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High-resolution flow cytometry of nuclear DNA in higher plants

Rapid communication

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Summary. High-resolution flow cytometry of nuclear DNA in higher plants has been performed from chopped plant tissues and plant protoplasts. A preparation and staining procedure with the DNA specific fluorochrome DAPI, successfully employed for precise flow cytometric DNA analysis of animal and human cells has been used in a slightly modified manner for the DNA analysis of plant cell material. High-resolution DNA histograms coefficients of variation about 1–1.5% have been obtained routinely from plant species with different DNA content. Staining of nuclei with DAPI in combination with the protein fluorochrome sulforhodamine 101 allows bi-parametric analysis of nuclear DNA and protein. The described simple and precise method might be very promising for the analysis of DNA in basic and applied cytogenetic investigations of plant cell research.

Keywords: Higher plants; Nuclear DNA; Nuclear protein; Flow cytometry; DAPI; SR 101.

Abbreviations: CV coefficient of variation; DAPI 4′,6-diamidino-2-phenylindole; SR 101 sulforhodamine 101.

Introduction

Shortly after the introduction of flow systems in quantitative cytometry the first use of this new technique for DNA analysis in plant cells was done by F. O. Heller (1973). But nearly one decade later flow cytometry became more interesting in the development of plant cell research (Galbraith 1989).

Because of the complex three-dimensional structure of plant tissues most experiments for DNA analysis with plants were done with isolated nuclei. Nuclei can be obtained either by mechanically chopping of tissues (Galbraith et al. 1983) or by lysis of protoplasts (Bergounioux et al. 1983, Puite and Ten Broeke 1983, Ulrich and Ulrich 1986). Staining of released nuclei with DNA specific fluorochromes demonstrated best results using the A-T base pairs binding fluorescent dyes DAPI or Hoechst (Ulrich et al. 1988). Flow cytometric studies with plant cell material had shown an improved resolution of DNA histograms when non-fixed cell materials have been investigated (Ulrich et al. 1988; Galbraith 1989, 1990; Bergounioux and Brown 1990). Employment of fixed cell material debased the resolution of DNA distribution curves with CVs > 4–5%. These measurements are often unsuitable for further cytokinetic studies (cell cycle analysis; Dean 1987, Galbraith 1989).

The aim of this work was to use a preparation and staining procedure for higher plant cell material, which has been successfully employed for high-resolution flow cytometry of DNA in animal cells and tissues (Otto et al. 1981, Otto 1990).

Material and methods

Different higher plant species have been investigated; in this paper the DNA analysis of material from *Allium cepa* (common onion), *Triticum durum* (wheat), *Vicia faba* (broad bean) and *Viscum album* (European mistletoe) are presented.

For preparation and staining of plant material a slightly modified method has been used (Otto et al. 1981, Otto 1990): To release nuclei plant tissues (leaves or roots) were cut into small pieces. After addition of 1 ml 0.1 M citric acid containing 0.5% Tween 20 (Serva, Heidelberg, Federal Republic of Germany) the pieces were chopped with a sharp razorblade and then incubated for 20–30 min by stirring gently. After filtration through a 56 µm nylon gauze filter the nuclei were fixed in a fresh ethanol-acetic acid solution (v/v 3 : 1; minimum

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fixation time 30 min) and centrifugated (200 × g 15 min). 1 ml of the citric acid/Tween 20 solution was added to the pellet and the nuclei resuspended for 5–10 min. Then a 5-fold volume either of 5 µg/ml DAPI (Partec GmbH, Münster, Federal Republic of Germany) in 0.4 M sodium hydrogen phosphate was added (for univariate DNA analysis) or a 5-fold volume of 5 µg/ml DAPI and 30 µg/ml SR 101 (Sigma, St. Louis, Mo., U.S.A.) in 0.4 M phosphate and 0.2 M NaCl (Stöhr et al. 1978) was added (for bivariate DNA and protein analysis).

Protoplasts were isolated from leaf material by enzymatic digestion as described previously (Ulrich and Ulrich 1986, Ulrich et al. 1988). The protoplasts were directly stirred in 1 ml citric acid/Tween 20 solution. All further steps were carried out as described for tissue probes.

