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REVIEW

# Partial genome transfer through micronuclei in plants

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## INTRODUCTION

The introduction of gene manipulation techniques has greatly extended the possibilities for plant breeding. New techniques are still emerging, and many are being used, for the introduction of foreign genes, chromosomes and organelles in order to improve the existing cultivars or to develop new varieties, and to localize genes on the chromosomes. The techniques presently available range from the transfer of single, cloned genes via DNA transformation (reviews in Negrutiu *et al.* 1987; Gasser & Fraley 1989; Potrykus 1990), to the addition of a complete genome of a donor species by somatic hybridization (Negrutiu *et al.* 1989). Until now, only identified and cloned genes can be transferred through DNA transformation. Traits which are polygenically determined, or with unknown biochemical and molecular background, e.g. many disease resistances, yield, etc. are not yet amenable

to this technique. In this regard, somatic hybridization may be a suitable approach, but it involves the fusion of whole protoplasts from two different parental species or genotypes, and thus adds two complete nuclear genomes and all cytoplasmic genomes from the chloroplasts and the mitochondria. This results in the production of highly complex somatic hybrids, with many unwanted additional genes. Moreover, when the genomes of the two parents are incompatible at the somatic level, random loss of chromosomes and organelle segregation or recombination will occur, leading to the formation of chimeric tissues. Several techniques have been used in recent years to eliminate or inactivate the unwanted chromosomes by treatment of the donor protoplasts prior to fusion. Commonly gamma or X-irradiation is used to obtain asymmetric hybrids. Although a large number of experiments have been carried out to transfer a limited number of chromosomes or chromosome fragments for integration into the recipient genome, so far only little success has been obtained through this technique. One of the major problems confronted by using irradiation is the stability of the introduced chromosomes or chromosome fragments, both at the cellular and the plant levels (Famelaer *et al.* 1989; Wijbrandi *et al.* 1990). The occurrence of chromosome breaks, deletions and rearrangements after irradiation makes this approach less suitable for transfer of large pieces of DNA with syntenic genes or intact chromosomes. The general application of gamma fusion in plant breeding is also hampered by the low frequency of plant regeneration as well as by the sterility of regenerated plants. Therefore, other alternatives for partial genome transfer were sought in plants. The two most widely used methods for chromosome transfer in mammalian cell biology, i.e. PEG-induced uptake and micro-injection, are being developed in plants. These methods are based on the application of isolated metaphase chromosomes, and are therefore called chromosome-mediated gene transfer (reviews in Klobutcher & Ruddle 1981, De Laat *et al.* 1989).

In the case of mammals, introduction of isolated metaphase chromosomes by PEG-induced uptake into recipient cells resulted in the fragmentation of the donor chromosomes, followed by incorporation of some of the fragments into the recipient genome. The integrated fragments or transgenomes can be maintained stably by applying selection pressure for marker genes. For this method, large numbers of chromosomes were isolated from metaphase arrested cells. By using a GC-specific fluorochrome combined with an AT-specific fluorochrome, it was possible to sort chromosomes by flow cytometry. This method enabled the construction of chromosome-specific transgenomes, which in turn made it possible to map genes on the donor chromosomes (Carrano *et al.* 1979).

In plants, however, PEG-induced uptake of chromosomes has met with limited success. Isolated metaphase chromosomes of carrot were introduced into recipient protoplasts by treatment with PEG, as was demonstrated cytologically (Szabados *et al.* 1981).

Micro-injection has been used to transfer *Petunia alpicola* chromosomes into protoplasts of *P. hybrida* (Griesbach 1987). Although no cytological evidence for the maintenance of the *P. alpicola* chromosomes was presented, it could be demonstrated by biochemical analysis that the calli produced some of the donor enzymes. Several enzymes of the flavonoid biosynthetic pathway of the donor were detected in the injected calli, and segregated in the backcross progeny as simple Mendelian traits. The method employed for the transfer of isolated metaphase chromosomes (the method that leads to the integration of specific DNA segments into the recipient genome) should considerably improve with the advancement of efficient cell-synchronization and chromosome-isolation techniques, sophisticated flow-cytometry for recognizing and sorting of chromosomes, the

introduction of selectable markers through transformation and optimization of protocols and techniques for micro-injection and chromosome uptake. When the potential of chromosome mediated gene transfer (CMGT) becomes available for plants, it will make chromosome transfer a valuable tool in plant breeding.

As an alternative to the transfer of isolated metaphase chromosomes, micronuclei containing only one or a few chromosomes can be used for partial genome transfer. Fragmentation of micronucleated cells yield so-called microcells, which consist of a small amount of cytoplasm containing one or more micronuclei, each surrounded by an intact cell membrane. In contrast to the transfer of isolated metaphase chromosomes, microcell hybridization does not lead to chromosome fragmentation, and is therefore suitable for the transfer of intact chromosomes. Thus, gene linkage is not disturbed after transfer, and chromosome morphology can be used to identify donor and recipient chromosomes. Several aspects of the application of micronuclei in plant cell research are reviewed in more detail in this article.

## MICROCELL HYBRIDIZATION: HISTORICAL AND METHODOLOGICAL SUMMARY

In mammalian cell cultures, treatments with ionizing radiation or chemical mutagens caused chromosome breakage, and occasionally the resulting fragments of chromosomes formed micronuclei. The frequency of micronucleation was considerably higher when dividing cells were exposed to metaphase-arresting agents during a prolonged treatment (Marshall & Bianchi 1983). The latter approach was especially important for the mass isolation of microcells, without genetic damage.

