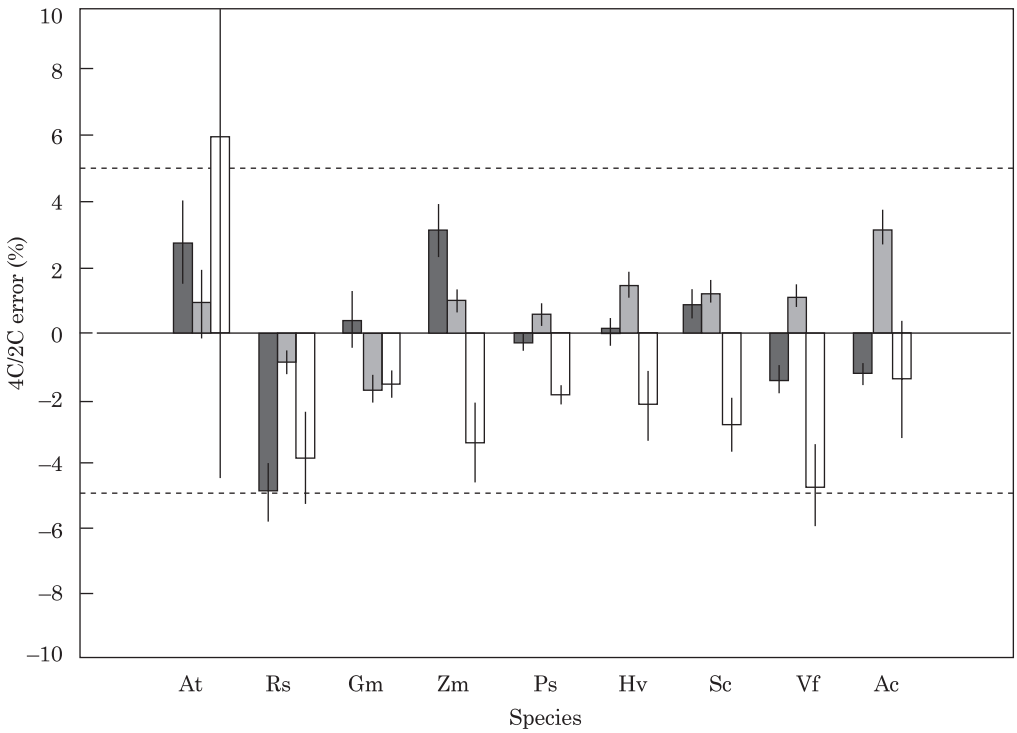


FIG. 3. Proportionality error (deviation of the 4C/2C ratio from the ideal value). Results for the three IC methods. [CIRES/MAA (■), CIRES/formaldehyde (▩) and KS 400 (□)] are shown for the nine plant species (mean 4C/2C error ± SEM). CIRES/MAA and CIRES/formaldehyde, 12 slides per bar ($N = 12$); KS 400, all slides of experiments 1 and 2 (N as indicated in Table 3). At, *Arabidopsis thaliana*; Rs, *Raphanus sativus*; Gm, *Glycine max*; Zm, *Zea mays*; Ps, *Pisum sativum*; Hv, *Hordeum vulgare*; Sc, *Secale cereale*; Vf, *Vicia faba*; Ac, *Allium cepa*. The species are ordered from the smallest 2C nuclear DNA content (left) to the largest 2C nuclear DNA content (right).

measured objects are diverse in terms of optical properties. Likewise, this may be the cause of a higher proportionality error in KS 400 when compared to both CIRES measurements. The nature of this discrepancy in CVp values and proportionality error should be verified by measurements with the interphase-peak and the prophase/telophase method on the same instrument and on the same slides. Should it be proven that the different range of CVp is indeed caused by the selection of nuclei on the slide, more stringent CVp criteria could be set for the prophase/telophase method than for the interphase-peak method.

