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ORIGINAL PAPER

A study of the process of synchronisation and micronucleation in *Beta vulgaris* and the monitoring of an isolation procedure for micro-nuclei and micro-protoplasts by confocal laser scanning microscopy and flow cytometry

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Abstract The process of synchronization and micro-nuclei induction in a suspension culture of *Beta vulgaris*, was induced by the sequential treatment with the DNA-synthesis inhibitor aphidicolin (30 μM, 24 h) and the spindle-toxin amiprophos-methyl (32 μM, 24 h). Mitotic arrest of divisions, spreading of G2-metaphase chromosomes, re-grouping of chromosomes, formation of a nuclear cell wall around single and re-grouped chromosomes and restitution of nuclei with a doubled DNA content was observed. The process of micro-nucleation was induced much less efficiently in *Beta vulgaris* than in *Nicotiana plumbaginifolia*. Cytological observations and monitoring of the process with flow cytometry and confocal laser scanning microscopy, was essential to follow up the course of events and to monitor the development of an efficient procedure for micro-protoplast isolation. Micro-nucleated protoplasts were fractioned by iso-osmotic Percoll gradient centrifugation to obtain heterogeneous micro-protoplast populations with cytoplasts and micro-protoplasts of different size. An enriched fraction with small sub-diploid micro-protoplasts was obtained with the equivalent DNA content of 1–4 chromosomes, as revealed by confocal laser scanning microscopy and flow cytometry. Sub-diploid micro-protoplasts with DNA amounts equivalent to 1–4 chromosomes were predominantly observed in the size classes of 1.8–10.2 μm at a frequency of 34.2–34.5%. The DNA measurements of micro-nuclei and micro-protoplasts, confirmed the hypothesis that the process of micro-nucleation followed the same course of cellular events as observed in *N. plumbaginifolia*. The correlation between DNA content and size of micro-nuclei and micro-protoplasts was not linear and affected by the degree of DNA condensation, total amount of DNA, and the presence of cytoplasm.

Keywords Sugar beet · Amiprophos-methyl · Synchronization · Micro-nucleated protoplast · Micro-protoplast · Micro-nuclei · Confocal laser scanning microscopy · Flow Cytometry

Abbreviations

APH	Aphidicolin
APM	Amiprophos-methyl
CA	Cremart
CB	Cytochalasin B
DAPI	4,6-Diamidino-2-phenylindole
CLSM	Confocal laser scanning microscopy

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FCM	Flow cytometry
HU	Hydroxy urea
MI	Mitotic index
MMCT	Microcell-mediated chromosome transfer
MNI	Micro-nuclei index
MPP	Micro-protoplast
Micro-nucleated cell	MN-cell

Introduction

Microcell-mediated chromosome transfer (MMCT) has been extensively used for partial genome transfer in human-mammalian cell systems (Lugo and Fournier 1986). The technique involves fusion between donor cells with one or a few chromosomes and recipient cells, and has been used to produce mono- or polychromosomal hybrids for chromosomal gene mapping and for analyzing the pattern of integration into the donor genome.

In plants, fusion between donor micro-protoplasts (MPPs) of potato (*Solanum tuberosum*) and recipient protoplasts of tomato (*Lycopersicon peruvianum*) has led to the transfer of single, specific donor chromosomes carrying alien genes (Ramulu et al. 1996). MMCT thus, can offer perspectives for applied and basic research. Important practical applications would be the transfer of desirable genes from sexually-incongruent wild to cultivated species, e.g. disease or stress resistance genes or genes controlling quality traits such as apomixis. Inter-specific or inter-generic transfer of specific chromosomes across sexual barriers and the production of addition and substitution lines could speed up introgressive hybridization (Sybenga 1992; Jacobsen et al. 1995). In view of recent developments in the field of mutagenesis and recombination (Ispas G, Famelaer I and Angenon G, patent application W2006032504; Li et al. 2006), MMCT would be a useful technology for the rapid introgression of foreign traits, as it avoids recurrent backcrosses or solves impossible hybridizations.

MMCT has advantages over classical para-sexual hybridizations with irradiated donor partner, as it avoids complications encountered with whole cell fusions, i.e. transfer of most of the cytoplasmic organelles and as it limits the amount of transferred DNA prior to the fusion event. This can reduce somatic incompatibility reactions in fusion products and enhance the regeneration ability and fertility of MPP hybrids.