Anti-microtubule agents inhibit the formation of the microtubules of the spindle apparatus, and prevent further polymerization of tubulins by binding directly to the tubulin monomeres. Colchicine, and its methylated derivative, colcemid, are potent inhibitors of spindle formation in mammalian cells, being effective at concentrations in the nanomolar range (Dustin 1984). Due to the absence of a functional spindle, the metaphase chromosomes do not separate into two sister chromatids, but become scattered throughout the cell. Eventually, a nuclear envelope forms around each group of chromosomes, resulting in a multinucleate cell (Fournier 1982).

Microcell fusion was developed in mammals as a tool for the transfer of single or a few intact chromosomes into recipient cells, thus overcoming the problem of chromosome fragmentation (Fournier 1982; Lugo & Fournier 1986). In brief, the protocol is based on the following steps:

(a) micronuclei are induced by prolonged treatment of cell cultures with a metaphase arresting agent;

(b) micronucleated cells are centrifuged to isolate microcells (i.e. subcells containing a micronucleus surrounded by a small rim of cytoplasm and an intact plasmamembrane);

(c) the smallest microcells are recovered by filtration;

(d) those microcells carrying only one or a few chromosomes, are fused with the recipient cells.

Several treatment protocols have been developed in order to increase the yield of micronucleated cells, such as sequential treatment with cytochalasin-B, which destroys the actin microfilaments of the cytoskeleton. Thus, from 60 to 90% of the treated cells

were induced to form micronuclei (review in Fournier 1982). After micronucleation, the cells are exposed to high *g*-forces on a continuous or discontinous gradient by centrifugation. Since the nuclei have a higher density than the surrounding cytoplasm, this will result in deformation of the cells, with the micronuclei on thin, thread-like extensions of the cytoplasm, ending with the separation of small portions of the cytoplasm, still surrounded by an intact plasmamembrane. This process is also facilitated by the pretreatment with cytochalasin-B, resulting in a higher yield of microcells. The smallest microcells, containing only one or a few chromosomes, could be purified by passing all microcells through a filter with a pore size slightly larger than the smallest microcells. The resulting microcell suspension contained no intact genomes, and was fused with the recipient cells using a PEG-based protocol. Recovery of hybrids with partial donor genomes is possible through selection for genes complementing recessive conditional lethal mutations present in the recipient cell, or for genes that confer dominant drug resistance carried by the donor chromosome. In this way, microcell hybrids containing one or a few donor chromosomes could be selected. Identification of heterokaryons and microcell hybrids could be carried out by cytology, because the chromosomes remained structurally and morphologically intact. This enabled the mapping of the complementation genes, and also other genes located on the donor chromosomes. Application of microcell fusion in human somatic cell genetics has resulted in the construction of a panel of hybrid lines, each one containing a different human chromosome (monochromosomal hybrid panels) or a few human chromosomes (polychromosomal hybrid panels) in addition to the mouse genome. Analysis of these lines has yielded detailed data on gene localization, gene linkage and regulation mechanisms of gene expression.

## MICRONUCLEATION IN PLANT CELLS

Micronucleation in plants has been reported after treatment with radiations (Marshall & Bianchi 1983) or chemical mutagens (Nuti Ronchi *et al.* 1986). In both cases, genetic damage is likely to occur, as they induce chromosome breakage and mutations. Therefore, colchicine was used in analogy with the treatments for mammalian cells. However, the concentration of colchicine required for metaphase blocking in plant cells appeared to be a thousand-fold higher than that in animal cells. This has been attributed to a lower affinity of colchicine to plant tubulin heterodimers (Dustin 1984). Therefore, colchicine and colcemid are not very effective in inducing micronuclei in plants. Recently, it was shown that some herbicides act directly on plant tubulin polymerization, and prevent cell cycle progression through mitosis (reviewed in Dustin 1984; Morejohn & Fosket 1984). The di-nitro-anilines trifluralin and oryzalin, and amiprophos-methyl (APM), a phosphoric amide, belong to this class of chemicals. Several of these inhibitors show very strong binding to plant tubulins, and arrest mitosis at concentrations of $10^{-7}$ M (Morejohn & Fosket 1986; Morejohn *et al.* 1987). Treatments of exponentially growing cell suspensions of *N. plumbaginifolia* and potato with APM resulted in metaphase arrest and chromosome scattering, finally resulting in the formation of micronuclei after decondensation of the scattered chromosomes and formation of nuclear membranes (Fig. 1a,b) (Verhoeven *et al.* 1986, 1987; De Laat *et al.* 1987, Sree Ramulu *et al.* 1991a). This process appeared to be identical to colcemid treatment of mammalian cells (Sree Ramulu *et al.* 1988a). Addition of APM or another herbicide with anti-microtubular action, e.g. oryzalin, resulted in a cumulative increase of the mitotic index (MI), due