Based on results for the nine plant species in this study, we propose that the following standards are applied to plant DNA IC: (1) CVp should be less than 6 %, and (2) the 4C/2C ratio should be between 1·9 and 2·1. These proposed standards need to be tested on a wider range of plant species. It is important to bear in mind that medical standards are very stringent and are adapted to properties of the human genome, whereas in botany, various species are measured, some of which have proven to be difficult to measure by methods other than IC. Nevertheless, should the properties of measured slides deviate substantially from the two proposed standards, every care should be taken to rule out any systematic methodological errors prior to acceptance of values measured in such slides as accurate estimates of nuclear DNA content. In such cases, the quality control procedures for instrumentation should be applied (Böcking *et al.*, 1995; Puech and Giroud, 1999) and correction for glare effect may be necessary (Goldstein, 1970; Kindermann and Hilgers, 1994). In this study, software procedures for glare correction were applied in the case of KS 400 measurements and these improved the precision of nuclear DNA content estimates (comparative results of measurement with and without glare correction are not shown).

Comparison of different IC methods

The estimates of 2C nuclear DNA content for the eight plant species, using *P. sativum* as the calibration standard, are shown in Table 2 for the software package CIRES and in Table 3 for KS 400.

Differences in measurement of 2C nuclear DNA content with different IC methods were investigated with regression analysis. For this purpose, one of the IC methods (the CIRES software, material fixed in formaldehyde) was used as the reference method and the other two methods were compared to this. A linear regression line showing correlation between the CIRES/formaldehyde method and the other two IC methods was plotted first (Fig. 4A and B). The regression equation for the CIRES/MAA method was $y = 1{\cdot}01x + 1{\cdot}72$ ($r = 0{\cdot}999$, $P < 0{\cdot}001$) and for the KS 400 method $y = 0{\cdot}92x + 4{\cdot}41$ ($r = 0{\cdot}997$, $P < 0{\cdot}001$). The point representing the calibration standard *P. sativum*, set at 100 % for all measurements, was excluded from regression analysis.

The linear regression analysis method has previously been used for inter-method comparison of C-value measurements (Doležel *et al.*, 1998; Johnston *et al.*, 1999).

TABLE 2. *Estimates of 2C nuclear DNA amounts with the CIRES software package after fixation in MAA and formaldehyde*

Species	Fixative: MAA 2C†	MAA SEM	MAA CV (%)	MAA 2C (pg)	Formaldehyde 2C†	Formaldehyde SEM	Formaldehyde CV (%)	Formaldehyde 2C (pg)	Ratio MAA/formaldehyde
A. thaliana	3·4	0·1	4·1	0·30	3·6	0·0	1·9	0·32	0·960 (ns)
R. sativus	11·2	0·2	3·3	0·99	12·9	0·3	5·2	1·14	0·873 (**)
G. max	26·3	0·3	2·6	2·32	26·3	0·7	5·4	2·32	0·999 (ns)
Z. mays	59·2	0·5	1·6	5·24	56·4	0·2	0·8	4·98	1·051 (**)
P. sativum‡	100·0	0·7	0·7	8·84	100·0	0·5	0·8	8·84	
H. vulgare	115·1	0·6	1·1	10·18	108·9	1·8	3·3	9·63	1·057 (*)
S. cereale	179·5	1·0	1·1	15·87	167·0	1·7	2·0	14·74	1·075 (***)
V. faba	295·3	6·7	4·5	26·10	293·5	7·1	4·9	25·94	1·006 (ns)
A. cepa	391·8	3·5	1·8	34·68	388·4	12·6	6·5	34·34	1·009 (ns)

In each experiment, three slides of each species were measured against three slides of *P. sativum* (ten late telophase nuclei per slide). The experiment was repeated four times for each fixative (total of 12 slides per species per fixative). The mean 2C nuclear DNA content, SEM and CV were calculated for the mean of the four repeats ($N = 4$). The 2C values were converted to estimates in pg DNA using *P. sativum* 2C = 8·84 pg as the standard value.

Ratio MAA/formaldehyde, Ratio between 2C values measured after fixation in MAA and in formaldehyde. The level of statistical significance of the difference between the two fixatives (*t*-test performed over the means of the four repeats of the experiment, $N = 4$ for each fixative) is indicated in parenthesis (ns, not significant; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$).

† The 2C values are given as a percentage of the 2C nuclear DNA content of *P. sativum*.

‡ For *P. sativum*, the mean 2C value, SEM and CV recorded in one measurement session are shown (number of slides, $N = 3$).

