Flow Cytometric DNA-Analysis of Plant Protoplasts Stained with DAPI

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Protoplasts prepared from leaves of various higher plants were stained with the specific DNA fluorochrome DAPI and measured with a pulse cytophotometer. DNA distribution curves of protoplasts showed DNA histograms like assays of animal cells stained with the same dye solution. The percentage of fractions of cell populations in G_0/G_1-, S- and G_2+M-phase of the cell cycle could be calculated. A simple method of protoplasts preparation and of flow cytometric estimation of DAPI stained plant protoplasts is described. This method allows cytogenetic and cytokinetic studies of cells from plant material and may be used for fluorescence activated cell sorting.

Introduction

Flow cytometry is a suitable technique for direct and quantitative evaluation of cellular DNA. This sensitive analytical method is often been used for cytogenetic and cytokinetic studies of cell populations, mostly employed for cells of animal source [1]. Because of the necessity having single cell suspensions for flow cytometric measurement including the difficulties of enzymatic digestion of plant material, there are only few reports of flow cytometric investigations of plant cells or plant protoplasts [2–6]. Several fluorochromes can be used for DNA staining. One of them, 4′-6-diamidino-2-phenylindole (DAPI), synthesized by Dann *et al.* [7], possesses specific binding properties with adenine-thymine rich DNA and is well documented as specific dye for cellular DNA of animal cells in the use of flow cytometry [8]. The fluorescence maximum of DAPI has a higher quantum efficiency than other DNA fluorochromes and should therefore be usable as specific dye for cytogenetic and cytokinetic studies and for employment with a fluorescence activated cell sorter [8].

Materials and Methods

Plant material and human cell cultures

Plant material for protoplasts isolation derived from freshly picked young leaves of greenhouse plants of following species: *Agapanthus* (Liliaceae); *Ceropegia* (Asclepidaceae); *Chlorophytum* (Liliaceae); Hippeastrum (Amaryllidaceae); *Lathyrus* (Leguminosae); *Nicotiana tabacum* (Solanaceae) haploid and diploid; *Petunia* (Solanaceae); *Triticum* (Gramineae); *Vicia faba* (Leguminoseae) var. Ackerperle and var. Herz Freya; and *Viscum album* (Loranthaceae).

For adjustment of the flow cytometer (FCM) and for comparative experiments following human cell cultures have been used: diploid fibroblasts from foreskin; aneuploid HeLa-cells (cervical carcinoma) and aneuploid Molt 4-cells (lymphoblastic leukemia).

Protoplasts isolation and DNA-staining

The lower epidermis of leaves was removed with forceps or if not possible the leaves were cut into thin sections. Leaf fragments were plasmolysed in 0.45 M mannitol-sorbitol-solution containing 10 mM $CaCl_2$, 2 mM KH_2PO_4 and 3 mM morpholinoethansulfone acid. For plant material derived from *Triticum* the mannitol-sorbitol concentration had to be raised to 0.6 M and for *Viscum album* to 0.9 M. The pH of this protoplast washing solution (PWS) was adjusted to 5.8. After 2 h plasmolysis leaf fragments were transferred to an enzyme solution of 2% Cellulase TC (w/v; Fa. Serva, Heidelberg FRG) and 2% Rohament P5 (w/v; Fa. Serva, Heidelberg FRG) dissolved in PWS at pH 5.8. Isolation of protoplasts from Viscum album made it necessary to use 2-fold enzyme concentration (4% w/v). Ca. 1 to 1.5 g plant material was transferred to 15 ml enzyme solution in PWS. Plant fragments were incubated for 16 h. This incubation time can be shortened by using higher enzyme concentrations. Within 2½ bis 3 h a sufficient quantity of protoplasts was released from leaf material

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when enzyme content was doubled (*e.g.* 4% w/v). The incubation mixture was filtered through stainless steel seaves (mesh size 100 and 50 μm) to separate protoplasts from debris. The protoplasts were collected by centrifugation ($100 \times g$; 3 min) and the pellet was resuspended in PWS. Washing procedure was repeated three times. To get intact protoplasts a purification step was carried out: 3 ml of protoplasts suspension was layered upon 5 ml of 20% sucrose in a 10 ml test tube and centrifugated ($100 \times g$; 5 min). Viable protoplasts float upon the sucrose and can be pipetted. The protoplasts were resuspended in a 5-fold amount of aqua bidest. for 10 min and were then centrifugated ($300 \times g$; 5 min). The supernatant was discarded and 7–8 ml EA (ethanol/acetic acid; v/v 3:1) was added and the pellet thoroughly resuspended. After 10 min fixation in EA the cells were collected by centrifugation ($2000 \times g$; 5 min) and were stained with a solution of 5 μg/ml DAPI in tris-HCl buffer pH 7.4 containing 4 mM $MgCl_2$ [8].

