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Isolation and characterization of microprotoplasts from APM-treated suspension cells of *Nicotiana plumbaginifolia*

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Summary. Subprotoplasts with a DNA content of less than the G1 level (microprotoplasts) were isolated from micronucleated cells of transformed *Nicotiana plumbaginifolia* ('Doba' line resistant to kanamycin) and characterized cytologically as well as by flow cytometry and Feulgen microdensitometry. Micronuclei were induced upon treatment of the suspension cells with the anti-microtubule drug amiprophos-methyl (APM). Protoplasts were fractionated on a continuous iso-osmotic gradient of Percoll; this resulted in several visible bands. Flow cytometric analysis of fluorescein and nuclear DNA contents after staining with fluorescein and DAPI respectively showed that the main band contained mostly evacuolated, intact (sub)protoplasts. Microprotoplasts contained one or a few micronuclei surrounded by a thin rim of cytoplasm and an intact plasma membrane. A maximum of 40% of the microprotoplasts in the fraction just below the main band had a DNA content less than the G1 level, in other fractions this maximum was 20%. Some of these contained an amount equivalent to that of one or a few chromosomes. The application of microprotoplasts for chromosome-mediated gene transfer in plants is indicated.

Key words: Cellular manipulation – Micronucleus – Microprotoplast – Flow cytometry – Chromosome-mediated gene transfer

Introduction

Recent progress in the field of plant genetic manipulation has resulted in a wide range of techniques for the transfer of agronomically important characters. Protoplast fusion can be used when polygenically determined complex characters are of interest or when the molecular basis of a particular trait is not yet known. However, since the entire donor genome is added to the recipient, the resulting hybrid constitutes a complex genetic system with several undesired chromosomes, which can not be analysed properly. Therefore, X-ray irradiation is often applied to induce chromosome elimination from the donor (Bates et al. 1987; Gleba et al. 1988). Recent work has shown that a large proportion of the fragmented genome is incorporated into the recipient genome as small fragments and that highly asymmetric hybrids occur only rarely (Imamura et al. 1987; reviewed in Negrutiu et al. 1989).

Mammalian somatic cell genetics employs two additional techniques for gene mapping: chromosome-mediated gene transfer (Klobutcher and Ruddle 1981) and microcell-mediated chromosome transfer (Fournier 1982). So far, only microcell-mediated chromosome transfer has resulted in the stable maintenance of donor chromosomes, while still transferring only a small fraction of the donor genome, even single chromosomes (Lugo and Fournier 1986). The procedure involves the induction of micronuclei in donor cell lines carrying selectable markers through modified C-mitosis after colchicine or colcemid treatment. Single or groups of chromosomes decondense and form micronuclei. Subsequently, microcells are prepared by either treatment with cytochalasin-B or by centrifugation in the presence of cytochalasin-B, and fused with the recipient cells (Ege et al. 1977; Fournier 1981).

Until recently, no treatment or agent (physical or chemical) was known for the efficient induction of micronuclei in plants, which prevented the development of a microprotoplast system analogous to the microcell sys-

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tem in mammals. Since the finding that the herbicide APM induces micronuclei at a high frequency in cell suspension cultures of *N. plumbaginifolia* (Verhoeven et al. 1986, 1987; De Laat et al. 1987), the application of microprotoplasts for limited genome transfer has become feasible. APM is efficient in the mass induction of micronuclei and the production of a high number of micronuclei per micronucleated cell. It interferes with the polymerization of plant cortical and spindle microtubules (Morejohn and Fosket 1984; Falconer and Seagull 1987). Normal mitosis and growth was observed to resume after removal of APM by washing and subsequent subculture of the cells, demonstrating that its inhibitory effect on the mitotic spindle is reversible (Sree Ramulu et al. 1988a). The induction of micronuclei by APM has been observed in a wide range of genotypes and species (Sree Ramulu et al. 1988b).

In this paper, results are presented on the isolation and characterization of microprotoplasts from APM-treated suspension cultures of *N. plumbaginifolia*. Some preliminary results have already been reported (Verhoeven et al. 1988).