TABLE 3. *Estimates of 2C nuclear DNA amounts with the KS 400 software package*

Species	Number of slides: Expt 1	Number of slides: Expt 2	Number of nuclei	2C*	SEM	CV (%)	2C (pg)
A. thaliana	4	2	88	3·8	0·6	21·5	0·33
R. sativus	3	5	236	11·5	0·7	8·7	1·02
G. max	3	5	252	26·5	0·3	1·7	2·34
Z. mays	4	5	207	60·1	0·9	2·1	5·32
P. sativum†	4	5	241	100·0	0·3	0·7	8·84
H. vulgare	4	5	387	104·0	1·1	1·5	9·19
S. cereale	4	5	285	166·7	4·2	3·6	14·74
V. faba	3	5	243	289·5	0·5	0·3	25·59
A. cepa	4	4	251	348·7	16·6	6·7	30·83

The mean 2C nuclear DNA content, SEM and CV were calculated for the mean of two repeats of the experiment ($N = 2$). The 2C values were converted to estimates in pg DNA using *P. sativum* 2C = 8·84 pg as the standard value. Number of slides, Number of slides measured per species in each of the two repeats of the experiment (experiment 1, experiment 2) are indicated. Number of nuclei, Mean number of nuclei measured per slide.

* The 2C values are given as a percentage of the 2C nuclear DNA content of *P. sativum*.

† For *P. sativum*, the mean 2C value, SEM and CV recorded in one measurement session are shown (experiment 1, number of slides $N = 4$).

However, Fig. 4 shows that the linear regression model may not be the most appropriate method by which to study such correlations. The discrepancy between the linear regression prediction values and real observations (measurements) is particularly acute in the case of KS 400, where the relatively low measurement value for *A. cepa* is overemphasized and markedly tilts the slope of the regression line, which in turn completely misses some of the points, especially in the low C-value range (*A. thaliana* by 106 %, *R. sativus* by 41 %, *G. max* by 8 %; Fig. 4B). Moreover, the intercept of the regression line at 4·4 % of the 2C value for *P. sativum* predicts an offset error (corresponding to approx. 0·39 pg DNA), which is higher than the measured value with KS 400 software for *A. thaliana* at 3·8 %. Again, a closer scrutiny of the regression graph in the low nuclear DNA range reveals that no such offset error exists. While the 2C measurement for the smallest of the genomes included in this study, *A. thaliana*, is 3·6 % for the CIRES/MAA method, the KS 400 software estimated this value to be 3·8 % of *P. sativum* 2C, and the real discrepancy between the methods at this measurement point is only 5 %.

To overcome such regression inconsistencies, we re-plotted the regression graphs, this time transforming the data logarithmically (Fig. 4C and D). For each nuclear DNA content value, the transformed value was calculated as ln(value in % of *P. sativum* 2C/100). This logarithmic regression model *a priori* predicts that the intercept of the regression line lies at the point $x,y(0, 0)$ when converted