Although stable transmission and introgression of genes was reported in very few para-sexual hybridization experiments (Famelaer et al. 1990), it is still expected that MMCT holds more promises because of its inherent possibilities. Nevertheless, very few studies on MMCT, reported the successful transmission of donor genetic material, i.e. chromosome additions (Ramulu et al. 1996; Binsfeld and Schnabl 2002), but none reported the stable introgression and transmission of the studied markers. Most reports concern the potential use of specific cells and optimization of the procedure with specific chemicals for micro-nucleation (Saito and Nakano 2002a, b) or the study of factors affecting the utility of cell types for MMCT (Matthews et al. 1999).

Two important aspects enabled the development of an efficient MMCT technique in mammals and the potato-tomato combination: (1) mass induction of micro-nuclei and (2) efficient isolation of small sub-diploid micro-cells or MPPs (Ramulu et al. 1996). However, although promising, the technology appeared to encounter, as shown above, a lot of technical hurdles, basic problems related to the use of cell types and tissue culture approaches or to the inducibility of the process of micro-nucleation, which altogether strongly reduce the general applicability of the method.

The present study aimed to investigate the process of synchronisation and micro-nucleation in a recalcitrant species, i.e. *Beta vulgaris*, and to deduce technical solutions from the observations for the development of a high yielding isolation procedure for MPPs and micro-nuclei. Flow Cytometry (FCM) and Confocal Laser Scanning Microscopy (CLSM) were essential tools in this investigation and enabled us to correlate DNA content with size of micro-nuclei and MPPs and to develop an efficient method for MPP or micro-nuclei isolation.

Materials and methods

Genotype and suspension culture

Cell line E of sugar beet (*Beta vulgaris*), kindly provided by Schlangstedt et al. (1992) was used in the present study. This cell line was a diploid line with

2n = 2x = 18 chromosomes. Cell suspensions were cultured on a gyratory shaker (120 rev. min^{-1}) in the dark at 28°C in liquid Pgo medium (De Greef and Jacobs 1979) with 5.4 pM NAA (naphthalene-L-acetic acid) and 0.1 μM Kin (kinetin). For sustained growth, subculturing was carried out at intervals of 3–4 days. The growth characteristics of the suspension culture were determined as described by Gilissen et al. (1983).

Synchronization and induction of micronuclei

Early log phase suspension cultures, at 8 h after subculture, were treated for 24 h with inhibitors of DNA synthesis, i.e. HU (hydroxy urea) at 5–10 mM or APH (aphidicolin) at 16–32 μM. Hydroxy urea and APH were obtained from Sigma Chemical Company, St Louis, MO, USA. The treatments with HU and APH were carried out as described by Ramulu et al. (1993). Afterwards, the cultures were washed repeatedly with culture medium. For induction of micronuclei, the suspension cells were subsequently treated for 24 h with APM (amiprophos-methyl; Tokunol M; 0-methyl-0-0-(4-methyl-6-nitrophenyl)-*N*-isopropyl-phosphoro thiamidate from Bayer Mobay Corporation, Agricultural Chemicals Division, Kansas City, MO., USA) or CA (Cremart; Butamiphos; Q-ethyl-Q-(3-methyl-6-nitro-phenyl) *N*-sec-butylphosphorothioamidate from Sumitome Chemical Comp., Osaka, Japan) at 15–30 μM or 2.25–36 μM respectively. These concentrations were chosen on the basis of our previous work with *Nicotiana plumbaginifolia* and potato (Ramulu et al. 1993, 1994).

Isolation of micro-nucleated protoplasts

After treatment with APH and APM or CA, suspension cells were incubated in a cell wall-digesting enzyme mixture with 3.3% cellulase (Onozuka-R10, Yakult, Honsha Corp., Japan), 0.5% driselase (Fluka), 0.1% pectolyase (Sheishin Carp., Japan), 0.4 M mannitol, 3 mM CaCl2 and 5 mM Mes (2(*N*-Morpholino-ethane-sulfonic acid). Prior to filter sterilization, the pH and osmolality of the enzyme mixture were adjusted to 5.6 and 550 mQsmal kg^{-1} respectively. Suspension cells were incubated in a 9 cm Petri dish (Greiner) with 15 ml of enzyme mixture on a gyratory shaker (30 rpm) and digested in the dark at 28°C for 6 h. To prevent the formation of microfilaments and fusion of micronuclei, CB (cytochalasin B) and APM were added to the enzyme mixture at 10–30 μM and 30 μM respectively. After enzyme treatment, the protoplast containing mixture was filtered through nylon sieves (Nybott, Swiss Silk Bolting Cloth Mfg. Co., Zurich, Switzerland) with a pore size of 88, 48 and/or 30 μm. Protoplasts were centrifuged at 100 *g*. for 15 min. The pellet, containing mono- and micro-nucleated protoplasts was re-suspended in W5 salt solution (Menczel et al. 1981) and recollected by centrifugation at 100 *g*. for 15 min. This step was repeated twice to remove the remaining enzymes.