Materials and methods

Cell culture

A suspension cell culture of *N. plumbaginifolia*, kindly provided by Dr. R. Shields, Unilever Research Laboratory, Colworth Home, Sharnbrook, Bedford, UK, was cultured in the dark at 28 °C in „Doba" medium (Bartfield et al. 1985) on a gyratory shaker (120 rpm). From this cell suspension, a kanamycin-resistance line was developed after transformation with *A. tumefaciens*, as has been described previously (de Laat and Blaas 1987). This cell line (designated as I-125-1) is a tetraploid and responds well to treatments with cell-synchronizing agents such as hydroxyurea (HU) and aphidicolin (APH).

APM treatments

Amiprophos-methyl (Tokunol M; *0*-methyl-*0*-*0*-(4-methyl-6-nitrophenyl-*N*-isopropyl-phosphorothioamidate, Kiermayer and Fedtke 1977) was obtained from Bayer Nederland B.V., Divisie Agrochemie, Arnhem, The Netherlands. Stock solutions of APM were prepared at 20 mg/ml in water-free DMSO, and could be stored for at least 6 months at −20 °C without any appreciable loss of activity.

Micronucleation

Micronuclei were induced by the addition of 32 μ*M* APM to cell suspensions on the 2nd day after subculture. For optimal synchronization, APM treatment was preceded by a 24-h incubation with 10 m*M* HU. Afterwards, the cells were washed free of HU, and then APM was added to the fresh culture medium. Hydroxyurea was obtained from Sigma chemical company.

Protoplast isolation

Protoplasts were isolated by treating 1 g of suspension cells with an enzyme solution containing 5% Cellulase R10 and 1% Macerozyme R10, and 0.4 *M* mannitol as osmoticum. To prevent the fusion of micronuclei during protoplast isolation, APM (32 μ*M*) was added to the enzyme mixture. Cytochalasin-B (20 μ*M*) was added to disrupt microfilaments during protoplast isolation. The protoplasts were purified as described previously (de Laat et al. 1987). Cytochalasin-B was obtained from Sigma chemical company.

Centrifugation

Continuous iso-osmotic gradients of Percoll were prepared by adding 7.2% (w/v) mannitol to the Percoll solution (Pharmacia), followed by centrifugation for, 30 min in a Christ Omega ultra centrifuge (170,000 g) using the 6 × 5 ml swing-out rotor. For calibration purposes, density marker beads (Pharmacia, code 17-0459-01) were used. The 2- to 5-mm top layer was removed from the preformed gradient, and the protoplast suspension containing 20 μ*M* cytochalasine-B was layered on top. Cell fractionation was achieved by centrifugation for 1 h at 170,000 *g* and 25 °C. The gradient was collected in small samples using a peristaltic pump. For cytological analysis of subprotoplasts, 3 μ*M* 4',6-diamidino-2-phenylindole (DAPI, Sigma chemical Co.) was added to the gradient.

Microdensitometry

Feulgen microdensitometry in combination with cytological analysis of the cell types was carried out to determine the relative nuclear DNA content of the individual protoplasts in each fraction. Samples were fixed by the addition of formaldehyde to a final concentration of 4%. Fixed protoplasts were collected onto a membrane filter (Millipore SCWP 02500) or on object slides both of which had been coated with egg protein-glycerine. Feulgen staining and microdensitometry were carried out as described previously (Sree Ramulu et al. 1984).

Flow cytometry

All measurements were made with a Partec PAS II flow cytometer equipped with a HBO-100 mercury lamp. Protoplast membrane integrity was assessed by incubation of the protoplasts with 25 μ*M* fluorescein-diacetate (FDA) prior to centrifugation. Samples were collected from the gradient and analysed for the fluorescein content of the fractionated protoplasts. For this analysis the FITC filter set (BP 450, dichroic mirrors TK500 and TK590 with BP520 as barrier filter) was used.

The nuclear DNA content of the subprotoplasts was determined by diluting the samples from the gradient with the Partec stain solution for milk cells (10 vol buffer on 1 vol of cells). The filter combination of UG5, TK420, TK590, and GG435 was used. For calibration of the histograms, nuclei isolated from leaves of control *N. plumbaginifolia* ($2n = 2x = 20$) plants stained with the same buffer were used.