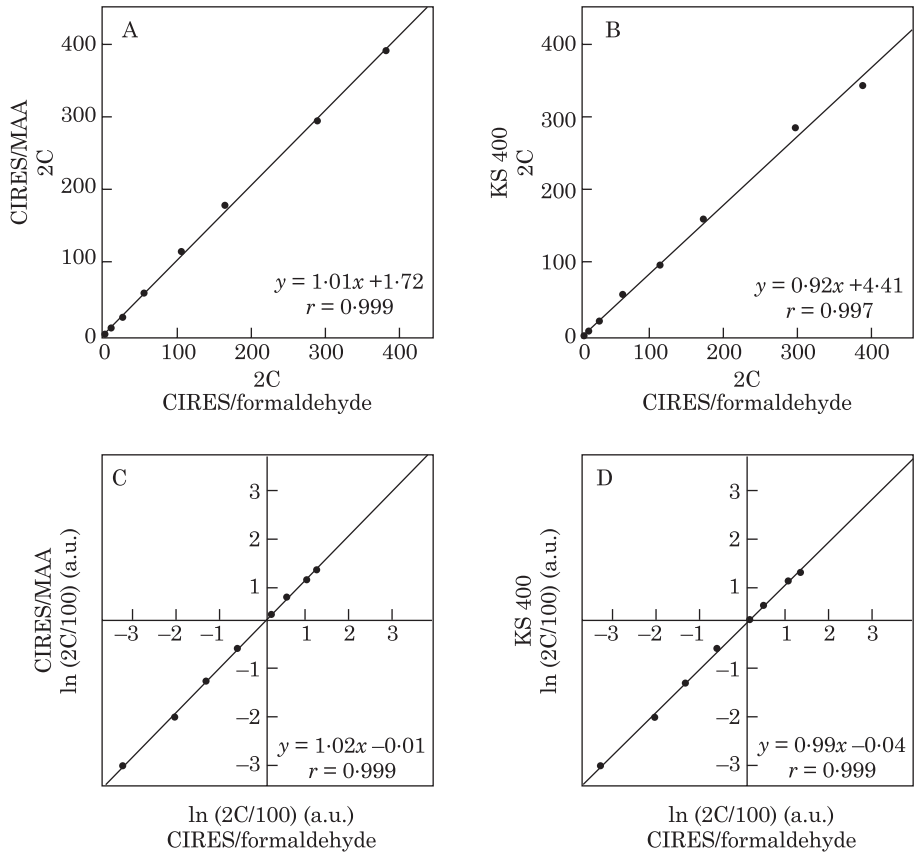


FIG. 4. Comparison of nuclear DNA content estimates measured with three IC methods (CIRES/MAA, CIRES/formaldehyde and KS 400). CIRES/formaldehyde was used as the reference method to which the other two methods were compared (A and C, CIRES/MAA; B and D, KS 400). Regression analysis was performed on raw data (A, B) or on logarithmically transformed data (C, D), using equation ln (2C/100) to calculate the transformed values. The 2C value was expressed as a percentage of the 2C value for *P. sativum*.

back to the linear scale ($y = e^{intercept}x^{slope}$, where e is the base of the natural logarithm). Case studies of regressions comparing IC methods among themselves, as well as comparing IC *vs* FC and PC methods, reveal that the offset error is indeed very close to zero in all cases studied.

With the logarithmic transformation described above, the calibration standard value for *P. sativum* is re-set at zero i.e. the *P. sativum* 2C value is 100 %, and ln(100/100) is zero. Hence, on each side of the calibration standard set at the zero intercept, we find four relatively evenly spread points corresponding to the other species measured. When a regression line is plotted over the logarithmically transformed data, any deviation from the ideal slope 1·00 now points to a systematical error, such as the measured 2C-values being generally too high for species with high C-values and too low for species with small C-values. Moreover, a high discrepancy in absolute 2C nuclear DNA content units between the compared methods in the high C-value range no longer has an undue influence on the slope of the regression line.

In the example given above, the comparison between the CIRES/formaldehyde and the KS 400 method reveals that when data are logarithmically transformed, the predicted 2C values deviate from the measured 2C values by 3·2 % for *A. thaliana*, 11·9 % for *R. sativus* and 1·6 % for *G. max* (Fig. 4D). The largest discrepancy between the regression-predicted values and the measured values is now 11·9 % for *R. sativus*, a marked improvement on the highest error for the linear regression (106 % for *A. thaliana*). Similar improvements in the accuracy of regression predictions following logarithmic transformation of the data prior to plotting the regression line were observed in all inter-method comparisons that we investigated.

Using the logarithmic-transformation model of regression, the slope of the regression line was 1·02 ($r = 0.999$, $P < 0.001$) when the CIRES/formaldehyde method was compared with KS 400 and 0·99 ($r = 0.999$, $P < 0.001$) when CIRES/formaldehyde and CIRES/MAA methods were compared. Both regression lines indicate a high correlation between the three IC methods studied. Moreover, in most cases, the discrepancy among the methods for nuclear DNA content of individual species was well below 10 % (calculated from data shown in Tables 2 and 3).