Human cell culture preparation and DNA-staining

Human foreskin fibroblasts and HeLa-cells were grown as monolayer cell cultures and collected by trypsinization. Molt 4-cells could be directly collected from cell suspension. After washing with phosphat buffered saline (PBS) the cells were centrifugated ($150 \times g$; 5 min) and fixed with 70% ethanol. After another centrifugation step cells were resuspended in 0,5% Pepsin-HCl to prevent clumping of cells [8]. Separating from Pepsin was carried out by centrifugation and then the cells were stained with the same dye solution as described above.

All steps of protoplasts isolation, human cell culture collection, fixation and staining were done at room temperature.

Flow cytometric DNA-measurement

The DAPI stained plant protoplasts and the human cell cultures were measured with a PAS-II pulse cytophotometer (Partec AG; CH-4144 Arlesheim Switzerland). Using filter combinations of UG1-; TK420-; TK560- and GG435-filters tens of thousands of cells were determined in a few minutes and the DNA distribution curves were automatically analysed and plotted by a computer. For cell cycle determination cumulative DNA-histograms [9] could be analysed and plotted by the FCM joined computer system.

Results

Preliminary experiments with various fixing agents had demonstrated best results for DNA determination using ethanol/acetic acid (v/v 3:1). For rapid analysis protoplasts can be directly stained with the DAPI dye-solution without fixation. The treatment with aqua bidest. is an essential step for getting evaluable DNA-histograms. In Fig. 1 a–l histograms

Table I. Percentage of cells in the various phases of the cell cycle of the DNA-histogram curves shown in Fig. 1 a–o.

Cell population	Amount of measured cells	Phases of cell cycle		
		G_0/G_1	S	G_2+M
Agapanthus	24.973	93.0%	2.3%	4.7%
Chlorophytum	28.700	69.5%	6.1%	24.4%
Ceropegia	64.415	80.4%	5.1%	14.5%
Hippeastrum	112.706	86.8%	1.3%	11.8%
Lathyrus	69.016	82.2%	10.2%	7.6%
Nicotiana tabacum haploid	34.015	91.9%	1.2%	6.9%
Nicotiana tabacum diploid	63.929	87.3%	3.5%	9.2%
Petunia	99.579	76.1%	6.8%	17.1%
Triticum	115.596	89.1%	2.1%	8.8%
Vicia faba var. Ackerperle	141.773	80.7%	1.0%	18.4%
Vicia faba var. Herz Freya	118.213	87.5%	4.1%	8.4%
Viscum album	34.759	88.7%	2.9%	8.4%
Human fibroblasts	55.843	72.4%	11.0%	16.6%
HeLa-cells	40.851	55.8%	27.2%	17.0%
Molt 4-cells	134.199	57.7%	27.1%	15.2%

