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Isolation of sub-diploid microprotoplasts for partial genome transfer in plants: Enhancement of micronucleation and enrichment of microprotoplasts with one or a few chromosomes

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Abstract. Results on the enhancement of the frequency of protoplasts with micronuclei, and on the isolation and enrichment of smaller sub-diploid microprotoplasts in transformed *Nicotiana plumbaginifolia* Viv. are reported. Suspension cells were treated with the spindle toxin amiprophos-methyl (APM) for 48 h, and subsequently incubated in a mixture of cell-wall-digesting enzymes in the presence of APM and cytochalasin-B. During enzyme incubation, the frequency of micronucleated protoplasts increased by a factor of 2–6. A shorter period (3 h) of incubation with a higher concentration of enzymes as well as a longer period (16 h) of incubation with a lower concentration of enzymes gave similar frequencies of micronucleated protoplasts and yields of micronuclei. Further, synchronization by sequential treatment with the DNA-synthesis inhibitor hydroxy urea, or aphidicolin, followed by APM and enzyme incubation, significantly increased the frequency of micronucleated protoplasts and the number of micronuclei. The suspension of protoplasts (mono- and micronucleated) obtained after enzyme incubation was fractionated throuh a continuous iso-osmotic gradient of Percoll, using high-speed centrifugation. This resulted in one large and a few small bands, which contained a heterogeneous population of microprotoplasts, protoplasts and cytoplasts. In contrast to the large band, the small bands contained a relatively higher frequency of small sub-diploid microprotoplasts. To separate the small sub-diploid microprotoplasts from the large microprotoplasts and protoplasts of the bands, discontinuous Percoll gradients and sequential filtration through nylon sieves of decreasing pore size (48 – 20 – 15 – 10 – 5 μm) were investigated. Compared with the former method, the latter gave a highly enriched fraction containing predominantly (≈80%) small sub-diploid microprotoplasts with DNA contents equivalent to that of one to four chromosomes, as revealed by microdensitometric and flow-cytometric analyses. The application of this technique for partial genome and limited gene transfer is discussed.

Key words: Amiprophos-methyl – Cytochalasin-B – Genome (partial, transfer) – Microprotoplast – *Nicotiana* – Synchronization

Introduction

Because of the existence of barriers to cross-breeding (pre- and post-fertilization), the transfer of desirable genes by conventional plant breeding is possible only between sexually compatible species. Genetic manipulation makes feasible the transfer of genes across sexual borders or taxonomic distance (reviews in Negrutiu et al. 1987; Gasser and Fraley 1989; Sybenga 1989; Potrykus 1990). In crop plants, several important agronomic and horticultural traits are polygenically controlled, and for many genes no protein product is known. These include genes for disease, insect and stress resistance. The identification, isolation and analysis of such genes are highly complicated, and therefore presently not amenable for manipulation by DNA-transformation techniques. In this regard somatic hybridization is a promising approach for transfer and genetic analysis of the traits, thus facilitating the isolation of the genes involved. However, in this case, the fusion of donor protoplasts with whole genomes, or after irradiation, has so far resulted in genetically complex hybrids with several donor chromosomes and unwanted genes (Famelaer et al. 1990; Piastuch and Bates 1990; Wijbrandi et al. 1990; Tempelaar et al. 1991; Wolters et al. 1991; Gilissen et al. 1992). Therefore, donor microprotoplasts containing one or a few chromosomes with the desired gene(s) (partial genome) are required for the production of asymmetric hybrids. This can offer an important alternative method for gene transfer, especially from a more or less related donor species to the recipient crop species that cannot be hybridized

Abbreviations: APH = aphidicolin; APM = amiprophos-methyl; Au = arbitrary units; CB = cytochalasin B; DAPI = 4,6-diamidino-2-phenylindole; FDA = fluorescein diacetate; HU = hydroxy urea

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sexually. In human and mammalian cell systems, genetically simple hybrid clones have been produced through fusion of so-called "microcells", containing one or a few chromosomes, with the recipient whole cells (reviewed in Fournier 1982; Lugo and Fournier 1986). Two important aspects enabled the efficient development of this technique in mammals: (i) mass induction of micronuclei by colchicine or colcemid and (ii) efficient isolation of small sub-diploid microcells.

Recently, we found that anti-microtubule agents, such as amiprophos-methyl (APM) and oryzalin induce a high degree of metaphase arrest and mass micronucleation in cell-suspension cultures or root meristems of various plant species (Verhoeven et al. 1990, 1991a; Ramulu et al. 1990, 1991). A high frequency of micronucleated protoplasts and a great yield of micronuclei are a prerequisite for mass isolation of microprotoplasts and fusion to achieve partial genome transfer. Therefore, not only efficient isolation of micronucleated protoplasts is necessary, but also enucleation from micronucleated protoplasts, and isolation and enrichment of smaller sub-diploid microprotoplasts are essential. In mammalian cell systems, individual microcells have been isolated from micronucleated cells, using high-speed centrifugation and cytochalasin B (CB; reviewed in Fournier 1982). Also in plants, viable evacuolated karyoplasts have been isolated through high-speed centrifiguation of protoplasts through a density gradient, and the presence of CB during centrifugation enabled efficient fractionation (reviewed in Wallin et al. 1989). However, when compared to mammalian cells, plant cells markedly differ in structure, consisting of cell wall, plastids and a large central vacuole. Therefore, protocols for the isolation of microprotoplasts have to be adapted differently. Recently, we reported that the evacuolation of mono- and micronucleated protoplast populations resulted in a heterogeneous mixture of karyoplasts, cytoplasts and microprotoplasts of various sizes (Verhoeven and Ramulu 1991; Ramulu et al. 1992). The present article reports data on the enhancement of the frequencies of micronucleated protoplasts and micronuclei after enzyme incubation of suspension cells treated with the DNA-synthesis inhibitor hydroxy urea (HU), or aphidicolin (APH), followed by APM treatment in transformed *Nicotiana plumbaginifolia*. Further, the results on the isolation and enrichment of sub-diploid microprotoplasts containing DNA amounts equivalent to that of one or a few chromosomes are presented.

Material and methods

Genotype and cell-suspension culture. A cell line of *Nicotiana plumbaginifolia* Viviani (Doba line, kindly supplied by Dr. R. Shields, Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, UK) transformed by *Agrobacterium tumefaciens* strain LBA 4404 pAL 4404 (pAGS 129; De Laat and Blaas 1987), was used in the present study. This cell line carrying kanamycin resistance is designated as Doba I–125–1, and is a tetraploid with $2n = 4x = 40$. Cell suspensions were cultured in the dark at 28° C in liquid Doba medium (Barfield et al. 1985) on a gyratory shaker (120 rev · min^{-1}). For sustained division activity, subculturing was carried out at intervals of 3–4 d.