When the same software package was used (CIRES), the use of either MAA or formaldehyde as the fixative did not affect measurement accuracy (Figs 5 and 6, and Tables 2 and 3). However, in general, plant material fixed in

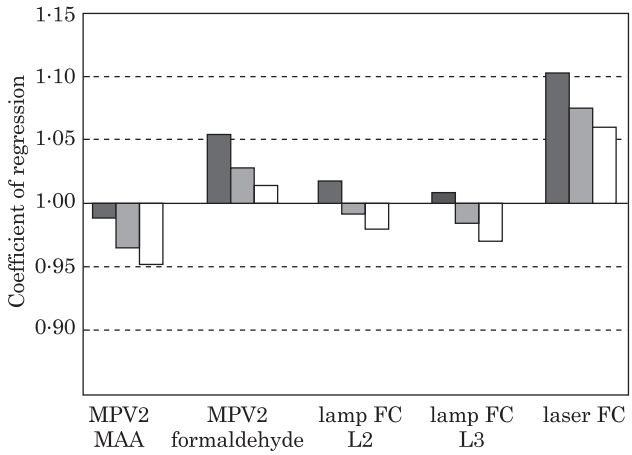


FIG. 5. Comparison of nuclear DNA content estimates measured with the three IC methods [CIRES/MAA (■), CIRES/formaldehyde (▒) and KS 400 (□)] and with PC and FC (data from Doležel *et al.* 1998, as explained in the section 'Comparison of image cytometry with other methods'). Regression analysis was performed on logarithmically transformed data, using equation ln (value in % *P. sativum* 2C/100) to calculate the transformed values. The coefficients of regression (slopes of the regression lines) are shown. PC and FC values were used as predictors in the regression analysis, and IC methods as the response.

formaldehyde gave narrower telophase (Fig. 2) and prophase peaks (data not shown), and this may be due to less tightly condensed chromatin in formaldehyde-fixed nuclei. Hence, formaldehyde is an acceptable fixative for plant material and gives results comparable to the more widely used MAA.

In conclusion, all three IC methods examined, CIRES/MAA, CIRES/formaldehyde and KS 400 gave comparable results (Tables 2 and 3, Fig. 4). Likewise, when measurements with the prophase/telophase method (the CIRES software) are compared to the interphase-peak method (KS 400), a high correlation between the two approaches is observed (Tables 2 and 3, Fig. 4). To our knowledge, the present study is the first systematical demonstration that the interphase-peak method can indeed be applied to plant material, and that this method is comparable not only to other IC methods, but also to PC and FC (Figs 5 and 6). Thus, the interphase-peak method may be reliably used in densitometry of plant material and is also the only densitometric method available when tissues with actively dividing cells are not present in examined tissues.

Reproducibility of results among operators

To investigate the reproducibility of results of plant DNA image cytometry, two operators prepared and measured slides independently, using the same experimental procedures and the same instrument (CIRES/MAA and CIRES/formaldehyde; Table 1).

The results were compared by linear regressions plotted over logarithmically transformed data. For telophase nuclei measured with the CIRES/MAA method, the slope of the regression line was 0·99 ($r = 1·000$), and the maximum and minimum differences between operators were 7·4 % and 0·9 % (*A. thaliana* and *S. cereale*). With the CIRES/formaldehyde method, the slope was 0·98 ($r = 0·999$), while the maximum and minimum differences between operators were 4·8 % and 0·7 % (*A. cepa* and *R. sativus*).

These data demonstrate a high reproducibility of measurements between operators using the same instrument. Moreover, the amount of divergence of results that can be expected with an experimental design essentially comparable to that used in the present study is usually up to 5 %, and with lower C-values up to 8 %.

Comparison of image cytometry with other methods

Nuclear DNA content estimates obtained using IC were compared with those obtained using PC and FC. Data for PC and FC methods were taken from Doležel *et al.* (1998) as the same plant material was used in both studies. Photometric cytometry involved measurements of root tips fixed in MAA or formaldehyde using a microspectrophotometer (Leitz MPV2) (Table 3 in Doležel *et al.*, 1998). For flow cytometry data obtained with two lamp flow cytometers (lamps FC L2 and FC L3 in Table 5 in Doležel *et al.*, 1998) and the mean of the data for two laser flow cytometers (lasers FC L1 and FC L4 in Table 7 in Doležel *et al.*, 1998) were included in the present study. All measurements were converted to a percentage of the 2C value for *P. sativum*.

Regression analysis performed over logarithmically transformed data was used to evaluate differences in nuclear DNA content estimates between the three IC methods (as defined in Table 1) and FC or PC (Fig. 5). The slopes of the regression lines were between 0·95 and 1·05 when the three IC methods and PC were compared. The correlation between all three IC methods and both lamp FC methods was extremely high, with the slope of the regression line being between 0·97 and 1·02.