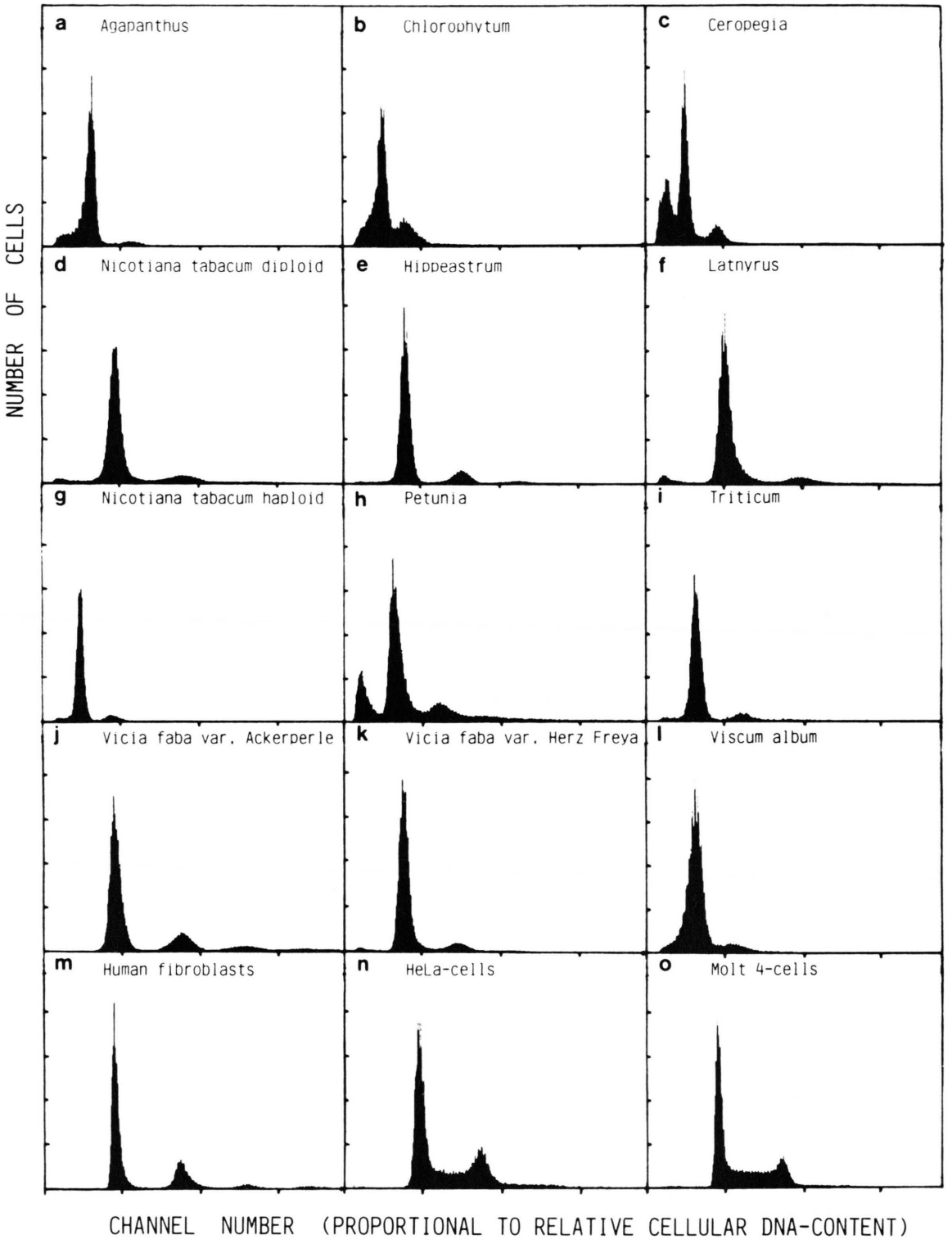

Fig. 1. DNA-histograms of DAPI stained plant protoplasts (a–l) and DAPI stained human cell cultures (m–o). The protoplasts of Ceropegia (c) and Petunia (h) were stained without fixation.

of DAPI stained plant protoplasts of various higher plants are shown. Fig. 1 m–o presents the comparative histograms of human cell cultures. Though in some cases an amount of cellular debris is detectable all histograms demonstrate the characteristic DNA distribution of proliferating cell populations with peaks for 2C-cells (cells in G_0/G_1-phase of the cell cycle) and 4C-cells (G_2+M-cells). Between both peaks the amount of cells with DNA-replication (S-phase) is recognizable. The power gain for the photomultiplier of the FCM had been varied to obtain excellent analyzable DNA histograms. In the two cases of *Nicotiana tabacum* protoplasts the fluorescence intensity of stained cells was the same. So it is possible to demonstrate the exact conformity of the 2C-niveau of diploid (Fig. 1d) and 4C of haploid cells (Fig. 1g). In Table I the single phases of the cell cycle of all tested cell populations are represented. The high amount of S-phase cells of the two aneuploid human tumor cell lines HeLa and Molt 4 results in the high proliferation activity of these cultures typical for permanent growing cell lines *in vitro*. The human diploid foreskin fibroblasts (Fig. 1m) were collected from confluent cultures. Therefore a lower amount of cells in the S-phase of the cell cycle is detectable.

Discussion

A simple method for DNA analysis of plant cells is presented. The described isolation procedure of plant protoplasts yield satisfactory quantity of single plant cells. An essential preparation step is the treatment of isolated protoplasts with aqua bidest. The isolated and swollen protoplasts can be stained directly with the specific DNA fluorochrome DAPI. As rapid method for DNA-analysis the time of enzymatic digestion of plant material can be reduced by using higher enzyme concentrations. Best results of DNA distribution analysis were obtained when ethanol/acetic acid fixed cells were used. These fixed cells can be stored at 4 °C for further studies.

The fluorochrome DAPI, first synthesized by Dann *et al.* [7], used as specific DNA dye for animal cells [8] seems also be suitable for plant cells. For FCM-measurement plant protoplasts and animal cells were stained with the same dye solution. This use of an identical dye solution for plant and animal cells might be a useful step for cytogenetical estimation of DNA content of various cells. Using internal standards for FCM-measurement like chicken red blood cells [10, 11] or other standards with known DNA per cell, the absolute DNA content (in pg DNA/cell) of plant cells can be calculated by flow cytometry. The ploidy level of cells from different plant genera and their species can be determined as demonstrated with protoplasts isolated from *Nicotiana tabacum* haploid and diploid. Flow cytometric DNA analysis shows characteristic DNA distribution curves of mammalian cells from proliferating cell populations with 2C- and 4C-peak. Between these two peaks the amount of cells in the S-phase of the cell cycle is detectable [1, 9, 12]. The DNA histograms of measured plant protoplasts in this paper show this characteristic distribution of cells during the cell cycle. The high amount of cells in G_0/G_1-phase and the small amount of the S-phase demonstrate that this used plant material (leaves) belongs to differentiated tissues. Like fibroblast cell cultures which have the attribute of topoinhibition when these cultures become confluent *in vitro,* the amount of cells with 2C-content is higher than cells with 2C to 4C or cells with 4C-content. This is a marker for cell populations with slow proliferating activity.

The DNA specific stain DAPI is suitable for flow cytometric DNA analysis as described for animal cells [8] and described in this paper for plant protoplasts. The use of this dye allows cytogenetic and cytokinetic studies of plant cells. The fluorochrome DAPI may also be used for employment with a fluorescence activated cell sorter.

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