Isolation of MPPs

To disrupt protoplasts and to obtain MPPs, the dense solution mixture of mono- and micro-nucleated protoplasts was loaded onto a continuous iso-osmotic Percoll gradient (Verhoeven and Ramulu 1991; Ramulu et al. 1993) and was centrifuged at 10^5 *g*. for 30 min to 1 h. After gradient centrifugation, the bands were collected and diluted 4- to 5-fold in W5 solution. The mixture with protoplasts of different sizes was filtered through a series of nylon sieves with 15, 8 and 5 μm pore size, and was centrifuged at 40 and/or 160 *g* for 15 min to collect the MPPs. MPPs were diluted in W5 solution to a density of about 10^6 ml^{-1} and could be stored for at least 36 h in protoplast culture medium.

Morphological and cytological analysis

The size (diameter) of MPPs was determined by a light microscope, equipped with an ocular lens bearing an ocular metre. To observe the frequency of dividing suspension cells and of micro-nucleated cells, samples of the control and treated suspension culture were stained with lacto-propionic-orcein, gently squashed on a slide and analysed with a light microscope. The mitotic index (MI) mainly included cells arrested in metaphase. About 400 cells were analysed per treatment. Micro-nucleated protoplasts were immobilized in thin agarose layers on a cover slip and stained in NIB-buffer (Nuclei Isolation Buffer; Saxeena and King 1989) with 10 μM EtBr (ethidium bromide). For a given treatment, more than 800 cells were analysed per sample with a fluorescence microscope.

Flow cytometry (FCM)

For FCM analysis of nuclear DNA contents, samples of suspension cells were digested and incubated in NIB buffer with the DNA fluorochrome DAPI (4,6-diamino-2-phenylindole, Sigma). The DNA content was measured as described by Verhoeven et al. (1990).

Observation of micro-nucleation and DNA analysis of micro-nuclei or MPPs by confocal laser scanning microscopy (CLSM)

MPPs or micro-nuclei, were immobilized in thin agarose layers and incubated in NIB buffer with 10 μM EtBr for about 1 h to stain the nuclear DNA. When stored in the dark at 4°C, the stained material remained stable for at least one week. The positions of individual immobilized MPPs were stored by using a computerized microscope stage. The DNA content of micro-nuclei and MPPs was analysed by confocal laser scanning microscopy, i.e. with a Biorad MRC500 combined with a Zeiss ICM405 inverted microscope by measuring the intensity of the fluorescence emitted by the micronuclei stained with the fluorochrome EtBr (Gilissen et al. 1994). The light intensity emitted by 2C interphase nuclei in G1 (2n = 2x = 18) from protoplast populations of untreated suspension cultures was considered as the standard value of the DNA content of 2C interphase nuclei. The DNA content of MPPs and micronuclei was expressed as a percentage of this standard value.

Results and discussion

Process of synchronisation and micro-nucleation

The maximum MI observed in a suspension culture of sugar beet in exponential growth phase was 2.5 ± 0.7%. The effect of initial packed cell volume (PCV) on the relative growth rate (RGR) of the cells was studied by varying the PCV of the inoculum within a range of 2.4–9.5 ml (data not shown). The RGR of cells was not affected by the PCV of the inoculum and, therefore, adjusted to 2.4 ml in subsequent experiments.