Treatments. Amiprophos-methyl (Tokunol M; O-methyl-O-O-(4-methyl-6-nitrophenyl)-N-isopropyl-phosphoro thioamidate) was obtained from Bayer Mobay Corporation, Agricultural Chemicals Division, Kansas City, Mo., USA. A stock solution was prepared at 20 mg · ml^{-1} in water-free dimethylsulfoxide (DMSO) and stored at −20° C. Hydroxy urea and APH were obtained from Sigma Chemical Company, St. Louis, Mo., USA. Hydroxy urea was applied to the cell-suspension culture as a concentrated, freshly prepared solution in the culture medium (40 mg · ml^{-1}) and APH from a stock solution (20 mg · ml^{-1}) in DMSO. Early log-phase suspension cells at 1 d after subculture were treated with HU at 10 mM or APH at 15 µM for 24 h. Afterwards, the suspension cells were washed free from HU or APH with the culture medium and treated with APM at 32 µM. These concentrations of HU, APH and APM were chosen, based on their effectiveness tested in previous experiments (Verhoeven et al. 1991b).

Incubation of cells in the enzyme mixture. After APM treatment, suspension cells were incubated in a cell-wall-digesting enzyme mixture consisting of Cellulase Onozuka-R10, Macerozyme-R10 (Yakult, Honsha Co., Tokyo, Japan) and mannitol as osmoticum for isolation of micronucleated protoplasts. For faster digestion of cell walls, suspension cells were incubated for a shorter period (3 h), using higher concentrations of Cellulase (5%), Macerozyme (1%) and mannitol (0.32 M). For slower digestion, incubation was carried out overnight (16 h) with lower concentrations of Cellulase (1%) and Macerozyme (0.2%), which were dissolved in half-strength V–KM medium (Bokelman and Roest 1983). This medium contained 0.2 M glucose and 0.2 M mannitol, but no hormones. In both shorter and longer periods of enzyme incubations, pH was adjusted to 5.6, and osmolality to 500 mOsmol · kg^{-1}. Enzyme incubation was carried out in a 9-cm Petri dish, containing 1.5 ml packed cell volume and 15 ml enzyme solution, on a gyratory shaker (30 rpm) at 28° C. Cytochalasin-B (20 µM) and APH (32 µM) were added to the enzyme incubation mixture at the time of incubation to prevent the formation of microfilaments and fusion of micronuclei, respectively, during the protoplast isolation.

Cytology. To analyse the frequencies of micronucleated protoplasts and micronuclei, the samples of control suspension cells and those treated with HU or APH followed by APM, or with APM alone were fixed in ethanol: acetic acid (3:1, v/v) for 24–48 h. The protoplasts isolated after enzyme incubation were washed free from enzymes, fixed by the addition of 30 vol. of 12% (v/v) formaldehyde in 0.2 M phosphate buffer (pH 6.0) for 4 h, followed by washing and fixation in ethanol: acetic acid (3:1, v/v) for 24 h. The fixed samples were stained by Feulgen reagent as described previously (Ramulu et al. 1984). For slide preparation, a more gentle squashing was used for the protoplasts isolated after enzyme incubation than was used for the suspension cells. The samples were analysed for mitotic index, chromosome behaviour and micronucleation. Mitotic index mainly includes the cells or protoplasts arrested in metaphase (>95%) and some prophases. The number of cells or protoplasts analysed was 1000 per sample for a given treatment in an experiment. The yield of micronuclei was calculated per total of 1000 cells or protoplasts. Statistical analysis of data from two to three experiments was carried out, using the Student *t*-test (comparison of two means), or analysis of variance and the Duncan test (comparison of more than two means), and χ^2 test.

Purification of protoplasts. After enzyme treatment, the protoplast suspension containing a mixture of micronucleate and mononucleate protoplasts, was filtered through 297-µm and 88-µm nylon mesh, and repeatedly washed with half-strength V–KM medium (Bokelmann and Roest 1983) with macro- and microelements and NaCl 0.24 M (pH 5.6). Protoplasts were further purified as described previously (de Laat et al. 1987).

Isolation of microprotoplasts. To isolate microprotoplasts, the micronucleated protoplasts have to be fragmented, while maintaining the integrity of the plasmamembrane. Therefore, the purified dense

suspension of intact micronucleate and mononucleate protoplasts collected from the surface of the sucrose solution, was loaded onto a continuous iso-osmotic gradient of Percoll and exposed to a high centrifugal force. The Percoll gradient was prepared by adding 7.2% (w/v) mannitol to the Percoll solution (Sigma) and was then centrifuged at 100000 · *g* for 30 min in a Christ Omega ultracentrifuge using a swing-out rotor (6 × 5 ml). The top 5-mm layer was removed from the gradient, and protoplast suspension containing CB (40 μM), was layered on the top. Centrifugation was carried out at 100000 · *g* for 1.5–2 h.

Enrichment of microprotoplasts. After ultracentrifugation, several bands containing a mixture of evacuolated protoplasts, microprotoplasts of various sizes and cytoplasts appeared in the gradient. To enrich the fractions with small microprotoplasts, two methods were used: (i) discontinuous Percoll gradients and (ii) sequential filtration.

Discontinuous Percoll gradients. The bands collected after ultracentrifugation were mixed well in 50% (v/v) Percoll in mannitol (0.44 M), successively layered in solutions of different Percoll percentages (20–50%; 50% Percoll solution being at the bottom of the centrifuge tube), and centrifuged at 40 *g* for 15 min. The fractions collected from various Percoll gradients were analysed for nuclear DNA contents by Feulgen microdensitometry (Ramulu et al. 1984).

Sequential filtration. The bands obtained after ultracentrifugation were sequentially filtered through a series of nylon sieves (Nybott; Swiss Silk Bolting Cloth Mfg. Co., Zürich, Switzerland) of decreasing pore size. The filtered fractions were collected as pellets in mannitol after centrifugation at 80 · *g* for 10 min, followed by recentrifugation of the supernatant at 160 · *g* for 10 min. The samples were analysed by light and UV microscopes, microdensitometer and flow cytometer.

DNA cytophotometry. Feulgen microdensitometry was used to analyse the nuclear DNA content of individual microprotoplasts or protoplasts. The fixation of samples, staining and slide preparation were carried out as described previously (Ramulu et al. 1984). Absorption values for the Feulgen-stained nuclei were measured at a wavelength of 548 nm with a Leitz (Wetzlar, FRG) MPV Compact microscope photometer and scanning stage, using a Hewlett Packard-85 computer. For comparison, in each experiment DNA contents of interphase nuclei of freshly isolated leaf mesophyll protoplasts of control diploid *N. plumbaginifolia* (2n = 2x = 20) were determined. The 2C DNA value was calculated from the measurements of interphase nuclei in G1, which was established by comparing with the values of prophases (4C) and telophases (2 × 2C). In addition, nuclei of chicken red blood cells (CRBC) were used as an internal standard because of their well-established DNA content (2.33 pg; Galbraith et al. 1983). The nuclear DNA values in arbitrary units (AU) were converted to picograms (pg), using the absolute DNA amount of CRBC nuclei (2.33 pg).