Culture of subprotoplasts

To determine viability, about 500 subprotoplasts collected from several fractions of the gradient were cultured at very low densities (1,000 protoplasts/ml) in Millicell-CM Culture plate Inserts (Millipore, Cat. No. PICM 01250). The Inserts were put in a 5-cm petri dish containing 3 ml of K3 culture medium (Kao et al. 1974) with 10^5 protoplasts/ml of *N. plumbaginifolia* as feeder.

Results

Isolation of micronucleated protoplasts

The prolonged exposure of suspension cells of *N. plumbaginifolia* to APM resulted in a high frequency of

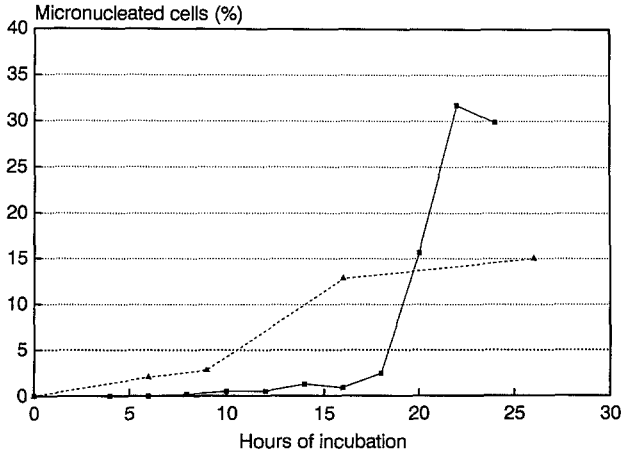

Fig. 1. Percentage of cells with micronuclei after treatment with APM (32 μ*M*) only (–▲–), or after synchronization with HU (10 m*M*, 24 h) followed by washing and APM (32 μ*M*) treatment (—■—) in cell suspension cultures of kanamycin resistant *N. plumbaginifolia*. The 0 h point refers to the starting of incubation time after the 24-h HU treatment and washing

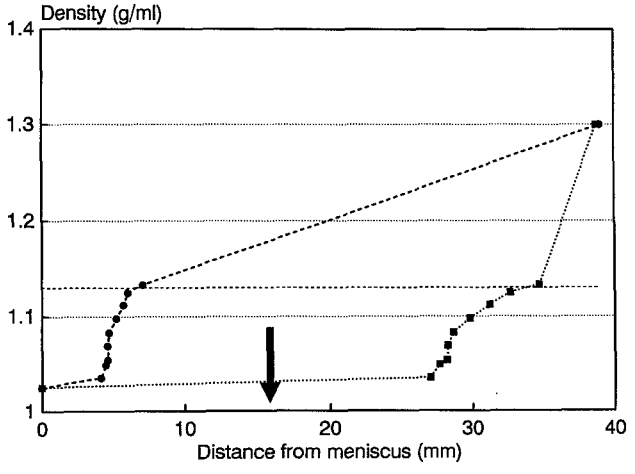

Fig. 2. Density profiles of Percoll solution before centrifugation (– – –), after 30 min performation at 170,000 *g* (—●—), and after protoplast fractionation (90 min, 170,000 *g*) (···■···). The *arrow* indicates the position of the main band of subprotoplasts after protoplast fractionation

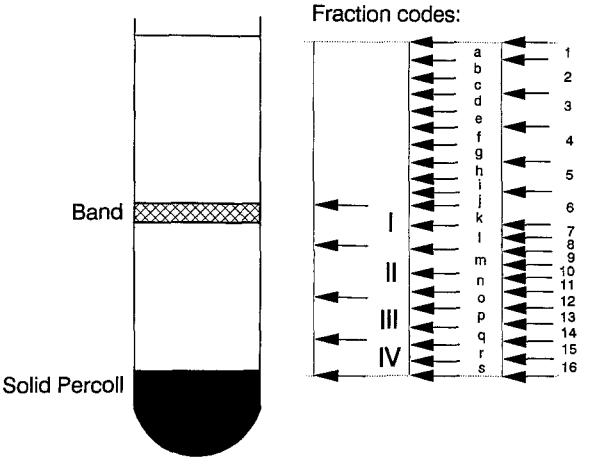

Fig. 3. Diagram of a centrifuge tube showing the position of the band in the Percoll gradient after protoplast fractionation. The *bars* illustrate the position of the various fractions and the codes used in the text for different experiments

micronuclei. With APM alone, up to 15% of the cells became micronucleated; after HU treatment, followed by the addition of APM, this value was more than doubled (Fig. 1), and the number of micronuclei per cell increased. During protoplast isolation, the addition of APM (32 μ*M*) to the enzyme mixture resulted in the maintenance of micronucleated cells at the same level as in the treated suspension cells. In the absence of APM, micronuclei in the protoplasts tended to fuse together, forming multilobed nuclei. The addition of cytochalasin-B induced a significant increase in the number of micronucleated protoplasts.