For synchronization of early log-phase cells, different concentrations of HU (5, 7.5 and 15 mM) or APH (15 and 30 μM) were applied. Cytological observations showed that a treatment with 30 μM APH resulted in the highest MI, i.e. 6.2 ± 0.6% (Table 1), compared to the best treatment with HU, i.e. 5.1 ± 0.4% (data not shown). To induce micro-nucleation (MN), synchronized suspension cells were washed free from APH and treated with different concentrations of APM (16 and 32 μM) or Cremart (2.25–36 μM) for 12 to 28 h. Cytological analyses showed that the sequential treatment with APH (30 μM, 24 h) followed by APM (32 μM, 24 h), gave a maximum frequency of MN-cells (Table 1). Although a sequential treatment with APH-CA (4.5 μM) resulted in the highest MI (8.5 ± 0.5%), a lower frequency of MN-cells was observed (5.5 ± 1%). Cytological observations and CLSM showed that the process of micro-nucleation and evolution of micro-nuclei followed the same course of events as observed in *N. plumbaginifolia* (Ramulu et al. 1993): i.e. mitotic arrest of dividing cells, chromosome spreading without centromere division as a consequence of APM or CA treatment, formation of nuclear walls around regrouping and decondensing chromosomes, formation of micro-nuclei and finally the restitution of intact nuclei by micro-nuclear fusions (Fig. 1a–f). Observations in suspension cells indicated that MN-cells contained 4.4 ± 0.8 micro-nuclei on average, which represents about 4–8 G2 chromosomes of a diploid or tetraploid suspension cell respectively. Multi-MN-cells with more than 6 MN were observed at a much lower frequency, i.e. 1 ± 0.3%. Compared to *N. plumbaginifolia*, much lower MI's and MNI's were observed for sequential treatments with the same compounds: i.e. MI's and MNI's of 6.2% and 8.3% respectively in *B. vulgaris* compared to up to 29% and 40% respectively in *N. plumbaginifolia*. The average number of micro-nuclei in MN-cells of *Beta* was also half of the number observed in *N. plumbaginifolia* (Ramulu et al. 1993). Different factors may account for the observed data: the genotype, cell division activity, cell growth rate (Lugo and Fournier 1986; Ramulu et al. 1991), the effectiveness of the used compounds to induce the process, which raised the question whether only few cells responded effectively to the induction or whether all synchronized cells responded badly to the treatment. The observations tend to support the first hypothesis: first, the process of synchronization and micro-nucleation follows the same course of events, as in the well responding species, i.e.

Table 1 Mitotic index and percentage of micronucleated cells after APH (30 pM) treatment for 24 h, followed by washing and APM (32 pM) treatment for 12–28 h

APM Treatment	Mitotic index (%)[a]	Micro-nuclei index (%)[b] (Total number)	% Cells with 4–6 micro-nuclei	% Cells with > 6 micro-nuclei	% Restituting nuclei
12 h	0.3 ± 0.2	1.3 ± 0.9	0.3 ± 0.2	0.2 ± 0.1	1.3 ± 0.3
16	6.2 ± 0.6	3.1 ± 0.6	2.1 ± 1.5	0	3.6 ± 1.9
20	4.8 ± 0.4	6.0 ± 0.4	4.3 ± 0.4	1.0 ± 0.3	4.5 ± 3.6
24	3.4 ± 0.6	8.3 ± 0.2	4.4 ± 2.2	0.7 ± 0.5	6.3 ± 2
28	4.2 ± 0.1	7.1 ± 1.2	4.6 ± 1.1	0.6 ± 0.3	12.4 ± 0.7

[a] Data from 3 different experiments (±SE)