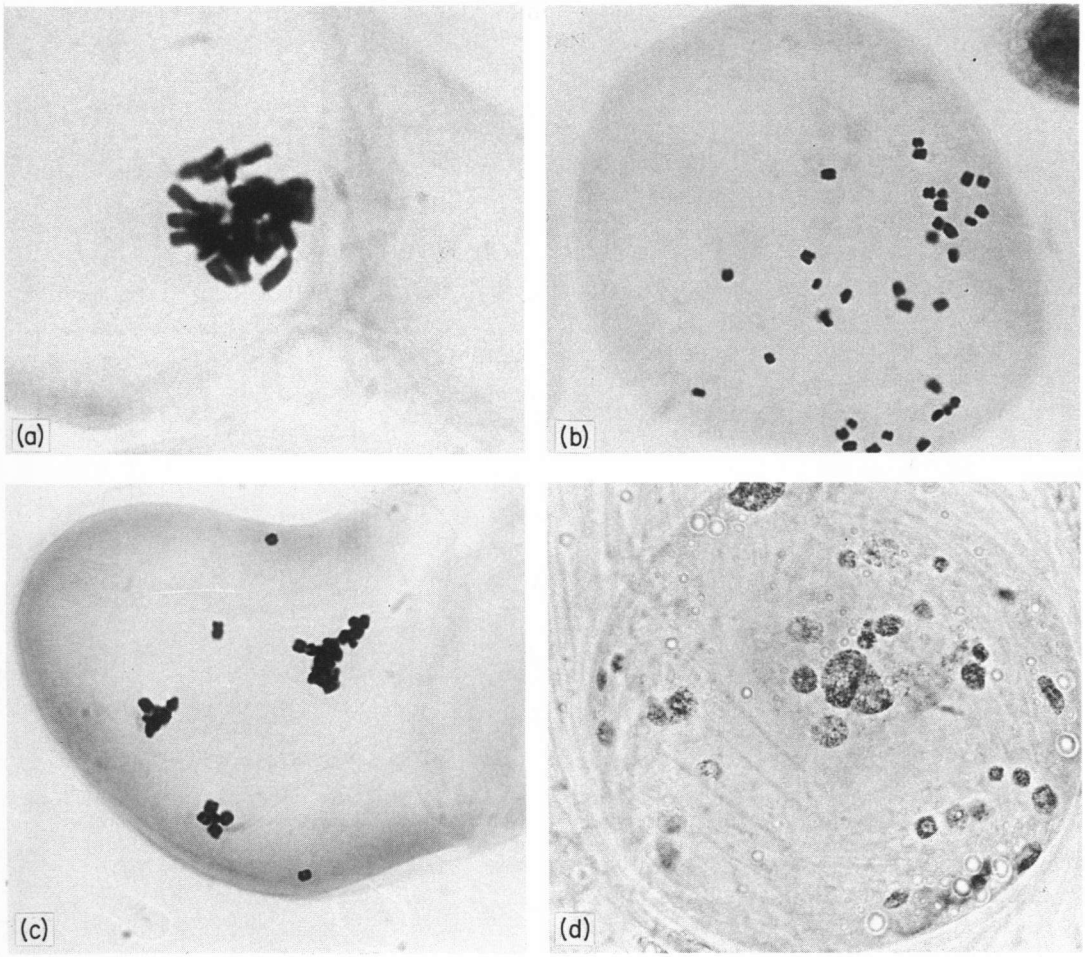


**Fig. 1.** (a) Photomicrographs of (a) *N. plumbaginifolia* cell with a ball metaphase (5 mM colchicine); (b) cell with scattered chromosomes (15 μM oryzalin); (c) cell with chromosome groups (32 μM APM); (d) cell with micronuclei (32 μM APM). (From Verhoeven *et al.* 1990.)

to the accumulation of cells in (prolonged) mitosis. Micronuclei appeared from 6 h onwards after the addition of the herbicide, resulting in a decrease in MI. The frequency of micronucleation rose sharply, however, and simultaneously micronuclei started fusing to form restitution nuclei, which eventually resulted in doubling of chromosome number (Figs 1 and 2) (Verhoeven 1989; Verhoeven, *et al.* 1990; Sree Ramulu *et al.* 1991a). Based on the finding that prolonged APM treatment induced micronuclei at a high frequency in plant cells compared to colchicine, it is suggested that the disturbance of the mitochondrial calcium uptake by APM (Hertel *et al.* 1980, 1981) is an important factor in the formation of micronuclei (Sree Ramulu *et al.* 1988a). Apart from the herbicide used, the frequency of micronucleation is influenced by many factors in plant cells. The most important factors, such as cell-division activity, species/genotype, plant tissue, cell synchronization, and sequential treatments are discussed below.