A software package (Partec) was used for the calculation of CV-values. Additional comparative measurements were carried out with chicken and trout red blood cells and with animal and human in vitro cell cultures prepared like protoplasts.

Flow cytometric measurements were performed with a PAS-II flow cytometer (Partec) equipped with a 100 W high pressure mercury lamp (Osram, Augsburg, Federal Republic of Germany) as described previously (Ulrich et al. 1988; Ulrich 1990, 1991).

Results

Cell nuclei from plant tissues or plant protoplasts were released by chopping and/or stirring in citric acid/Tween 20 solution at low pH (1.8). After addition of the dyes DAPI or DAPI/SR 101 in sodium hydrogen phosphate solution (pH 9.0) the pH increased to neutral values and the nuclei were stained. Flow cytometric analysis of plant material yielded high-resolution DNA histograms with CVs about 1–1.5%. Best results were obtained in investigations with protoplasts with CV values up to 1% and less (Fig. 1 a and b). These results are in agreement with data obtained from animal or human cell material (Fig. 1 c). Analysis of nuclei released from chopped tissues shows little bit higher CVs about 1.1–1.5% as presented in Fig. 1 d–f.

Simultaneous staining of nuclear DNA and protein for bivariate analysis after staining with DAPI/SR 101 can be done with nearly the same precision as obtained in univariate measurements after staining with the DNA fluorochrome alone (Fig. 2). The CV values of the DNA parameter in bi-parametric measurements had been about 1.3–1.6%.

Discussion

High-resolution nuclear DNA analysis of higher plants can be obtained using the DNA specific fluorochrome DAPI after pretreatment of cell material (tissues or protoplasts) with citric acid and the detergent Tween

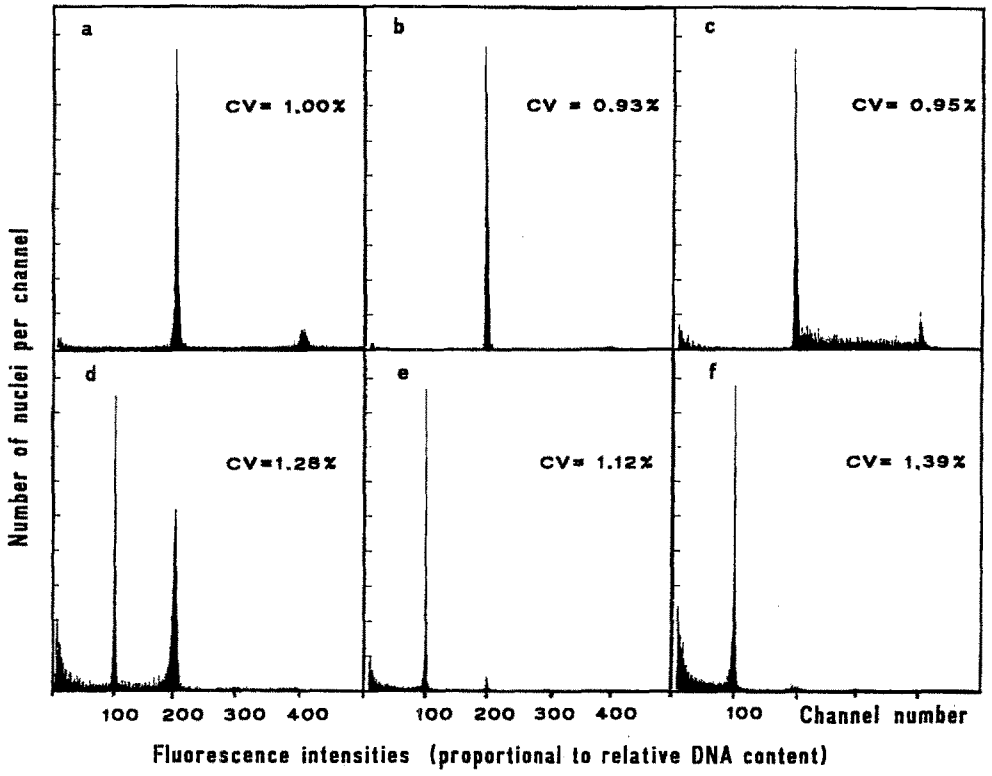

Fig. 1. High-resolution DNA histograms of DAPI stained nuclei from protoplasts of *Allium cepa* (**a**), of *Vicia faba* (**b**), and a comparative histogram from nuclei of a human in vitro leukaemic T cell line (**c**). High-resolution DNA histograms of nuclei from chopped plant tissues: **d** roots of *Allium cepa*, **e** leaves of *Triticum durum*, and **f** leaves of *Viscum album*