[b] Micro-nuclei index, including cell with 3 nuclei

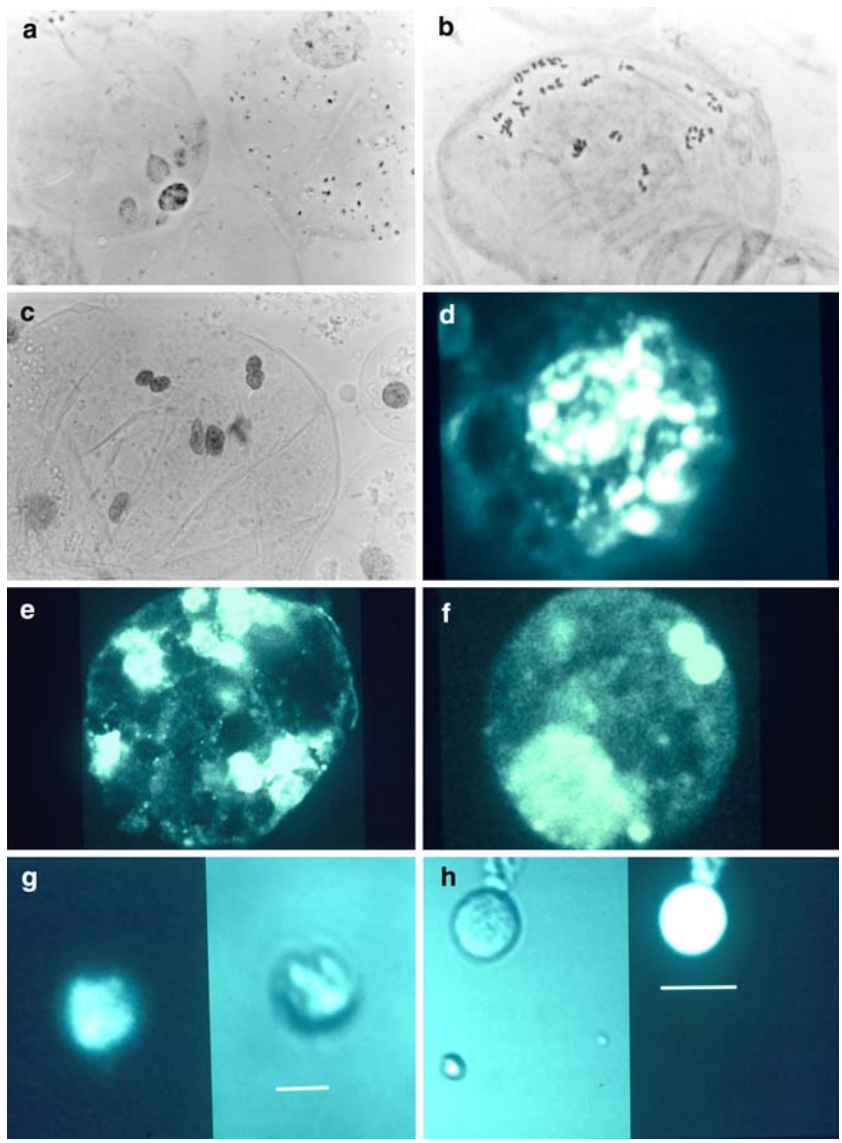

Fig. 1 Classical cytological observations and CLSM of the process of synchronisation and micronucleation: chromosome spreading in a suspension cell, induced by APH and APM (**a**); re-grouping of chromosomes (**b**); formation of micro-nuclei (**a**) and fusion of micro-nuclei (**c**); multi-micro-nucleated cell, observed by CLSM (**d**); gradual fusion of micro-nuclei (**e**, **f**); observation of DNA content in isolated MPPs (**g**, **h**) with left part of the image observed under normal light and the right part under UV. Small cytoplast without DNA (**h**) can be observed under normal light. Bar (not to scale = 2.25 μm)

N. plumbaginifolia; second, additional cytological observations showed that cell size may affect the micro-nucleation process, e.g. by enhancing micro-nuclear fusions and the restitution of intact nuclei. Size measurements, confirmed on MN protoplasts (see below), showed that multi-MN-cells (>6 MN/cell) were consistantly larger than average (dividing) suspension cells, i.e. up to 40 μM compared to 15 to 30 μM respectively. Hence, the high frequency of cells with restituting nuclei observed in the course of the experiments, i.e. up to 12.4 ± 0.7% (Table 1), and consequently the lower average number of micro-nuclei, could be caused by the small size of the average suspension cell.

Monitoring of the process of micro-nucleation and MPP isolation with FCM and CLSM

To evaluate the effectiveness of the sequential APH-APM treatment on micro-nucleation, and to confirm the hypothesis that only a minor fraction of suspension cells responded effectively to this treatment, the relationship between size and DNA content of micro-nuclei had to be analysed. According to the expected observations these should be small and contain 1 to a few chromosomes (Ramulu et al. 1994, data above). An effective isolation procedure had, therefore, to be developed based on the previous observations with FCM and CLSM.

Isolation of MN-protoplasts and MPPs

Based on the observation that MN-protoplasts with more than 6–7 micronuclei (37.5 ± 1.3 μm), were significantly larger than protoplasts with up to 4 micronuclei (26.7 ± 0.8 μm), and that micro-nuclei fused more rapidly in smaller protoplasts, the following factors were investigated for their effect on the yield of MPPs: (a) filtration of protoplasts prior to Percoll gradient centrifugation for enrichment of a subpopulation of larger protoplasts; (b) duration of Percoll gradient centrifugation and cytochalasin-B treatment to prevent premature fusions of micro-nuclei; (c) sequential filtration and centrifugation of MPPs.

Filtration prior to gradient centrifugation

In order to obtain an enrichment of larger protoplasts, containing more micro-nuclei, protoplasts were filtered prior to gradient centrifugation through a nylon sieve with a pore size of 30 μm. The results in Table 2 show that the filtration of protoplasts increased the yield of MPPs, i.e. $10.2 \pm 1.1 \times 10^4$ per 10^6 protoplasts, compared to non filtration, i.e. $7.2 \pm 0.2 \times 10^4$. Variance analysis on log-transformed data indicated that filtration did not affect the distribution frequency of MN-protoplasts or the number of micro-nuclei per protoplast.

Duration of Percoll gradient centrifugation and cytochalasin-B treatment

High speed (10^5 *g*) Percoll gradient centrifugation for 60 min., resulted in significantly higher yields of MPPs, i.e. $3.5 \pm 0.3 \times 10^4$ per 10^6 protoplasts, compared to centrifugation for 30 min, i.e. $1.2 \pm 0.2 \times 10^4$. The highest concentration (30 μM) of cytochalasin B was used in the experiments, as it did not affect the yield of MPPs.