Fractionation of protoplasts

After a short incubation in 0.4 *M* mannitol with 20 μ*M* cytochalasine-B, the protoplasts were layered on top of the preformed Percoll gradient. The preformed gradient was analysed using the Density Marker Beads. After centrifugation the pattern of the beads was again recorded (Fig. 2). In this way, each band was related to the specific density of the gradient at the position of the band. Figure 3 gives the relationship between fraction number and position in the gradient. The fractionation of the protoplasts was carried out in three different experiments. For microdensitometric and cytological analyses, the lower fractions of the gradient (I-IV) were used; for more detailed analysis, the whole gradient was separated into 16 fractions (1–16). Flow cytometric analysis was performed with yet another gradient separated into 19 fractions (a–s).

Flow cytometric analysis of fluorescein and nuclear DNA contents

Protoplasts stained with FDA were analysed by flow cytometer directly after isolation. More than 95% of the protoplasts were intensely stained, and thus intact. Each of the 19 fractions (a–s) was split into two equal parts: one was used for analysis of the fluorescein content and the other for determination of the nuclear DNA content. The visible bands were collected into different samples. The band in fraction c was only visible after FDA staining on a UV-box and contained only vacuoles or vacuoplasts with very few contaminating nuclei (Fig. 4a). The next band (fraction k) consisted of evacuolated protoplasts with varying DNA contents. This band contained