Flow cytometry. For flow cytometric analysis of nuclear DNA contents, the nuclei of microprotoplasts or protoplasts were stained with the DNA fluorochrome 4,6-diamidino-2-phenylindole (DAPI; Sigma) and measured using a Partec Pas-II flow cytometer (Partec, Arlesheim, Switzerland), with a UG5 excitation filter, TK420 and TK520 dichroid mirrors, and GG435 longpass filter. The details of flow cytometry were published earlier (Verhoeven et al. 1990).

Results

Enhancement of micronucleation in protoplasts. Micronucleation in the protoplasts isolated after enzyme incubation occurred through modification of mitosis under the influence of the spindle toxin, APM. The metaphase-blocked protoplasts with single or groups of two or more chromosomes showed no anaphase separation of chromosomes, i.e. no centromere division or chromatid separation, but the chromasomes eventually decondensed and developed nuclear membranes, forming several micronuclei (Fig. 1a–c).

Two different factors were investigated for their influence on the enhancement of micronucleation during the incubation: i) synchronization by sequential treatment with the DNA-synthesis inhibitor HU, or APH followed by APM, prior to the enzyme incubation, and ii) duration of enzyme incubation.

i) Sequential treatment preceding the enzyme incubation. Table 1 shows that the sequential treatment with HU, or APH, followed by APM and enzyme incubation, resulted in a significant increase in the percentage of micronucleated protoplasts and the yield of micronuclei compared with values obtained after APM treatment alone, or after treatment with HU or APH followed by APM. The mitotic index and the percentage of metaphases with chromosome groups increased significantly after the sequential treatment (HU or APH followed by APM) compared with values obtained with APM alone. However, both parameters decreased during the 3-h incubation in the enzyme mixture, indicating a rapid change of metaphases to micronucleated cells.

ii) Duration of enzyme incubation. Table 2 shows data on mitotic index and micronucleation in protoplasts of *N. plumbaginifolia* after 3 or 16 h enzyme incubation, preceded by 48 h APM treatment. For comparison, the data obtained after APM treatment of suspension cells for 51 and 64 h are also given. As can be seen from the

Table 1. Effect of sequential treatment with HU or APH and APM preceding the enzyme incubation on mitotic index and micronucleation in the Doba cell line of *Nicotiana plumbaginifolia*

Treatments	Mitotic index (%)	% Metaphases with chromosome groups	% Micronucleated cells or protoplasts	Yield of micronuclei	No. of micronuclei per micronucleated cell or protoplast
APM 51 h	19.4 ± 3.1	12.4 ± 1.6	7.8 ± 0.7	468 ± 58[a]	6.0 ± 0.2[a]
HU 24 h → APM 27 h	33.0 ± 5.2***	16.6 ± 1.9**	14.8 ± 1.7***	1278 ± 50[b]	8.6 ± 0.5[b]
HU 24 h → APM 24 h → Enz. 3 h	25.4 ± 4.3**	11.2 ± 0.8	34.0 ± 4.1***	2764 ± 421[b]	8.1 ± 0.4[ab]
APH 24 h → APM 27 h	29.2 ± 4.7**	13.4 ± 1.1	17.0 ± 1.4**	1594 ± 368[b]	9.4 ± 0.7[b]
APH 24 h → APM 24 h → Enz. 3 h	16.2 ± 2.0**	8.8 ± 0.4	40.4 ± 5.2***	3054 ± 565[b]	8.1 ± 0.3[ab]

** Significant at $P < 0.001$; *** significant at $P < 0.0001$ compared with APM alone, in this and in Table 2

[a, b] Different letters refer, within each column, to significant differences at $P = 0.05$, in this and in Table 2