Table 2 Effect of filtration of protoplasts prior to gradient centrifugation on the frequency distribution (%) of micronucleated protoplasts and yield of MPPs

Treatment	Frequency distribution (%) of mononucleate and micronucleate protoplasts			Yield of micro- protoplasts[a] ($\times 10^4$) per 10^6 protoplasts
	Mono-nucleated protoplasts	Micro-nucleated protoplasts with		
		2–3 micro-nuclei	≥6 micro-nuclei	
Without filtration	88.0 ± 1.4	8.7 ± 1.3	3.4 ± 0.2	7.2 ± 0.2
Filtration	78.5 ± 1.1	15.2 ± 0.9	6.2 ± 0.4	10.2 ± 1.1

[a] MPPs were isolated by procedure 4 as described in Table 3. Data from two experiments (±SE)

Sequential filtration and centrifugation of MPPs

Percoll gradient centrifugation of mono- and MN-protoplasts (10.9×10^6 protoplasts ml^{-1}), was performed for 1 h. Centrifugation bands containing protoplasts and MPPs were filtered using 4 different procedures (Table 3). In procedure 1 and 2, MPPs were sequentially filtered through sieves with 15, 8 and 5 µm pore size. Then, the MPP population was centrifuged once at 40 *g* (procedure 1) or it was centrifuged at 40 *g* and the supernatant additionally centrifuged at 160 *g* (procedure 2). In procedure 3 and 4, the micro-protoplast population was only filtered through sieves with 15 and 8 µm pore size and centrifuged at 40 *g* (procedure 3) or it was first centrifuged at 40 *g* and the supernatent re-centrifuged of 160 *g* (procedure 4).

The isolation procedures had a significant effect ($P < 0.05$) on the distribution pattern of the MPPs in different size classes (Table 3). Enhanced frequencies of MPPs of the smaller size classes were observed, when the MPPs were filtered through sieves with 15, 8 and 5 µm pore size (i.e. with procedure 1 and 2). The highest yield of MPPs was obtained with procedure 4. However, the highest number of MPPs, smaller than 9.6 µm, was obtained with procedure 2 (Table 3). FCM data showed that the fraction obtained after differential sieving (15, 8, 5 µm), centrifugation at 40 *g* and re-centrifugation of the supernatant at 160 *g*, was highly enriched with small sub-diploid MPPs, eliminating almost all large MPPs or protoplasts with 2C and 4C DNA content (Fig. 2).

Analysis of DNA content of isolated micro-nuclei with CLSM

The analyses of DNA content of micro-nuclei, showed that the smallest and largest amount of DNA observed in micro-nuclei was about 5.2% to 50% respectively of a 2C nuclear DNA content (Fig. 3a). This represents respectively the equivalent DNA content of 1 small sized chromosome up to 5 average sized two-chromatid (G2) chromosomes. The lowest value of DNA content and size, i.e. 5.2% and 2.25 µM respectively, probably also represents the under limit for a single and small, highly condensed chromosome. The correlation between DNA content and size was not strictly linear, as the DNA content varied more with increasing size (Fig 3a), which reflects the degree of chromosome condensation, the amount of total DNA, and DNA content of individual chromosomes. The observations confirm that small sub-diploid micro-nuclei are induced in *Beta vulgaris* following sequential APH-APM treatment.

Analysis of the DNA content of isolated MPPs with CLSM

The non-linear correlation between DNA content and size of MPPs indicates that MPPs also contain cytoplasm and organellar DNA (Fig. 3b). The DNA content of an average two-chromatid chromosome would be approximately 11.1% based on the DNA content of a G1 (2C) nucleus of B. *vulgaris* (2n = 2x = 18) with 2n mono-chromatid chromosomes.