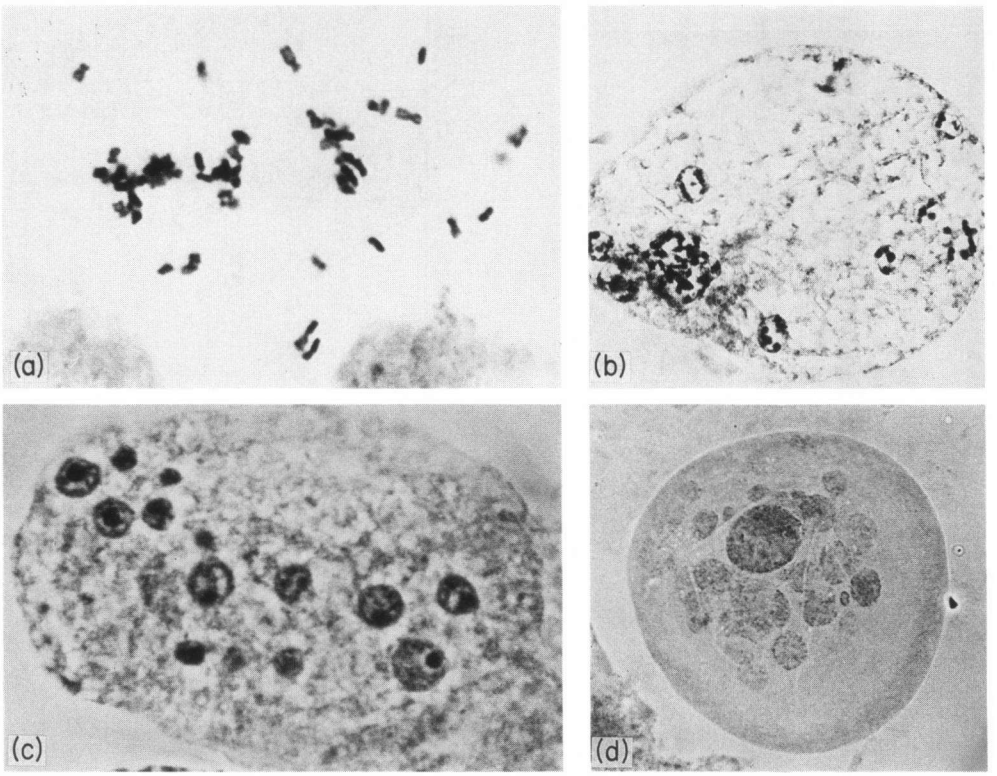


**Fig. 1.** (b) Chromosome scattering and micronucleation in potato: (a) Metaphase cell showing scattering and grouping of chromosomes (30 μM oryzalin); (b) cell showing chromosome decondensation and development of micronuclei (5 mM colchicine); (c) micronucleated cell showing nucleoli within the micronuclei (32 μM APM); (d) cell with several micronuclei (30 μM oryzaline). (From Sree Ramulu *et al.* 1991a.)

*Cell-division activity*

The growth rate of the cells is the most important parameter in the determination of the rate of micronucleation. The frequency of mitotic division of the suspension cells showed a strong correlation with the level of micronucleation (Sree Ramulu *et al.* 1988b,c). As micronuclei developed during division, a high division activity is obviously beneficial for the formation of a high number of micronucleated cells. The division frequency can be manipulated in several ways. Many cell suspension cultures responded to subculture by a spontaneous increase in mitotic index (MI) (which is a reflection of the momentary division activity) (reviewed in King 1980; Gould 1984). Starvation during the previous culture period increased the effect of subculture, as has been shown for *N. plumbaginifolia* (King 1980; Verhoeven 1989). Another method for increasing the MI is to maintain the cell suspension continuously in the logarithmic growth phase by subculturing for short intervals, e.g. 3 or 4 days in the case of *N. plumbaginifolia* (H. A. Verhoeven, unpublished results).

*Species/genotype*

Extensive comparisons have been made between cell suspension cultures of different species and genotypes for micronucleation after APM or oryzalin treatment (Sree Ramulu

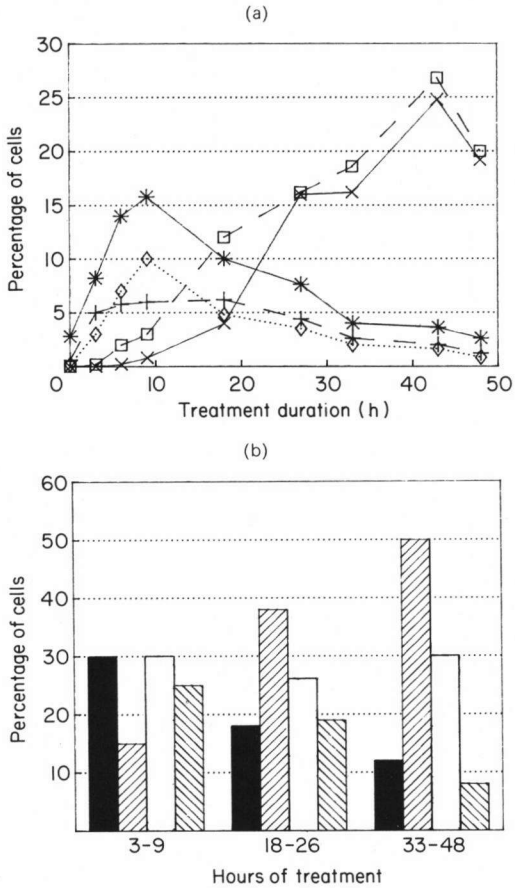


**Fig. 2.** (a) Time course of induction of micronuclei in relation to other mitotic events after the treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM). In this figure, C-metaphases (+) are blocked metaphase cells with well spread or partially clumped chromosomes arranged in a 'single group'; chromosome-groups (◇) are metaphase cells showing separate groups of chromosomes; micronuclei (□) are cells showing separate micronuclei; fused nuclei (×) are cells with lobed micronuclei. Mitotic index (MI): (*). (Based on data from Sree Ramulu *et al.* 1998a; see Verhoeven 1989.)

**Fig. 2.** (b) Relative percentage of micronucleated cells showing 2 (■), 3–5 (▨), 6–9 (□) and more than 9 (▧) micronuclei per cell after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for 3–9h (left), 18–26 h (middle) and 33–48 h. (Based on data from Sree Ramulu *et al.* 1988a; see Verhoeven 1989).