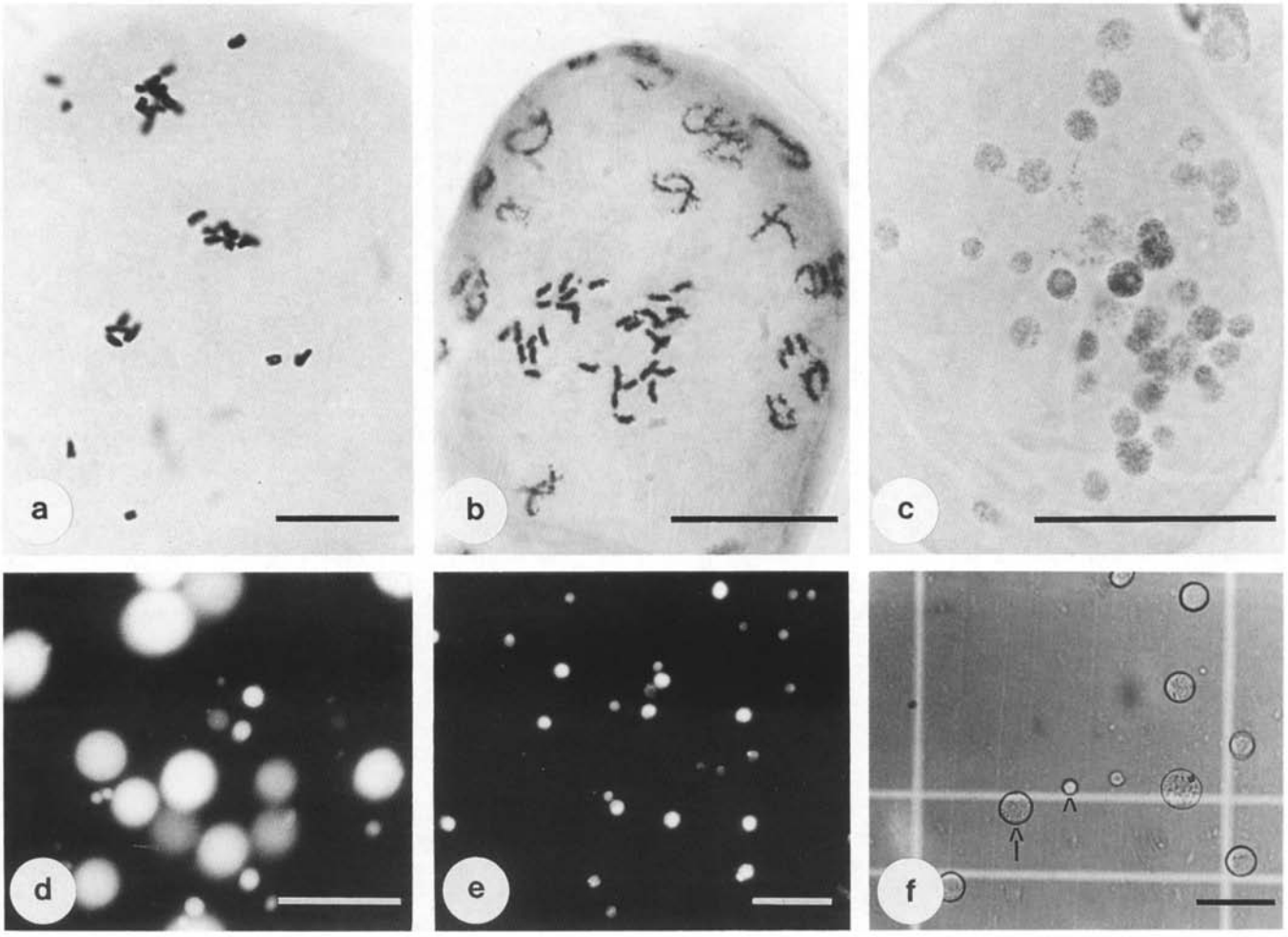

Fig. 1a–f. Microphotographs showing: **a** Metaphase arresting of groups of two or more chromosomes of *N. plumbaginifolia;* **b** decondensation of metaphase chromosomes; **c** protoplast with several micronuclei after enzyme incubation in the presence of APM and CB; **d** fractions of protoplasts and microprotoplasts (stained with DAPI) in the bands obtained after ultracentrifugation; **e** fractions of microprotoplasts (stained with DAPI) after enrichment through sequential filtration of the bands; and **f** different sizes of protoplasts, evacuolated protoplasts (indicated by an *arrow*) and microprotoplasts (indicated by a *arrowhead*). *Bars* = 50 μm; × 330 (**a, d**), × 450 (**b**), × 670 (**c**), × 210 (**e, f**)

Table 2. Effect of the duration (3 h versus 16 h) of enzyme incubation (Enz.) after APM treatment on mitotic index and micronucleation in Doba cell line of *Nicotiana plumbaginifolia*

Treatments	Mitotic index (%)	% Metaphases with chromosome groups	% Micronucleated cells or protoplasts	Yield of micronuclei	No. of micronuclei per micronucleated cell or protoplast
APM 51 h	29.4 ± 0.4	11.6 ± 1.4	9.6 ± 0.8	602 ± 26[a]	6.3 ± 0.2[a]
APM 48 h → Enz. 3 h	18.6 ± 1.4***	6.8 ± 0.7***	27.5 ± 0.7***	1889 ± 183[b]	6.9 ± 0.5[b]
APM 64 h	31.4 ± 1.2	12.6 ± 1.8	6.3 ± 0.1	435 ± 15[a]	7.0 ± 0.1[ab]
APM 48 h → Enz. 16 h	14.2 ± 0.8***	5.4 ± 0.6***	26.2 ± 5.8***	1669 ± 395[b]	6.3 ± 0.1[ab]

data, after enzyme incubation, the percentage of micronucleated protoplasts and the yield of micronuclei increased significantly (i.e. three- to fourfold), while the mitotic index and the percentage of metaphases with chromosome groups considerably decreased. Both shorter (3 h) and longer (16 h) enzyme incubations gave approximately similar results.

Isolation of microprotoplasts. To isolate microprotoplasts from micronucleated protoplasts, the suspension of protoplasts containing the mixture of mononucleate and micronucleated protoplasts obtained after enzyme treatment of suspension cells (eight Petri dishes, each with 1.5 ml packed cell volume cells and 15 ml enzyme mixture) was purified and subsequently fractionated using high-

speed centrifugation. Two methods were investigated for fractionation. In the first method, the protoplast-suspension mixture was layered onto an iso-osmotic gradient of Percoll and centrifuged at 100000 · *g* for 1.5 h. The staining tests with DAPI showed that the resulting bands contained a high frequency (≈15%) of unfragmented micronucleated protoplasts. In the second method, Percoll added with mannitol was first centrifuged at 100000 · *g* for 0.5 h for preformation of the Percoll gradient and then the protoplast suspension was layered on top of the gradient and centrifuged at 100000 · *g* for 2 h. In this case, the resulting bands contained only a low frequency (<5%) of unfragmented micronucleated protoplasts, showing a better fractionation than in the first method (Fig. 1d). Therefore, the second protocol was used in all the subsequent experiments.

Characterization of bands obtained after ultracentrifugation. After centrifugation, one large and three small bands appeared in the Percoll gradient at various distances (usually from 16 to 25 mm) from the top of the centrifuge tube. The bands contained successively, from top to bottom, vacuoplasts or cytoplasts, evacuolated protoplasts and microprotoplasts. Using a peristaltic pump, the large and small bands were collected separately and analysed for nuclear DNA contents of protoplasts and microprotoplasts.

The data on DNA content of interphase nuclei from leaf mesophyll protoplasts of control diploid *N. plumbaginifoila* (2n = 2x = 20) were compared with those for the nuclei of chicken red blood cells (CRBC). Nuclear DNA contents of leaf mesophyll protoplasts were distributed in two peaks, one at 15 AU and the other at 30 AU, corresponding to 2C (Gl) and 4C (G2) DNA levels, respectively, as determined by Feulgen microdensitometry. The 2C and 4C nuclei of *N. plumbaginifolia* contained DNA amounts of 7.5 and 15.0 pg, respectively, based on the DNA content (2.33 pg) of CRBC nuclei. For classification of microprotoplasts obtained after ultracentrifugation, 0.75 pg DNA content is taken as equivalent to that of one G2 or metaphase chromosome of *N. plumbaginifolia.* A micronucleus forms directly from a metaphase chromosome (without centromere division or chromatid separation), under the influence of the spindle toxin APM, and therefore the interphase micronucleus in a microprotoplast contains a DNA amount which is equivalent to that of a G2 or metaphase chromosome. The small sub-diploid microprotoplasts in the bands were classified into two types on the basis of the DNA content of the micronucleus present in them: type 1 which had a DNA content of 0.75–3.00 pg (equivalent to that of one to four G2 or metaphase chromosomes) and type 2 containing 3.75–6.00 pg (~ five to eight G2 or metaphase chromosomes). Large microprotoplasts with high amount of DNA and G1 or G2 protoplasts were classified into "others" (Table 3). The small bands 1–3 contained a considerably higher percentage of type-1 microprotoplasts than the large band. The total yield of microprotoplasts and protoplasts in the small bands was, however, much lower than that of the large band.