The frequency distribution of MPPs in different classes of size (diameter) and DNA content, can

Table 3 Effect of sequential filtration and centrifugation procedures on the cumulative, distribution of MPPs in different size classes

Procedures of sequential and centrifugation		Filtration	MPPs			
			Cumulative distribution (%) in different size classes (diameter, µm)			Yield ($\times 10^4$) per 10^6 protoplasts
Procedure	Filtration pore size (µm)	Centrifugation	≤4.8	≤7.2	≤9.6	
1	15 → 8 → 5	40 *g*	38.6 ± 3.3	66.1 ± 1.3	74.5 ± 2.0	1.3 ± 0.2
2	15 → 8 → 5	40 → 160 *g*	36.9 ± 0.7	62.6 ± 1.7	80.7 ± 2.2	2.0 ± 0.3
3	15 → 8	40 *g*	27.4 ± 2.0	45.9 ± 5.8	60.4 ± 0.2	2.8 ± 0.1
4	15 → 8	40 → 160 *g*	34.3 ± 1.6	49.1 ± 0.1	65.6 ± 1.9	4.4 ± 0.1

Variance analysis indicated a significant interaction of sieves and centrifugation, sieves and size and centrifugation and size, on the distribution of MPPs in different size classes ($P < 0.05$)

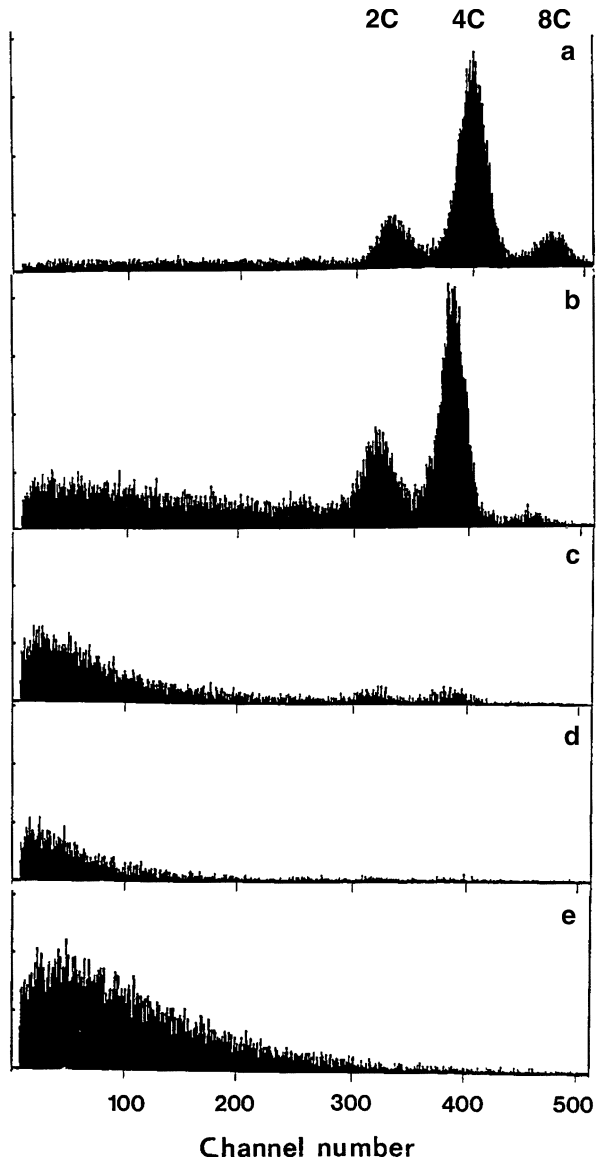

Fig. 2 Flow cytometric analysis of nuclear DNA content in sugar beet: 2C, 4C and 8C nuclei of a suspension culture synchronized with APH for 24 h (**a**); mixture of MPPs and protoplasts of the unfiltered bands obtained after ultracentrifugation (**b**); the fraction obtained after filtration through a 15 μm sieve showing the presence of some 2C and 4C nuclei (**c**); the pellet of the enriched fraction obtained after sequential filtration (15–8–5 μm) and centrifugation at 40 *g* for 10 min (**d**); and the supernatant recentrifuged at 160 *g* for 10 min (**e**). The Y-axis represents the logarithm of the fluorescence intensity and the X-axis the number of nuclei (80 channels doubles fluorescence intensity)

reveal more accurately the relationship between size and DNA content (Fig 1g,h; Fig. 3b; Table 4). The data show that MPPs up to the size of 1.7 μm appeared to be predominantly cytoplasts. High frequencies of cytoplasts were also observed among MPPs with a diameter of 1.8 to 3.4 μm: i.e. 60.8 ± 4.8%. In the size classes of 1.8 to 10.2 μm, MPPs with DNA amounts equivalent to that of one to four metaphase (G2) chromosomes, were observed at a frequency of 34.2–34.5% (Table 4). These MPPs with 5.3–22.2% and 22.3–44.4% DNA of a 2C nucleus, should contain 1–2 and 3–4 metaphase chromosomes respectively. The frequency of MPPs with the equivalent DNA content of more than 4 metaphase chromosomes, i.e. with more than 44.5% DNA of a 2C nucleus, increased