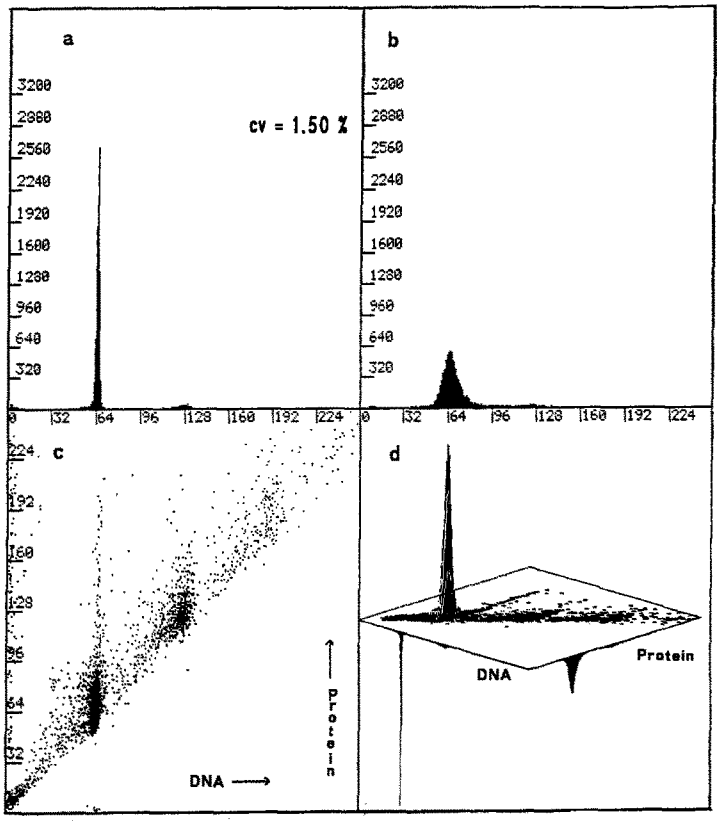

Fig. 2. Bi-parametric analysis of DAPI/SR 101 stained nuclei of leaf protoplasts from *Vicia faba*. **a** Univariate DNA-histogram, **b** univariate protein histogram, **c** contour plot of the simultaneous analysis, **d** three-dimensional presentation of the bi-parametric measurement of nuclear DNA and nuclear protein with projection of the two parameters

20. DNA histograms of plant cell material demonstrate the same resolution as described for animal cells and tissues. The presented method guarantees routinely obtainable CVs about 1.5% and less. These low values make it possible to estimate small changes in nuclear DNA content. The higher CV values in the measurements of chopped plant tissues seem to be artefacts of mechanical destruction during chopping. As shown in the DNA histogram of chopped leaves from *Triticum durum* (Fig. 1 e) the mechanical working up does not necessarily debase the high-resolution DNA analysis. Although single plant species have a wide range in cellular DNA content and therefore a great variety in nucleus sizes the amount of DNA per nucleus does not debase significantly the resolution of DNA histograms, as demonstrated with the presented plant species *Triticum durum* (estimated DNA content/nucleus 16 pg DNA), *Vicia faba* (described DNA content 23.9 pg), *Allium cepa* (33.5 pg DNA) and *Viscum album* (180 pg DNA; Bennett and Smith 1976, Ulrich et al. 1988).

An advantage of the described method for DNA analysis with plant cells is that fixed material can be analyzed without debased resolution of the DNA histograms as performed by using other known preparation procedures (Ulrich et al. 1988; Galbraith 1989, 1990; Bergounioux and Brown 1990). The fixed nuclei can be stored in the cold for several weeks without loss of resolution in later measurements. This might be of importance if a great quantity of probes has, to be analyzed as, for example, in investigations of cultivated plant material in plant breeding.