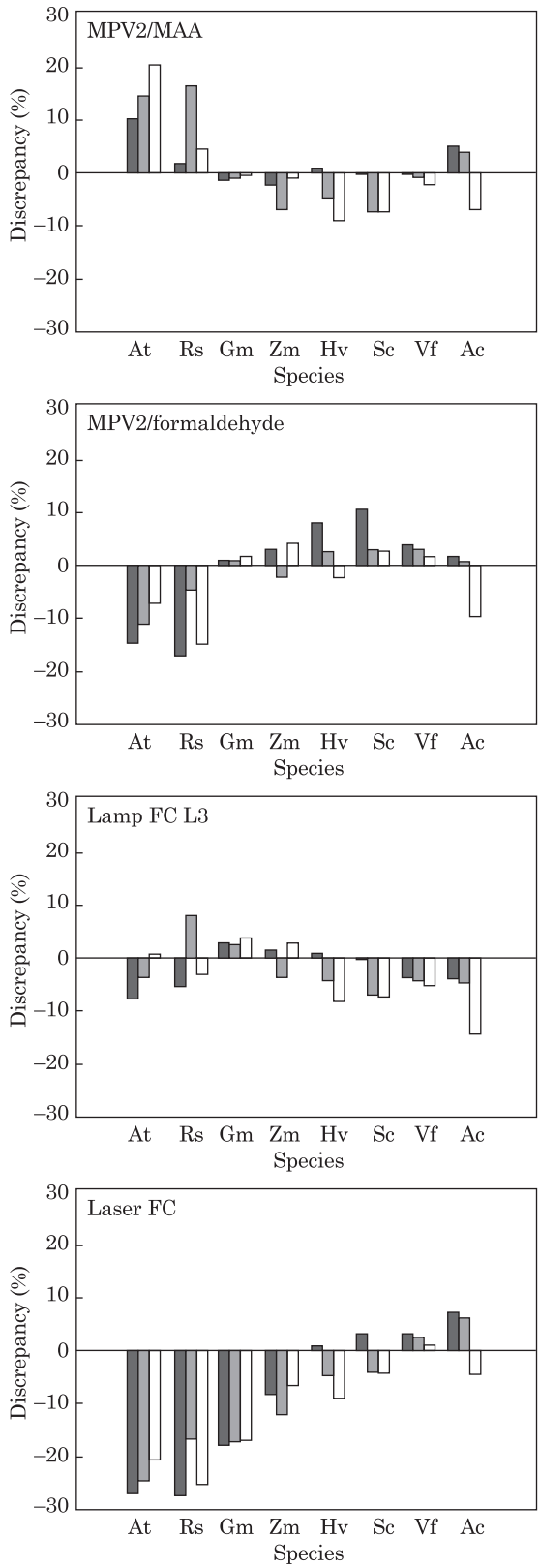
A different situation emerged when the three IC methods were compared to laser FC; the regression slopes were between 1·06 and 1·10 (Fig. 5). These discrepancies point to a systematical error occurring in one or other of the methods compared.

In all the cases mentioned above, the correlation coefficient, r, for inter-method comparisons of the logarithmically transformed data was between 0·998 and 1·000, and regression was highly significant ($P < 0·001$).

Discrepancies among different methods for nuclear DNA content measurement were further investigated by comparison of nuclear DNA content estimates for individual plant species (Fig. 6). Disagreement between any of the IC methods and PC was mostly below 10 % and often below 5 %, except for a higher discrepancy in measurements of the two species with the smallest C-value, *R. sativus* and *A. thaliana*.

The three IC methods also agreed very well with lamp FC measurements, with discrepancies again being less than 10 % in all eight species (Fig. 6). This level of discrepancy between different methods has previously been observed for comparisons between PC and FC (Doležel *et al.*, 1998; Johnston *et al.*, 1999). The IC methods are thus as reliable

as any other method and should be added to the list of accepted methods for nuclear DNA content measurement of plant species.