Discontinuous Percoll gradient. The effect of a discontinuous Percoll gradient was investigated on the separation of small sub-diploid microprotoplasts from the mixture of large microprotoplasts and protoplasts in the

Table 3. Percentage of small sub-diploid microprotoplasts, large microprotoplasts and protoplasts, and the total yield in various bands obtained after ultracentrifugation. In this, as in Tables 4 and 5, 200–400 nuclei were measured for nuclear DNA contents by Feulgen microdensitometry in two experiments, and the mean percentage ± standard error was calculated

Bands obtained after ultra centrifugation	% Small sub-diploid microprotoplasts		% Others[c]	Total yield (× 10^6)
	Type 1[a]	Type 2[b]		
Large band	5.5 ± 0.5	14.5 ± 3.5	80.0 ± 3.0	2.27 ± 0.37
Small band 1	32.0 ± 4.0	13.0 ± 1.0	55.0 ± 3.0	0.29 ± 0.10
Small band 2	24.5 ± 2.5	11.5 ± 1.5	64.0 ± 1.0	0.21 ± 0.09
Small band 3	19.0 ± 2.0	28.5 ± 3.5	52.5 ± 1.5	0.14 ± 0.07

[a] DNA content of micronucleus = 0.75–3.00 pg (equivalent to that of one to four G2 or metaphase chromosomes)
[b] DNA content of micronucleus = 3.75–6.00 pg (~five to eight G2 or metaphase chromosomes)
[c] Large microprotoplasts and G1 or G2 protoplasts with DNA contents of 6.75–30 pg (~9–40 G2 or metaphase chromosomes) in this, as in Tables 4 and 5

Table 4. Percentage of small sub-diploid microprotoplasts, large microprotoplasts and protoplasts in various fractions obtained from different Percoll gradients

Fractions	% Percoll in the gradients	% Small sub-diploid microprotoplasts		Others
		Type 1	Type 2	
1	20	28.0 ± 2.0	15.5 ± 1.5	56.5 ± 4.5
2	30	25.5 ± 1.5	29.5 ± 2.5	45.0 ± 4.0
3	40	24.5 ± 2.5	17.5 ± 2.5	58.0 ± 5.0
4	50	21.0 ± 2.0	18.5 ± 3.5	60.5 ± 5.5
Bands obtained after ultracentrifugation		25.5 ± 4.0	10.0 ± 2.0	64.5 ± 6.5

bands obtained after ultracentrifugation. Protoplasts with high amounts of nuclear DNA should appear at higher density compared with microprotoplasts with low DNA contents. After ultracentrifugation, the large and small bands were collected together, layered onto discontinuous Percoll gradients containing 20–50% of Percoll and centrifuged for 15 min at 40 · *g*. The data in Table 4 on nuclear DNA contents show that, compared with the bands obtained after ultracentrifugation, the fractions (1–4) collected from Percoll gradients showed a higher percentage (15–24%) of small sub-diploid microprotoplasts of type 2. However, these fractions still contained a high frequency (45–60%) of other types, i.e. large microprotoplasts and protoplasts.

Enrichment of small sub-diploid microprotoplasts by sequential filtration. During sequential filtration of the bands (obtained after ultracentrifugation) through nylon sieves of decreasing pore size, faster filtration was achieved by repeated washing with mannitol (0.4 M). Samples collected from fractions obtained after filtration through each mesh were analysed for nuclear DNA contents. The data in Table 5 show that, compared with the bands, the fractions collected after filtration through 20-μm (i.e. 48–35–20 μm) and 10-μm (i.e. 48–35–20–10 μm) sieves contained higher frequencies of small sub-diploid microprotoplasts of type 2 (21 and 38% respectively), whereas 5 μm (i.e. 48–35–20–10–5 μm) sieves gave a highly enriched fraction, containing predominantly small sub-diploid microprotoplasts of type 1 (compare Fig. 1d with Fig. 1e). Compared with the bands obtained after ultracentrifugation (Fig. 2a), the fraction collected after sequential filtration (Fig. 2b) contained a higher frequency (80%) of small sub-diploid microprotoplasts with DNA contents of 0.5–0.6 AU (i.e. 0.75–3.0 pg), equivalent to that of one to four chromosomes. About 65% of these microprotoplasts had a DNA content equivalent to that of one chromosome (Fig. 2b). Also, flow -cytometric data confirm that the fraction obtained after sequential filtration is highly enriched with small sub-diploid microprotoplasts, eliminating almost all large microprotoplasts, or protoplasts with 2C or 4C DNA content (Fig. 3).

Further, the data on the measurements of the size of microprotoplasts in the enriched fraction obtained after sequential filtration (48–20–15–10–5 μm) show that it contained predominantly (90%) small microprotoplasts with diameters ranging from 2.5 to 10.0 μm (Table 6). Thus, there seems to be a correlation between pore size of filtration sieves, size of protoplasts and microprotoplasts, and DNA content. The size of microprotoplasts was several times smaller than that of the protoplasts of the control *N. plumbaginifolia* cell line (maximum of 55 μm in diamter). Also, the evacuolated protoplasts of the bands obtained after ultracentrifugation were smaller (maximum of 35 μm in diameter) than the control proto-

Table 5. Percentage of small sub-diploid microprotoplasts, large microprotoplasts and protoplasts in the fractions obtained after sequential filtration through nylon gauze of decreasing pore size (48–35–20–10–5 μm)

Fraction No.	Pore size in μm	% Small sub-diploid microprotoplasts		Others
		Type 1	Type 2	
1	48	18.5 ± 3.5	6.5 ± 2.5	75.0 ± 6.0
2	35	17.0 ± 3.0	5.5 ± 2.5	77.5 ± 5.5
3	20	22.5 ± 1.5	21.0 ± 3.0	56.5 ± 4.5
4	10	21.0 ± 1.0	38.5 ± 2.5	40.5 ± 3.5
5	5	80.0 ± 6.0	6.5 ± 1.0	13.5 ± 2.5
Bands obtained after ultracentrifugation		20.5 ± 3.5	5.0 ± 2.0	74.5 ± 5.5

Table 6. Comparison of the size of microprotoplasts in the fraction obtained after sequential filtration (48–20–15–10–5 μm) of the bands (pooled data from three experiments)

Materials	Total no. of protoplasts or microprotoplasts	Percentage distribution of protoplasts and microprotoplasts, varying in size								
		Size in μm[a]								
		55	45	35	25	20	15	10	5	2.5
Control[b]	102	13.7	27.4	45.2	13.7					
Bands[c]	196			26.0	22.9	19.9	11.7	10.2	8.2	1.1
Enriched fraction[d]:										
80 · *g*	102					2.0	5.9	47.0	40.2	4.9
160 · *g*	74						2.8	37.8	44.6	14.8