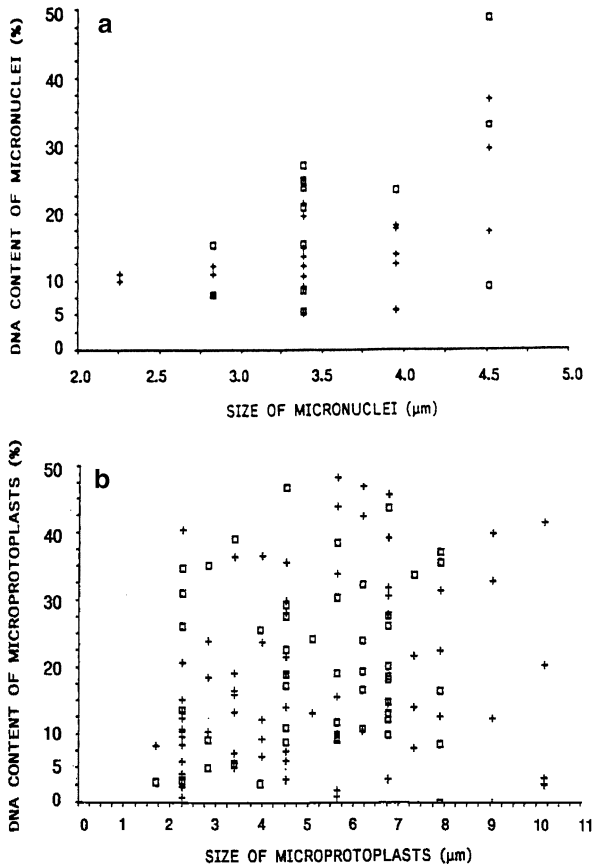

Fig. 3 Scatter diagram showing the correlation between size (μm) and DNA content of micro-nuclei (**a**) or MPPs (**b**). Observations are made by CLSM. DNA content is expressed as a percentage of the DNA content of a 2C interphase nucleus in a diploid cell (2n = 18)

Table 4 Frequency (%) distribution of MPPs with variable DNA content in correlation with size

Size (μm) class of MPPs	Frequency distribution (%) of MPPs with variable DNA content, expressed as a percentage of 2C DNA content[a]			
	% of 2C DNA content			
	0–5.2	5.3–22.2	22.3–44.4	≥44.5
0.1–1.7	85.8 ± 3.6	4.2 ± 3.6	0	0
1.8–3.4	60.8 ± 4.8	24.5 ± 7.0	9.7 ± 4.4	5.3 ± 2.7
3.5–5.7	31.2 ± 8.9	31.0 ± 5.0	9.0 ± 2.4	28.8 ± 11.6
5.8–7.9	18.4 ± 4.7	22.9 ± 4.2	16.5 ± 2.7	31.3 ± 8.7
8.0–10.2	11.7 ± 4.5	21.4 ± 7.4	13.1 ± 2.5	50.8 ± 5.1
10.3–12.4	2.4 ± 1.9	2.4 ± 1.9	4.8 ± 3.9	90.5 ± 5.1
12.5–14.7	0.0	0.0	0.0	100

[a] Data from 4 different experiments (±SE)

with the increasing size of MPPs. MPPs larger than 10.2 μm predominantly belong to this class of DNA content.

For technological applications, the frequency of MPPs containing 1–4 metaphase chromosomes could be further increased using different sets of sieves for

sequential filtration, e.g. with a pore size of 12, 6 and 4 μm (instead of 15, 8 and 5 μm). In addition, higher yields of protoplasts from the sugar beet suspension culture can increase the final yield of sub-diploid MPPs as well.

Conclusions

The present study confirms that the process of synchronization and micro-nucleation in a suspension culture of *B. vulgaris*, follows the same pattern of cellular events as observed in *N. plumbaginifolia*, though at a much lower frequency (Ramulu et al. 1993). Micro-nucleation was effectively induced, especially in larger suspension cells In smaller sized cells, size probably imposes a physical constraint on the spreading of chromosomes, which is essential for the formation of micro-nuclei with reduced numbers of chromosomes, and affects the efficiency of micro-nucleation by enhancing nuclear restitution through micro-nuclear fusions. The choice of other genotypes or chemical compounds (Saito and Nakano 2002a, b), could possibly enhance the process of micro-nucleation. Detailed cytological observations, and a keen monitoring of the micro-nucleation process with FCM and CLSM, was essential to overcome the initial technical limitations of the isolation procedure in order to develop an enhanced MPP isolation procedure for the applicability of this technology in a recalcitrant species as *Beta vulgaris*.

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