Addition of the protein fluorochrome SR 101 to the DAPI staining solutions allows simultaneous measurement of nuclear DNA and acid-detergent resistant nuclear protein with nearly the same precision as performed for univariate DNA analysis. This method can be used distinguish to single cell cycle phases G_1, S, and G_2 + M into the sub-compartments G_0, G_1A, G_1B, S, G_2Q, G_2A, and G_2B (Sgorbati et al. 1988, Ulrich 1991). Therefore flow cytometry can be employed for cytokinetic studies of proliferative activities in plant cell systems. As a non-radioactive method for cell cycle analysis flow cytometric measurements require CVs smaller than 4–5% (Dean 1987, Galbraith 1989) for an exact determination of the single phases of the cell

cycle ($G_{0/1}$-, S-, and G_2M-phase). High CV values produce to much overlapping of the amount of S-phase cells with the 2C cell- ($G_{0/1}$-cells) and the 4C cell-distributions (G_2 + M-cells), whereby estimation of the single cell cycle phases are impaired.

The presented simple method for high-resolution flow cytometry of nuclear DNA in higher plant cell material, its reproducibility and its practicability might be very promising for basic and applied cytogenetic studies in plant cell research.

References

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Philos Trans R Soc Lond 274: 227–274

Bergounioux C, Brown SC (1990) Plant cell cycle analysis with isolated nuclei. In: Darzynkiewicz Z, Crissman HA (eds) Methods in cell biology, vol 33. Academic Press, San Diego, pp 563–573

– Perennes C, Miege C, Gadal P (1983) Rapid flow cytometric analysis of male sterility on protoplast division in *Petunia hybrida*. Protoplasma 130: 138–144

Dean PN (1987) Data analysis in cell kinetics research. In: Gray JW, Darzynkiewicz Z (eds) Techniques in cell cycle analysis. Humana Press, Clifton, pp 207–253

Galbraith DW (1989) Analysis of higher plants by flow cytometry and cell sorting. Int Rev Cytol 116: 165–228

– (1990) Flow cytometric analysis of plant genoms. In: Darzynkiewicz Z, Crissman HA (eds) Methods in cell biology, vol 33. Academic Press, San Diego, pp 549–562

– Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049–1151

Heller FO (1973) DNA-Bestimmungen an Keimwurzelzellen von *Vicia faba* mit Hilfe der Impulszytophotometrie. Ber Deutsch Bot Ges 86: 437–441

Otto FJ (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Darzynkiewicz Z, Crissman HA (eds) Method in cell biology, vol 33. Academic Press, San Diego, pp 105–110

– Oldiges H, Göhde W, Jain VK (1981) Flow cytometric measurement of nuclear DNA variations as a potential in vivo mutagenicity test. Cytometry 2: 189–191

Puite VJ, Ten Broeke WRR (1983) DNA staining of fixed and non-fixed plant protoplasts for flow cytometry with Hoechst 33342. Plant Sci Lett 32: 79–88

Sgorbati S, Lucretti S, Lister A, Moretti F, Brown SC (1988) Biparametric DNA-nuclear protein analysis of quiescent, non-cycling differentiated and cycling cells of plant tissues. Basic Appl Histochem 32: 389

Stöhr M, Vogt-Schaden M, Knobloch M, Vogel R, Futterman G (1978) Evaluation of eight fluorochormes combinations for simultaneous DNA-protein flow analysis. Stain Technol 53: 202–215

Ulrich I, Ulrich W (1988) Flow cytometric DNA-analysis of plant protoplasts stained with DAPI. Z Naturforsch 41 c: 1052–1056

– Fritz B, Ulrich W (1988) Application of DNA fluorochromes for flow cytometric DNA analysis of plant protoplasts. Plant Sci 55: 151–158

Ulrich W (1990) Aneuploid and polyploid cellular heterogeneity in insect cell material and diptera species analyzed by flow cytometry. Z Naturforsch 45 c: 1027–1030

– (1991) Simultaneous measurement of DAPI-Sulforhodamine 101 stained nuclear DNA and protein in higher plants by flow cytometry. Biotechnic Histochem (in press)

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