In fact, the 5 % disagreement among methods seems to be a general limit of accuracy of the present techniques for nuclear DNA content measurement and this should be considered when data are interpreted in terms of absolute DNA units (pg). Nevertheless, a higher accuracy and reproducibility of measurements can be achieved on each individual instrument, particularly with non-problematic plant species.

The only exception to the generally good agreement of IC with other methods was the comparison of IC with laser FC, where a systematical error was detected (Figs 5 and 6). Namely, compared to the laser FC, the three IC methods generally gave higher readings for the two species with the largest C-values, *V. faba* and *A. cepa*, and lower readings for species with smaller C-values (*S. cereale* to *A. thaliana*). Moreover, the discrepancy between IC and laser FC systematically increased with decreasing nuclear DNA content (Fig. 6).

The laser FC has previously been shown to disagree to some extent with both PC and lamp FC methods (Doležel *et al.*, 1998), with the pattern of disagreement being similar to that found here for comparisons between laser FC and IC. We also compared our results to another study in which laser FC was used to measure nuclear DNA content in five of the nine species investigated here (Johnston *et al.*, 1999). Again, a similar pattern of discrepancies emerged (results not shown). Therefore, laser FC measurements seem to deviate from other methods in a systematical way.

Species with high and, in particular, with very low C-values seem to pose problems for all methods. In IC, the very low optical density of the pixels in nuclei of plants with small genomes gives problematic readings against the background and accurate segmentation is hard to achieve. On the other hand, very dark nuclei may also be difficult to measure precisely, as the glare effect increases for dark objects. Furthermore, few grey values are available to measure very dark spots, while in this range a difference of one grey value results in a relatively large difference in optical density due to the logarithmic nature of the calculation of optical density. Similar methodological problems arise when extreme nuclear DNA contents are measured using any of the methods, and measurements in these ranges severely strain the technical capability of the measurement instruments.

In our study, we used *P. sativum* as the primary standard. Several authors have argued that this species is an adequate primary standard for both PC and FC (Greilhuber and Ebert, 1994; Baranyi and Greilhuber, 1995, 1996; Johnston

FIG. 6. Comparison of relative discrepancies in 2C nuclear DNA content estimates measured for eight plant species with the three IC methods [CIRES/MAA (■), CIRES/formaldehyde (▒) and KS 400 (□)] and with PC and FC (data from Doležel *et al.* 1998, as explained in the section 'Comparison of image cytometry with other methods'). The bars show the relative discrepancy between the IC method and PC or FC methods as a percentage. The values for PC and FC were set at 100 for each species. At, *Arabidopsis thaliana*; Rs, *Raphanus sativus*; Gm, *Glycine max*; Zm, *Zea mays*; Ps, *Pisum sativum*; Hv, *Hordeum vulgare*; Sc, *Secale cereale*; Vf, *Vicia faba*; Ac, *Allium cepa*. The species are ordered from the smallest 2C nuclear DNA content (left) to the largest 2C nuclear DNA content (right).

et al., 1999). The use of *P. sativum* gained further support in this study, due to its low CVp (Fig. 2), low proportionality error (Fig. 3) and low variation among samples in one experiment (Tables 2 and 3), indicating that *P. sativum* may indeed be a good standard for the emerging IC method.

CONCLUSIONS

Data show that image cytometry is comparable to both photometric and flow cytometry, given that the instruments and procedures are carefully optimized. After a thorough comparative analysis of different methods, no preference can be given in terms of accuracy to any of the three IC methods examined. Furthermore, the interphase-peak method gives results comparable to the classical prophase/telophase approach.

We hereby demonstrate that DNA IC gives reliable and reproducible results over a range of plant species with different nuclear DNA amounts and nuclear sizes, and with various chromatin properties. Thus we strongly recommend this method to be considered as an alternative to PC and FC in plant nuclear DNA content measurement. Moreover, IC is *per se* a densitometric method, and may soon be the only method of this type available due to gradual retirement of microspectrophotometers (Bennett *et al.*, 2000).

Due to the absence of any botanical guidelines for DNA IC, we adopted two standards from international medical consensus on DNA image cytometry for use in plant nuclear DNA content measurement. These standards have been tested in nine plant species in the present study, and only further scrutiny of the proposed criteria applied to other plant species will reveal the acceptable level of stringency in plant nuclear DNA content measurements.

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