[a] Two diameters were measured per protoplast or microprotoplast in the suspension mounted on hemocytometer
[b] Protoplasts isolated from control Doba cell suspension culture
[c] Bands obtained after ultracentrifugation
[d] Filtered fractions collected as pellets after centrifugation at 80 · *g* for 10 min, followed by recentrifugation of the supernatant at 160 · *g* for 10 min

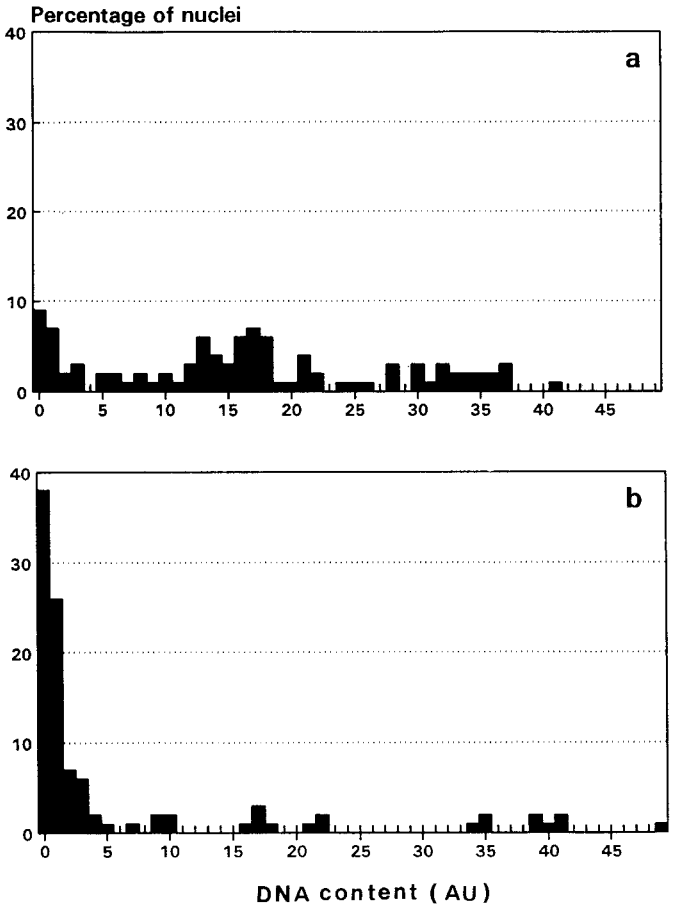

Fig. 2a, b. Feulgen microdensitometric determination of DNA contents of micronuclei in microprotoplasts, or nuclei in protoplasts, of the bands obtained after ultracentrifugation (**a**), and in the enriched fraction obtained after sequential filtration (48–20–15–10–5 μm) of the bands (**b**). Two hundred nuclei were measured per sample in two experiments

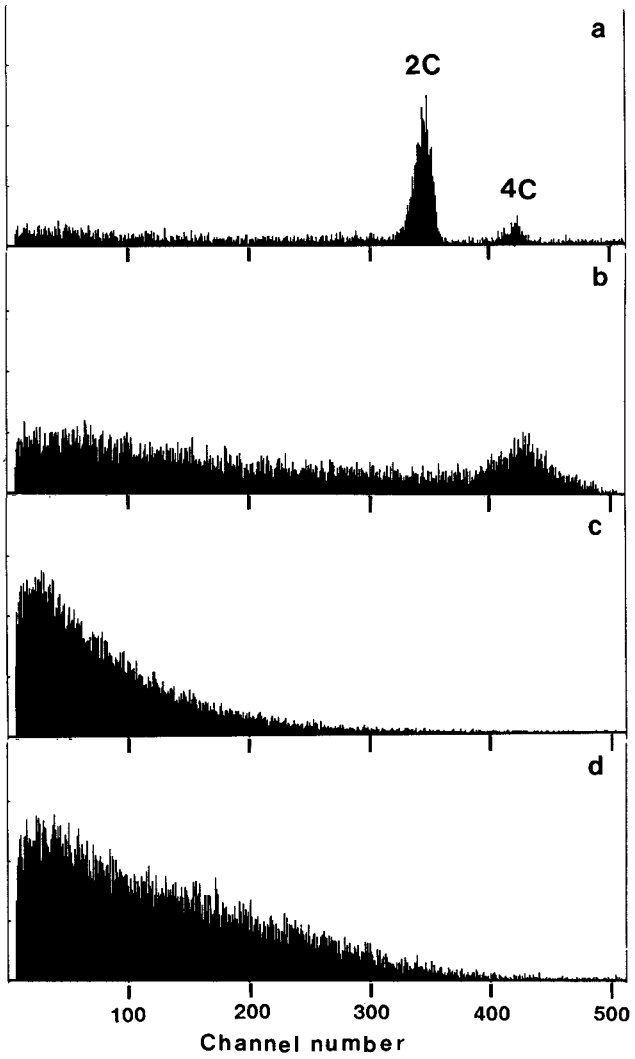

Fig. 3a–d. Flow-cytometric analysis of nuclear DNA contents of interphase nuclei of freshly isolated leaf mesophyll cells of diploid *N. plumbaginifolia* (**a**), microprotoplasts or protoplasts of the unfiltered bands obtained after ultracentrifugation (**b**), and the enriched fraction obtained after sequential filtration (48–20–15–10–5 μm) collected as a pellet after centrifugation at $80 \cdot g$ for 10 min (**c**), and after recentrifugation of the supernatant at $160 \cdot g$ for 10 min (**d**). About 50000 nuclei were measured per sample in two experiments. The X-axis gives fluorescence intensity, and the Y-axis the number of nuclei. The DNA contents of leaf nuclei of control diploid *N. plumbaginifolia* ($2n = 2x = 20$) were measured for comparison of the 2C and 4C DNA values

plasts (Fig. 1f). The yield of the enriched fractions of smaller sub-diploid microprotoplasts varied from $0.4 \cdot 10^6$ to $1.9 \cdot 10^6$, and of the unfiltered bands (containing protoplasts and microprotoplasts) ranged from $2.5 \cdot 10^6$ to $12.7 \cdot 10^6$ in various experiments (12 ml packed cell volume of suspension cells used per experiment). The staining tests with fluorescein diacetate (FDA) and DAPI revealed that 60–70% of the microprotoplasts (with micronuclei) were FDA-positive.