*et al.* 1988c, 1990, 1991a; Verhoeven *et al.* 1990). So far, all species tested responded with varying yields of micronuclei. The results show that identical treatments of different species or genotypes can result in different maximum levels of micronucleation. For example, *Solanum brevidens* responded to micronucleation with a low frequency, although the division activity of the suspension tested was sufficiently high (K. Sree Ramulu *et al.*, unpubl. results). This is probably due to a high rate of fusion of micronuclei forming restitution nuclei, indicating a shorter duration of the micronucleation stage in this species. The genotype-dependence of micronucleation is most strikingly illustrated by the different responses of various transformed potato cell lines, yielding, in general, higher frequencies of micronucleation, especially after *Agrobacterium rhizogenes* transformation (Sree Ramulu *et al.* 1990). This is probably related to the presence of the increased levels

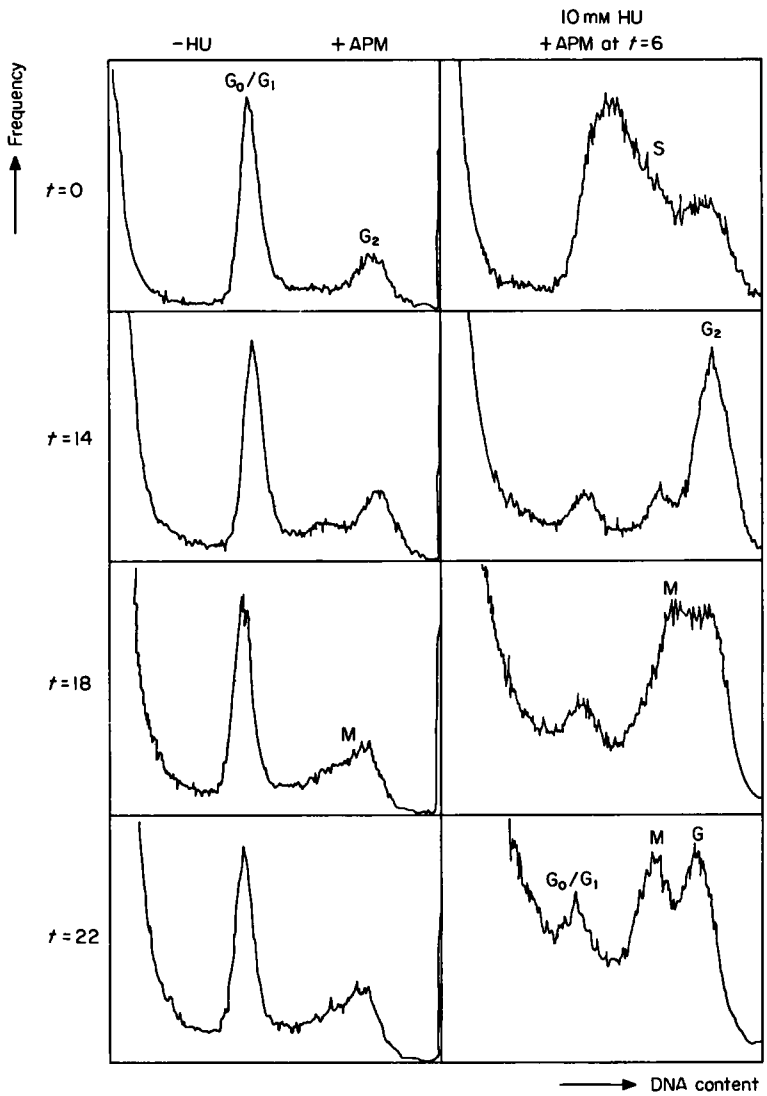


**Fig. 3.** Flow cytometric histograms of the nuclear DNA content of a cell suspension, without hydroxyurea (HU) treatment (left column), and a HU-treated cell suspension (right column). The DNA content is expressed in arbitrary units on the *x*-axis, and the number of nuclei is on the *y*-axis. One day after subculture, 10 mM HU was added to a suspension culture. After 24 h, inhibition was released by washing the treated culture and an untreated control suspension culture at $t=0$. Cycling cells were accumulated in metaphase after the addition of 32 μM APM to the washed cultures. Nuclei were isolated from small samples of the cell suspension at $t=0$, $t=14$, $t=18$ and $t=22$, and stained for flowcytometric DNA-content analysis. At the moment of washing ($t=0$), the untreated control (left half of the diagram) showed about 85% of the cells in $G_1/G_0$ (as estimated from the area under the peaks), whereas 10% was in $G_2$, and 5% in S-phase. The HU-treated culture (right half of the diagram) showed a completely different pattern, with at least 50% of the cells in S-phase, indicated by the appearance of many nuclei between $G_1$ and $G_2$. At $t=14$ h after washing, the control showed almost the same pattern as at $t=0$. The HU-treated culture, however, showed a great transition (i.e. more than 75%) of the cells into $G_2$-phase, with a small peak at lower DNA-contents, due to the extreme condensation of DNA in metaphase chromosomes. At $t=18$ and $t=22$, the number of mitotic cells becomes even more prominent, and exceeds the number of cells in $G_1$ and $G_2$ together. (From Verhoeven *et al.* 1991b.)

of hormones in the transformed tissues, favouring a higher division activity, and micronucleation.

*Plant tissue*

In addition to cell suspension cultures, plant tissues can also be used for micronucleation, and especially meristematic regions, such as shoot tips and root tips, are suitable. In most tissues, however, problems of chemical penetration and effective concentration arose due to the tight organization of tissues as compared to suspension cells. As a consequence, tissues must be treated for a longer period, and with higher concentrations of anti-microtubule agents than cell suspension cultures. When intact tissues are not required, it is possible to cut the tissue into small pieces, which can be treated more efficiently. Under appropriate treatment conditions, micronucleation can be induced at a high frequency in the meristematic zones of root tips (Sree Ramulu *et al.* 1990).