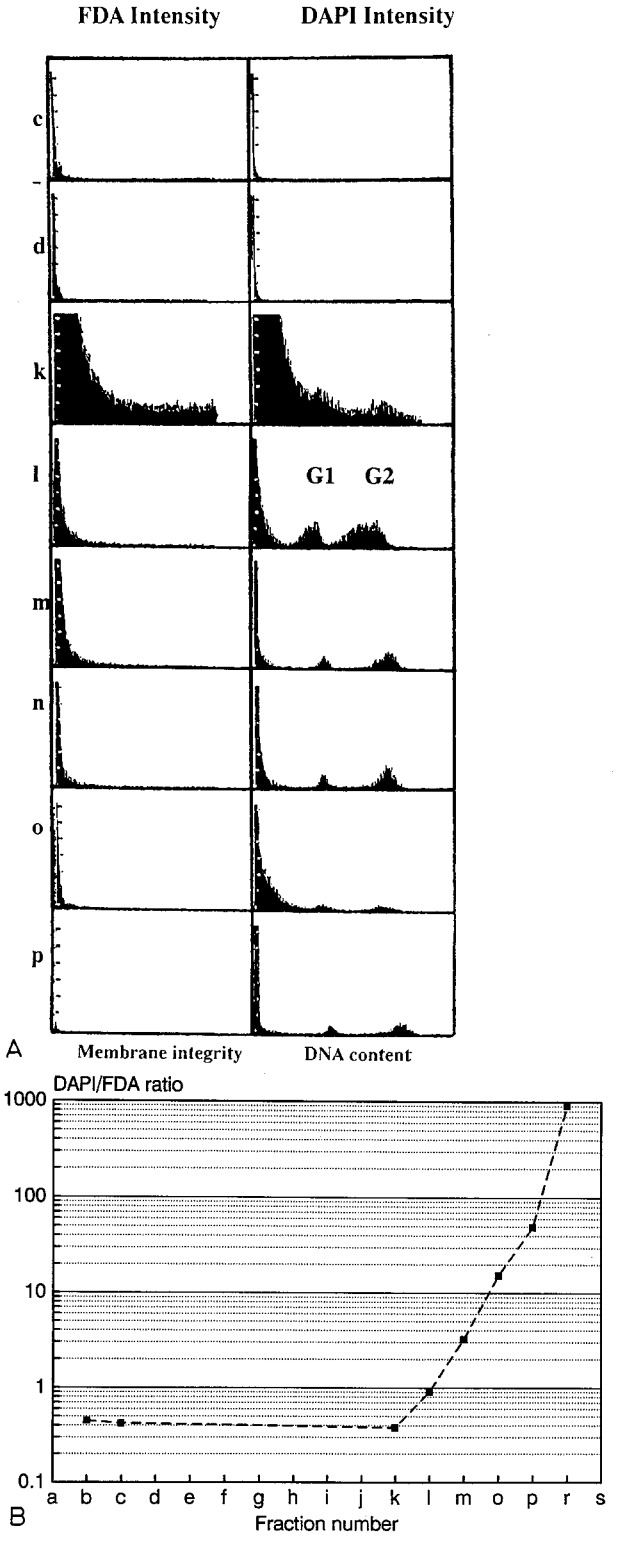

Fig. 4A, B. **A** Intact protoplasts, determined by FDA-staining of subprotoplasts (*left column*), and DNA analysis of subprotoplasts after lysis and DAPI-staining (*right column*). Intact subprotoplasts show intense FDA staining, whereas lysed ones hardly show any fluorescence. The position of the G1 and G2 levels of nuclear DNA content are indicated in the DAPI intensity histograms. **B** Ratio of the number of DAPI-positive subprotoplasts and the number of FDA-positive subprotoplasts. DAPI stains only nuclei, and FDA stains only subprotoplasts with an intact plasma membrane

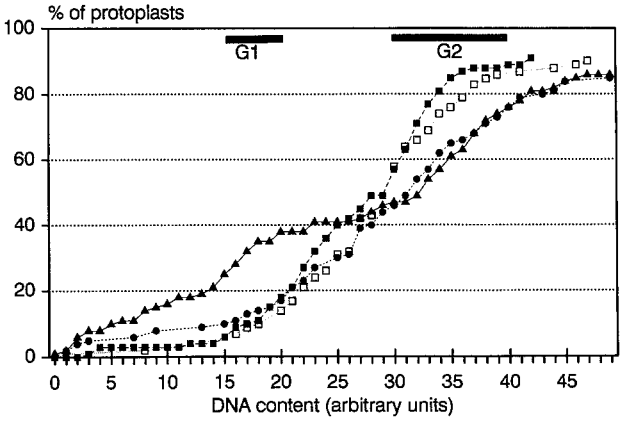

Fig. 5. Cumulative distribution of nuclear DNA content of subprotoplasts isolated from gradient fractions I–IV. The frequency (%) of each value of DNA content was added to the preceding value to obtain cumulative DNA distribution. The *bars* indicate the G1 and G2 DNA levels as calculated from the DNA histograms of leaf nuclei from diploid *N. plumbaginifolia*, measured by microdensitometry under the same conditions. –▲– Fraction I, –■– fraction II, ···□··· fraction III, ––●–– fraction-IV

the highest number of subprotoplasts. The nuclear DNA content of the subprotoplasts was determined by DAPI staining in combination with Triton-X100. Triton-X100 makes the membranes permeable to DAPI, which cannot cross an intact plasma membrane. The number of particles still containing fluorescein after centifugation was greater than the number of DAPI-positive particles in fractions a to k (fraction k contains the major band), while these numbers were equal in fraction I (Fig. 4a). In fractions below fraction I, fewer FDA-positive structures were observed as compared to the number of DAPI-positive structures. The ratio of DAPI-positive particles to FDA-positive particles is presented in Fig. 4b. The results showed that all subprotoplasts in the main band, and in the fractions just below the main band (i.e. fractions a to l), still possessed an intact plasma membrane (Ratio < 1). Fractions m–r contained increasing numbers of damaged subprotoplasts or isolated nuclei.