Discussion

Enhancement of micronucleation in protoplasts by APM and CB. Our results show that after incubation of APM-treated suspension cells in an enzyme mixture containing CB and APM, the percentage of micronucleated protoplasts and the yield of micronuclei increased by a factor of 2–6. On the other hand, the mitotic index and the percentage of metaphases with chromosome groups considerably decreased compared with those obtained prior to the enzyme incubation. During the incubation in enzyme mixture, various cellular structures, such as the cell wall, the protein systems and the stability of the plasmamembrane will be disturbed or lost. Because of digestion of the cell wall by enzymes, changes also occur in the orientation and organization of various elements (microtubules, microfilaments, intermediate filaments) of the cytoskeleton as well as the cell polarity, influencing cell division (Traas 1990; Simmonds 1991). Especially, APM and CB present in the enzyme mixture influence microtubule and chromosome decondensation, and microfilaments, respectively. Therefore, micronuclei formed prior to enzyme incubation were maintained in a stable state without undergoing fusion, and additionally the chromosomes in the remaining metaphases decondensed forming micronuclei, thus enhancing the per-

centage of micronucleated protoplasts and the yield of micronuclei.

Further, it was observed that the percentage decrease in mitotic index and in metaphases with chromosome groups was not of the same extent as that of the enhancement of the percentage of micronucleated protoplasts after enzyme incubation. It is likely that, during incubation, prophases changed to metaphases, and metaphases to micronucleated protoplasts under the influence of APM and CB. Also, our previous studies on APM treatment of cell suspensions, or root and shoot meristem cells in various plant species indicate that the chromosomes in metaphase-blocked cells decondensed and developed nuclear membranes around them, forming micronuclei (Ramulu et al. 1988, 1990; Verhoeven et al. 1990).

Increase of yield of micronuclei by synchronization treatment. The yield of micronuclei which is important for the isolation of large numbers of microprotoplasts (Verhoeven and Ramulu 1991), depends not only on the frequency of micronucleated protoplasts, but also on the number of micronuclei per micronucleated protoplast. The present data show that, compared with APM treatment, the synchronization by the sequential treatment with HU or APH followed by APM and enzyme incubation resulted in a significant increase in the percentage of micronucleated protoplasts and in the number of micronuclei per micronucleated protoplast. The sequential treatment with HU or APH followed by APM results in a synchronous progression of cells through S-phase to G2/M, enhancing the frequency of micronucleation (Verhoeven et al. 1991b). Further, the presence of CB in the enzyme mixture can positively influence the micronucleation of protoplasts. Also, previous studies showed that CB enhances the frequency of micronucleation of cells, when applied as a sequential treatment after colcemid in human or mammalian cells (Fournier 1982), or after APM in *N. plumbaginifolia* and potato cell lines (Ramulu et al. 1990). Cytochalasin-B also ensures synchrony of micronucleated cells and viability of microcells (Fournier 1982).

Prerequisite of APM and CB for fractionation of micronucleated protoplasts and isolation of microprotoplasts. The presence of APM and CB during ultracentrifugation was a prerequisite for efficient fractionation of micronucleated protoplasts and isolation of microprotoplasts, while maintaining the integrity of the plasma membrane. The presence of CB disrupts the microfilaments, and APM avoids reformation of microtubules in micronucleated protoplasts. The resulting evacuolated protoplasts (micronucleated) are devoid of cytoskeleton, and hence highly susceptible to deformation by the gravity field. During ultracentrifugation, micronuclei form as small beads on cytoplasmic strands, which eventually separate, giving rise to individual microprotoplasts (Verhoeven and Ramulu 1991). Also, high-speed centrifugation at $100000 \cdot g$ was required for a longer duration (2 h) to obtain a better fractionation and separation of microprotoplasts, unlike in human and other mammalian cell systems where centrifugation at $27000 \cdot g$ for 1 h was sufficient for fractionation of microcells (Landolph and Fournier 1983).

Enrichment of smaller sub-diploid microprotoplasts by sequential filtration. Sequential filtration with nylon sieves of decreasing pore size (48–20–15–10–5 μm) gave a highly enriched fraction containing predominantly small sub-diploid microprotoplasts with DNA contents equivalent to that of one to four chromosomes. A repeated washing of micronucleated protoplasts with a washing medium containing mannitol (0.4 M) was highly essential to avoid clogging of sieves during the purification step, thus increasing the total yield of micronucleated protoplasts. In mammalian cell systems, sequential filtration has been successfully used for enrichment of fractions with small microcells, although discontinuous Ficoll density gradients have been occasionally utilized for separating the small microcells from the large microcells and normal cells (Ege et al. 1977; Fournier 1982; Athwal and Dhar 1984). The same also holds true in the present study for the separation of small microprotoplasts from the large microprotoplasts and evacuolated protoplasts. The enrichment of the fraction with small microprotoplasts by discontinuous Percoll gradients was not optimal, as several intermediate types of microprotoplasts with various chromosome numbers occurred.

Further, various fractions which contained a mixture of mono- and micronucleate protoplasts or microprotoplasts showed sustained division and growth in vitro, indicating that the isolation procedures are not harmful (data not shown). Previous studies on the isolation of karyoplasts or cytoplasts also indicate that the evacuolation procedures used for various plant species resulted in viable products (reviewed in Wallin et al. 1989). The enriched fraction of microprotoplasts that contained a partial genome (predominantly one to four chromosomes) were FDA-positive and intact for several days in culture, but did not regenerate cell wall or undergo cell division. The lack of cell-division activity of the donor microprotoplasts with a partial genome can be an added advantage in fusion experiments, because it avoids contamination of the donor partner while selecting the fusion products, e.g. via kanamycin resistance. The small sub-diploid microprotoplasts with partial genome isolated in the present study can be used for transfer by a polyethylene-glycol-based mass-fusion method (Gleba and Sytnik 1984) or one-to-one microfusion or microinjection (Verhoeven et al. 1991), offering an important perspective in plant breeding for limited gene transfer across sexual barriers.

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References

Athwal, R.S., Dhar, V. (1984) Transfer of specific chromosomes by microcell fusion and gene mapping in mammalian cells. In:

Gene transfer and cancer, pp. 21–29, Pearson, M.L., Sternberg, N.L., eds. Raven Press, New York

Barfield, D.G., Robinson, S.J., Shields, R. (1985) Plant regeneration from protoplasts of long term haploid suspension cultures of *N. plumbaginifolia*. Plant Cell Rep. **4,** 104–107

Bokelmann, G.S., Roest, S. (1983) Plant regeneration from protoplasts of potato (*Solanum tuberosum* cv. Bintje). Z. Pflanzenphysiol. **109,** 259–265

De Laat, A.M.M., Blaas, J. (1987) An improved method for protoplast microinjection suitable for transfer of entire plant chromosomes. Plant Sci. **50,** 161–169

De Laat, A.M.M., Verhoeven H.A., Ramulu, K.S., Dijkhuis, P. (1987) Efficient induction by amiprophos-methyl and flow cytometric sorting of micronuclei in *Nicotiana plumbaginifolia*. Planta **172,** 473–478

Ege, T., Ringertz, N.R., Hamber, H., Sidebottom, E. (1977) Preparation of microcells. In: Methods in cell biology, 15, pp. 339–358, Prescott, D.M., ed. Academic Press, New York