*Synchronization*

When a cell suspension or tissue progresses through the cell cycle synchronously, theoretically a maximum MI can be obtained. As non-cycling cells ($G_0$ phase of the cell cycle: many cells of plant cell suspensions are arrested in $G_0$) do not participate in mitosis, micronucleation will also be maximal in synchronous cell suspension. Synchronous division can be achieved by utilizing the naturally occurring cell synchrony in gametophytic tissues, such as anthers or meiocytes (Malmberg & Griesbach 1980), and root tips which show a peak in division activity around the beginning of daylight. Also freshly isolated protoplasts occasionally show a high degree of synchrony in their first division, as has been reported for *Brassica* protoplasts (Weber & Schweiger 1985).

As the block at metaphase by herbicide treatment is a temporary one (arrest at metaphase finally yields micronuclei, which fuse to form restitution nuclei that can enter S-phase, even in the presence of the herbicides), their action cannot be considered as a synchronization treatment. Synchronization can only be induced by an absolute blockage of cell cycle progression during at least one cell cycle. Treatment with DNA-synthesis inhibitors, such as hydroxyurea or aphidicolin, resulted in a blockage at the beginning of S-phase, which can persist during several cell cycles (Kihlman *et al.* 1966; Guri *et al.* 1984; Verhoeven *et al.* 1991a). After treatment, all actively cycling cells become arrested at the beginning of S-phase. When the block is released by washing the cultures free from the inhibitor, most of the cells resume the cell cycle, and enter S-phase simultaneously (Fig. 3). Due to differences among cells in the duration of G2-phase, this synchrony is reduced to some extent when the cells finally reach mitosis. In most cases, however, a considerable increase in the MI, together with a simultaneous occurrence of mitotis, has been observed. The metaphase inhibitor, which has been added to the fresh medium after washing the DNA-synthesis inhibitor, accumulates all cells in metaphase. In the case of herbicide treatments, this will finally lead to the synchronous transition of scattered metaphases to micronuclei in the treated cell suspension (see details in Verhoeven *et al.* 1991b).

*Sequential treatments*

As has been described earlier in mammalian cells, sequential treatment of cells with colcemid and cytochalasin-B resulted in an increase of the number of micronucleated cells. Human cells which are normally difficult to micronucleate with colcemid, can undergo micronucleation in the presence of cytochalasin-B. In both *N. plumbaginifolia* and potato, the frequency of micronucleated cells considerably increased after sequential treatment of

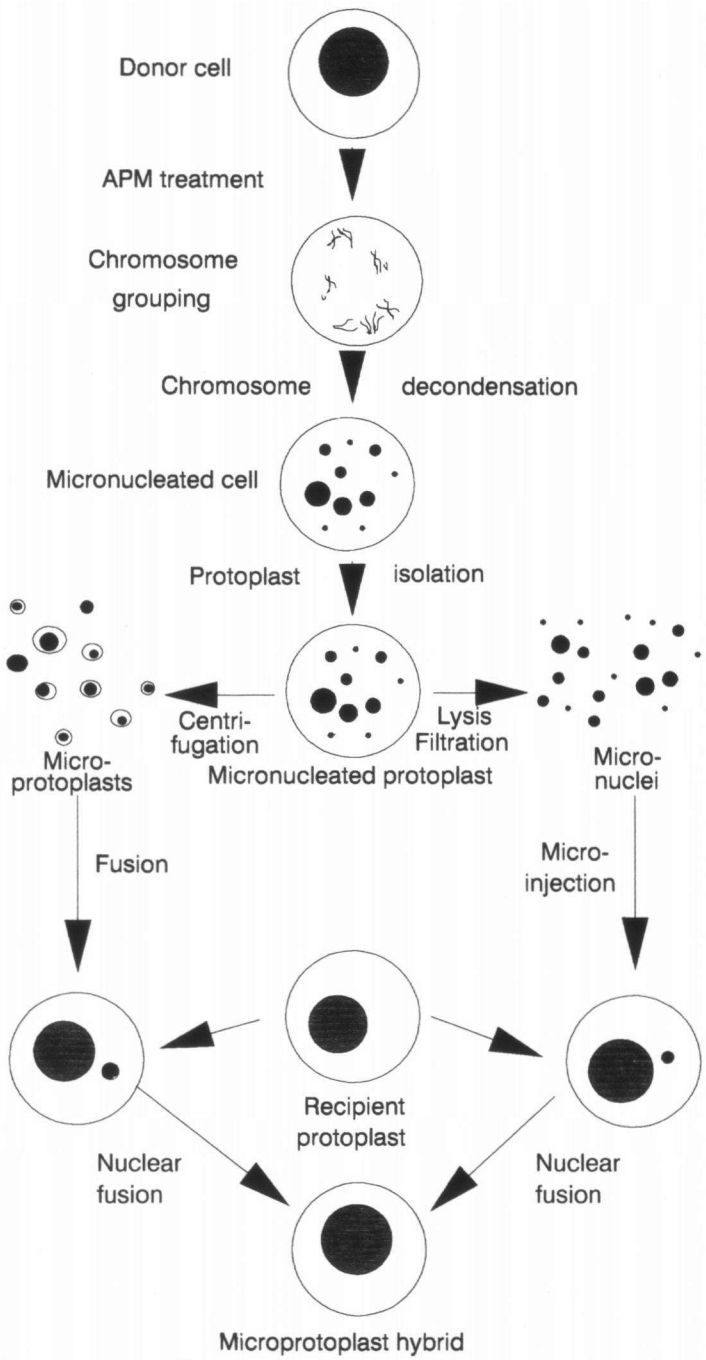


**Fig. 4.** A general scheme for partial genome transfer through micronuclei in plants. After inducing micronucleated cells by treatment of donor cell suspensions with an anti-MT agent (e.g. APM), microprotoplasts can be isolated and fused with the recipient protoplasts (left part of the figure), or micronuclei can be isolated and transferred by micro-injection (right part of the figure) in order to produce microcell hybrids (modified after Sree Ramulu *et al.* 1988c).