Microdensitometric determination of nuclear DNA content in individual protoplasts

Microdensitometric analysis revealed the presence of relatively high numbers of microprotoplasts in or just below the dense band, depending on the size of the fraction. Figure shows the cumulative distribution of the DNA content of intact-looking subprotoplasts of the band (fraction I) and the three lower regions (fractions II, III, and IV), and Fig. 6 presents data of this area divided into 11 samples, i.e. fractions 6–16 (see Fig. 3 for the details of codes). These data show that just below the main band (fraction 6) a high proportion (about 40%) of intact microprotoplasts with less than G1 DNA content was

Table 1. Frequency of various types of subprotoplasts isolated from gradient fractions I–IV and the number of microcalli developed from cultured subprotoplasts

	Percentage distribution of various types of subprotoplasts in gradient fraction			
Types of subprotoplasts	Fractions			
	I	II	III	IV
Cytoplasts	7	9	6	9
Evacuolated protoplasts	70	84	86	78
Microprotoplasts <G2	2	3	4	4
Microprotoplasts <G1	21	4	4	9
	Number of microcalli			
Microcalli	–	90	49	35

–: Lost due to contamination

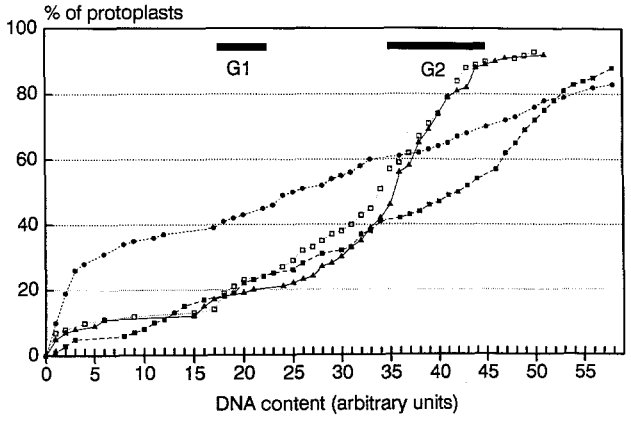

Fig. 6. Cumulative distribution of nuclear DNA content of subprotoplasts isolated from gradient fractions 6–16. Fractions 13–16 gave similar patterns of DNA distribution, and therefore only the data on fraction 13 are presented as a typical example. *Bars* indicate the DNA content of the G1 and G2 DNA levels. –■– Fraction 6, –●– fraction 7, ···□··· fraction 8, –▲– fraction 13

observed, i.e. in fraction 7 (Fig. 6). In Figure 5 a lower frequency (20%) of microprotoplasts with a DNA amount below the G1 level was found due to the simultaneous presence of the major band in fraction I. The other fractions also contained microprotoplasts showing less than G1 DNA content, but at lower frequencies (5%–10%) (Fig. 6).

Cytology

All cell types were recorded, together with their nuclear DNA content, allowing the analysis of the different subprotoplasts (Table 1). The percentage of cytoplasts is almost constant in all four fractions, whereas the frequency of subprotoplasts with a DNA content less than the G1 level is high only in fraction I, which contains the dense band of the gradient.

The analysis of the DAPI-stained subprotoplasts showed the presence of microprotoplasts with single micronuclei as well as some with multiple micronuclei (Fig. 7a, b). Figure 7c shows an incompletely fractionated protoplast already missing its vacuole. Micronuclei can be seen on a thin thread of cytoplasm, pulled out of the evacuolated protoplast.

Culture of subprotoplasts

From the fractions I, II, III and IV, 1,000, 1,000, 200, and 35 FDA-positive subprotoplasts respectively, were isolated and cultured in Millicell-CM Plate Inserts, with *N. plumbaginifilia* cell suspension-derived protoplasts as feeder in the petri dish. In this way microcalli were obtained from each fraction tested, except for fraction I, which was lost due to contamination (Table 1). The microcalli were transferred to solid MS-medium with 1% sucrose when they were 0.5–1 mm in diameter.

Discussion

Previous work with the evacuolation of protoplasts has demonstrated the viability of the products obtained after