Famelaer, I., Negrutiu, I., Mouras, A., Vaucheret, H., Jacobs, M. (1990) Asymmetric hybridization in *Nicotiana* by "gamma fusion" and progeny analysis of self-fertile hybrids. Theor. Appl. Genet. **79,** 513–520

Fournier, R.E.K. (1982) Microcell-mediated chromosome transfer. In: Techniques in somatic cell genetics, pp. 309–327, Shay, J.W., ed. Plenum Press, New York, London

Galbraith, D.W., Harkins, K.R., Maddox, J.M., Ayres, N.M., Sharma, D.P., Firoozabady, E. (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science **220,** 1049–1051

Gasser, G.S., Fraley, R.T. (1989) Genetically engineered plants for crop improvement. Science **244,** 1293–1299

Gilissen, L.J.W., Van Staveren, M.J., Verhoeven, H.A., Ramulu, K.S. (1992) Somatic hybridization between potato and *Nicotiana plumbaginifolia*. 1. Spontaneous biparental chromosome elimination and production of asymmetric hybrids. Theor. Appl. Genet. **84,** 73–80

Gleba, Y.Y., Sytnik, K.M. (1984) Protoplast fusion – Genetic engineering in higher plants. In: Monographs on theoretical and applied genetics, vol. 8, pp. 1–220, Shoeman, R., ed. Springer, Berlin, Heidelberg

Landolph, J.R., Fournier, R.E.K. (1983) Microcell-mediated transfer of carcinogen-induced ouabain resistance from C3H/10 T1/2C1 8 mouse fibroblasts to human cells. Mut. Res. **107,** 447–463

Lugo, T.G., Fournier, R.E.K. (1986) Microcell fusion and mammalian gene transfer. In: Gene transfer, pp. 79–93, Kucherlapati, R., ed. Plenum publishing Corp., New York

Negrutiu, I., Mouras, A., Horth, M., Jacobs, M. (1987) Direct gene transfer to plants: present developments and some future prospectives. Plant Physiol. Biochem. **25,** 493–503

Piastuch, W.G., Bates, G.W. (1990) Chromosomal analysis of *Nicotiana* asymmetric somatic hybrids by dot blotting and in situ hybridization. Mol. Gen. Genet. **222,** 97–103

Potrykus, I. (1990) Gene transfer to cereals: an assessment. Bio Technology **8,** 535–542

Ramulu, K.S., Dijkhuis, P., Roest, S., Bokelmann, G.S., De Groot, B. (1984) Early occurrence of genetic instability in protoplast cultures of potato. Plant Sci Lett. **36,** 79–86

Ramulu, K.S., Verhoeven, H.A., Dijkhuis, P. (1988) Mitotic dynamics of micronuclei induced by amiprophos-methyl and prospects for chromosome-mediated gene transfer in plants. Theor. Appl. Genet. **75,** 575–584

Ramulu, K.S., Verhoeven, H.A., Dijkhuis, P., Gilissen, L.J.W. (1990) A comparison of APM-induced micronucleation and influence of some factors in various genotypes of potato and *Nicotiana*. Plant Sci. **69,** 123–133

Ramulu, K.S., Verhoeven, H.A., Dijkhuis, P. (1991) Mitotic blocking, micronucleation and chromosome doubling by oryzalin, amiprophos-methyl and colchicine in potato. Protoplasma **160,** 65–71

Ramulu, K.S., Dijkhuis, P., Verhoeven, H.A., Famelaer, I., Blaas, J. (1992) Microprotoplast isolation, enrichment and fusion for partial genome transfer in plants. Physiol. Plant. **85,** 315–318

Simmonds, D.H. (1991) Microtubules in cultured plant protoplasts. Acta Bot. Neerl. **40,** 183–195

Sybenga, J. (1989) Genetic manipulation. Generative versus somatic. In: Biotechnology in agriculture and forestry, 9: Plant protoplasts and genetic engineering II, pp. 26–53, Bajaj, Y.P.S. ed. Springer, Heidelberg

Tempelaar, M.J., Drenth-Diephuis, L.J., Saat, T.A.W.M., Jacobsen, E. (1991) Spontaneous and induced loss of chromosomes in slow-growing somatic hybrid calli of *Solanum tuberosum* and *Nicotiana plumbaginifolia*. Euphytica **56,** 287–296

Traas, J.A. (1990) The plasma membrane-associated cytoskeleton. In: The plant plasma membrane, pp. 269–292, Larsson, C., Moller, I.M., eds. Springer, Berlin, Heidelberg

Verhoeven, H.A., Ramulu, K.S. (1991) Isolation and characterization of microprotoplasts from APM treated suspension cells of *Nicotiana plumbaginifolia*. Theor. Appl. Genet. **82,** 346–352

Verhoeven, H.A., Ramulu, K.S., Dijkhuis, P. (1990) Comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginifolia*. Planta **182,** 408–414

Verhoeven, H.A. Ramulu, K.S., Gilissen, L.J.W., Famelaer, I., Dijkhuis, P., Blaas, J. (1991a) Partial genome transfer through micronuclei in plants. Acta Bot. Neerl. **40,** 97–113

Verhoeven, H.A., Ramulu, K.S., Blaas, J., Dijkhuis, P. (1991b) Control of cell cycle progression. In: A laboratory guide for cellular and molecular plant biology, pp. 346–355, Negrutiu, I., Ghatri-Chhetri, G.B., eds. Birkhäuser, Basel

Wallin, A., Waara, S., Eriksson, T. (1989) Miniprotoplasts and their use in genetic engineering. In: Biotechnology in agriculture and forestry, 9: Plant protoplasts and genetic engineering II, pp. 480–493, Bajaj, Y.P.S., ed. Springer, Berlin, Heidelberg

Wijbrandi, J., Zabel, P., Koornneef, M. (1990) Restriction fragment length polymorphism analysis of somatic hybrids between *Lycopersicon esculentum* and irradiated *L. peruvianum*: Evidence for limited donor genome elimination and extensive chromosome rearrangements. Mol. Gen. Genet. **222,** 270–277

Wolters, A.M.A., Schoenmakers, H.C.H., Van der Meulen-Muisers, J.J.M., Van der Knaap, E., Derks, F.H.M., Koornneef, M., Zelcer, A. (1991) Limitied DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor. Appl. Genet. **83,** 225–232

Note added in proof

Our recent investigations show that, also in potato, it is possible to isolate and enrich small sub-diploid microprotoplasts containing DNA amount equivalent to one or a few chromosomes. Furthermore, the results on PEG-based microprotoplast fusions revealed limited gene (partial genome) transfer from the potato donor line to tomato, and the phenotype of the regenerated plants to be normal-looking, which resembled the recipient parent. The details will be published elsewhere.