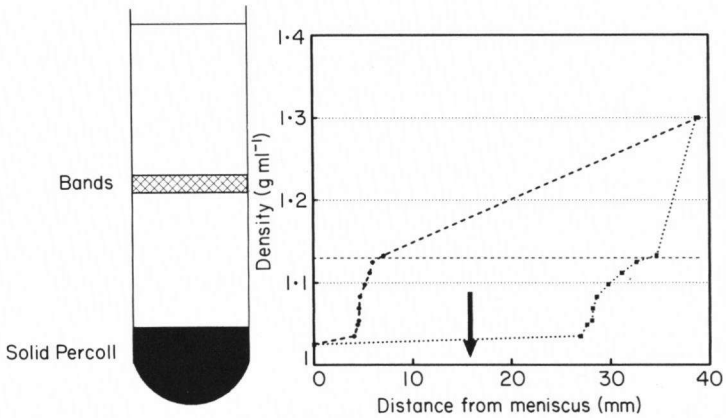


**Fig. 5.** Diagram of a centrifuge tube showing the position of the multiple bands containing microprotoplasts in a Percoll gradient after fractionation at 100,000 *g* (left part), along with the density profile (right part). The dashed line indicates the density profile of the gradient after 30 min preformation at 100,000 *g*, whereas the dotted line indicates the density profile after 60 min, at the end of the protoplast fractionation. The arrow illustrates the position of the band(s). (From Verhoeven & Sree Ramulu 1991.)

APM and cytochalasin-B (Sree Ramulu *et al.* 1990). In accordance with the hypothesis that endogenous calcium concentration plays a role in the formation of nuclear membranes after division (Hepler 1980; Wolniak *et al.* 1983; Wolniak & Bart 1985), treatments with chelating agents increased the frequency of micronucleation in human cells (Matsui *et al.* 1982). A similar effect has been observed in plant cells treated with EGTA and APM (K. Sree Ramulu *et al.*, unpublished results).

## METHODS

A general protocol of partial genome transfer through fusion of microprotoplasts or micro-injection of isolated micronuclei is schematically presented in Fig. 4.

### *Isolation of micronucleated protoplasts*

Protoplasts were isolated from a micronucleated cell suspension after 3 h incubation in a cell-wall degrading enzyme mixture. The herbicide used for the induction of micronuclei, was added to the enzyme mixture to prevent fusion of micronuclei during cell-wall degradation (De Laat *et al.* 1987). After cell-wall digestion, protoplasts were purified by repeated washing with iso-osmotic mannitol or saline solutions, all containing the herbicide. The purified protoplast suspension was cleared from damaged protoplasts by flotation on an iso-osmotic sucrose solution. Intact protoplasts were collected from the surface of the sucrose as a highly dense suspension. The micronucleation index increased significantly during protoplast isolation, due to the influence of the treatment on the cytoskeleton and chromosome decondensation (K. Sree Ramulu *et al.*, unpublished results).

### *Micronuclei*

*Isolation.* Incubation of micronucleated protoplasts in ice cold modified nuclear isolation buffer (Saxena & King, 1989) induced further scattering of micronuclei in the protoplasts.

Protoplasts were lysed by gentle passage through a hypodermic needle. Release of micronuclei was facilitated by including low concentrations of cytochalasin-B in the nuclear isolation buffer, which effectively destroyed the microfilaments of the protoplasts, resulting in immediate interruption of cytoplasmic streaming. This treatment yields cleaner nuclei, i.e. micronuclei with less adhering organelles.

*Enrichment.* The isolation protocol results in a suspension containing different types of nuclei: $G_1$ or $G_2$ nuclei, isolated metaphase chromosomes, separated sister chromatids and micronuclei. Subdiploid micronuclei contain a variable amount of DNA, equivalent to different numbers of chromosomes. For partial genome transfer through micronuclei, the $G_1$ and $G_2$ nuclei should be removed. This was achieved by passing the nuclear suspension through a series of nylon gauze with decreasing mesh sizes. Flow cytometric analysis of the fractions stained with a stoichiometric DNA fluorochrome confirmed that $G_1$, $G_2$ and the bigger sub-diploid micronuclei (i.e. micronuclei with a DNA less than the $G_1$-level) were separated from the smaller sub-diploid micronuclei.

*Transfer.* Protocols are being developed for micro-injection of micronuclei into a whole cell or protoplast. Micronuclei can be taken up from a suspension of isolated micronuclei, or removed from micronucleated donor protoplasts by direct cell to cell transfer. Micro-injection, combined with confocal laser scanning microscopy and vital staining (Van der Valk *et al.* 1988), offers certain unique advantages, i.e. direct observations of the early events leading to fusion of the introduced micronucleus, followed by the behaviour of the donor chromosomes during the first cell division. In this way, it becomes possible to study the interactions of the two genomes by direct observation, provided the morphology of the donor and the recipient's chromosomes can be distinguished.