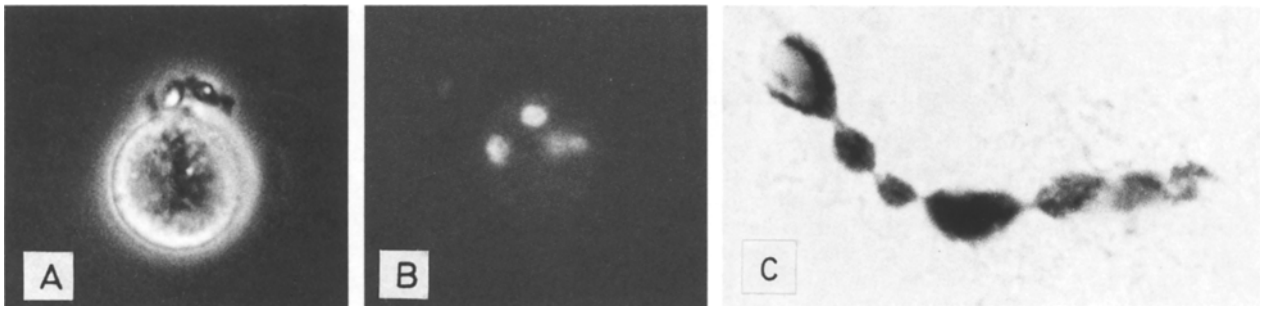

Fig. 7A–C. Photomicrograph of a microprotoplast after centrifugation. **A** Phase contrast image; **B** fluorescence image, showing the DAPI-stained micronuclei; **C** incompletely fractionated microprotoplast, with the nuclei visible on the strands of cytoplasm

evacuolation (Wallin et al. 1979; Lörz et al. 1981; Griesbach and Sink 1983; Burgess and Lawrence 1985; Lesney et al. 1986). The different stages of the evacuolation procedure, as reported previously by Griesbach and Sink (1983), also apply to the fractionation of subprotoplasts presented in this paper. First, the protoplasts band at their corresponding density, becoming oblong due to the accumulation of organelles and nuclei at the lower end of the protoplast. The vacuole pinches off and rises to the top of the gradient. The evacuolated protoplasts are completely devoid of a cytoskeleton due to the action of APM and cytochalasin-B, and consequently are more susceptible to deformation by the gravity field (Fig. 7c). The micronuclei can be seen as small beads on the strand of cytoplasm. With continued centrifugation these cytoplasm strands divide to give rise to individual microprotoplasts. Due to a decrease in the cytoplasm to nucleus ratio, the microprotoplasts band at a higher density in the gradient below the main band.

The main difference between the procedures employed by the previous authors and the one presented in this paper is the use of cytoskeleton-disrupting agents, namely APM for the microtubules and cytochalasin-B for the microfilaments. The negative effects of cytochalasin-B on the regeneration of subprotoplasts (Lörz et al. 1981) are compensated for by the higher yield of evacuolated, intact subprotoplasts and better fractionation under the present experimental conditions. The main band appeared to consist of 100% evacuolated, intact (sub)-protoplasts, as can be deduced from the flow cytometry data. The upper band, with a very low percentage of nuclei, contains only vacuoplasts. This is evident from the fact that only vacuoles surrounded by an intact protoplast membrane retain FDA efficiently (Lesney et al. 1986). From the main band downwards, the number of FDA-positive structures decreases strikingly. In fraction I, the ratio FDA-positive: DAPI-positive particles is about one, and this decreases in fraction m and lower. Also, particles with a lower DNA content than the G1 level are still present in fraction I, whereas they completely disappear from the lower fractions. This pattern is confirmed by the cytological data, which show a high proportion of subprotoplasts containing micronuclei in fraction I. This finding is also consistent with the results of two different microdensitometric analyses of the gradients: the highest frequency of subprotoplasts with DNA content less than G1 level is found in or just below the main band, depending on the size of the sample. The formation of microcalli from subprotoplasts demonstrates their viability. Microprotoplast culture might have a valuable application in the production of cell lines with partial genomes and in studies of genome function in cell differentiation.

With the procedure for the isolation of microprotoplasts described in this paper, a system for the transfer of a limited number of chromosomes by microprotoplast fusion becomes feasible. Up to 40% of the subprotoplasts isolated from the fraction just below the main band have a DNA content less than the G1 level, some showing amounts equivalent to that of one or a few chromosomes. This means that 60% of the protoplasts still contain at least the normal DNA content. Consequently, it is desirable to fuse and culture the microprotoplasts in a controllable way. The fusion of individual protoplasts, such as developed by Koop et al. (1983), provides an elegant system for the fusion of microprotoplasts with recipient protoplasts. This would open up the possibility to transfer either single or a few chromosomes for gene mapping and expression studies in plants, and for the direct construction of addition lines important for use in plant breeding.

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