Recent studies in plants suggest that it is also possible to introduce isolated nuclei by PEG-induced uptake, and that part of the DNA becomes integrated in the recipient genome (Saxena & King, 1989). PEG-induced uptake is a process, in which fusion of the outer nuclear membrane with the plasmamembrane takes place, resulting in direct DNA uptake. The process is different from transfer by micro-injection because the latter method introduces a (micro)nucleus with an intact nuclear envelope into the recipient. The feasibility of micro-injection in plants has already been demonstrated for the direct cell to cell transfer of mitochondria (Verhoeven & Blaas 1988), and the micro-injection of isolated metaphase chromosomes (De Laat & Blaas 1987; Griesbach 1987). As the transfer of isolated micronuclei into mammalian cells did not result in chromosome fragmentation, it is envisaged that the same applies to transfer of micronuclei into plant cells. These aspects are currently under investigation.

*Microprotoplasts*

*Isolation.* In order to isolate microprotoplasts from micronucleated protoplasts, the protoplasts have to be fragmented, while maintaining the integrity of the plasma membrane. The presence of microfilaments opposes changes of shape of protoplasts under the influence of high ***g***-forces during ultracentrifugation, resulting in a low yield of subprotoplasts (Lörz *et al.* 1981). Therefore, the microfilaments have to be disrupted by incubation with cytochalasin-B, combined with a herbicide treatment to avoid reformation of microtubules in micronucleated protoplasts. For fractionation, protoplasts were loaded on top of a continuous gradient of iso-osmotic Percoll. Density marker beads were included in the tarra tube, allowing the calibration of gradient density profile

at the end of the run. Protoplasts were fractionated by centrifugation in a swing out rotor at 100,000 *g*, resulting in the formation of several bands in the gradient (Fig. 5). The bands contained successively vacuoplasts, evacuolated protoplasts, and microprotoplasts from top to bottom. Microdensitometric analysis of various fractions revealed that there were up to 40% microprotoplasts in the lowest visible band, whereas there were about 10% microprotoplasts in the largest band. This protocol of microprotoplast isolation, initially developed for *N. plumbaginifolia* (Verhoeven *et al.* 1988, 1991b; Verhoeven & Sree Ramulu 1991), is also suitable for other plant species, such as potato (Sree Ramulu *et al.* 1991b; K. Sree Ramulu *et al.* unpubl. results) and sugarbeet (I. Famelaer, unpublished results).

*Enrichment.* Although there are sufficient microprotoplasts in the various fractions of the gradient, the presence of protoplasts or subprotoplasts with intact or larger genomes is undesirable in mass fusion experiments. Subprotoplasts with larger genomes can be removed by centrifugation on a discontinuous gradient. Due to the presence of relatively more DNA, subprotoplasts with larger genomes or microprotoplasts with a high number of chromosomes will have a higher specific density. As a consequence, the smaller subdiploid microprotoplasts (i.e. subprotoplasts containing micronuclei with a low chromosome number) will be collected at a lower density than the larger ones. Therefore, the choice of gradient densities is a critical parameter for the quality of separation. However, efficiency in terms of yield and purity, (i.e. the frequency of smaller subdiploid microprotoplasts) is low due to the occurrence of many intermediate subprotoplasts, with varying amounts of cytoplasm (Sree Ramulu *et al.* 1991b). Microprotoplasts can also be separated by sequential filtration through nylon gauze of different mesh sizes (Sree Ramulu *et al.* 1991b). By choosing for the last filter, a mesh size slightly larger or equal to the average size of $G_1$-nuclei, it was possible to eliminate virtually all protoplasts or subprotoplasts with intact genomes, maintaining a high yield of small microprotoplasts, i.e. without loss due to filter clogging.

*Fusion.* In mammals, either inactivated Sendai virus, or PEG has been used as fusogen for mass fusion of microcells of donor cell lines with whole cells of recipient cell lines (reviewed in Fournier 1982). PEG at 40% has been applied successfully with fusion frequencies equivalent to those obtained after whole cell fusions, and is being used routinely (Fournier 1982; Fournier & Frelinger 1982; Fournier & Moran 1983; Landolph & Fournier 1983; Killary *et al.* 1986; Schultz *et al.* 1987). In plants, investigations are in progress using a similar method of mass fusion using PEG (K. Sree Ramulu & P. Dijkhuis; I. Famelaer, unpublished results). In these experiments, the availability of genetic markers, including selectable markers such as kanamycin resistance, are highly desirable for selection of heterokaryons and microprotoplast hybrids as well as for gene localization. Therefore, several genetic markers have been recently introduced into potato (De Vries-Uijtewaal *et al.* 1989; Gilissen *et al.* 1991) and *N. plumbaginifolia* (De Laat & Blaas 1987) which are being used as donor parents for fusion. In addition, experiments are in progress on other intergeneric fusion combinations, using microprotoplasts of sugarbeet as donor and a nitrate reductase deficient line of *N. plumbaginifolia* as the recipient partner (I. Famelaer, unpublished results). Electrofusion is less suitable to fuse donor microprotoplasts with recipient whole protoplasts, because of the large difference in size (the diameter of a microprotoplast is about 10% of that of a normal protoplast). Since the effect of a high frequency electric field is proportional to protoplast diameter (Zimmermann 1982), alignment is poor. Even if pearl chain formation occurs, the

interaction between the large recipient protoplast and the small donor microprotoplast is so weak, that any recipient protoplast will replace the microprotoplast. Moreover, the effect of DC-pulses on the microprotoplast membrane will be insufficient to cause membrane breakdown, which is a prerequisite to induce fusion. Electrofusion with highly inhomogeneous fields, e.g. caused by needle electrodes, may circumvent these problems. This approach is feasible only in combination with individual, single cell fusion systems (Koop *et al.* 1983).

## PERSPECTIVES

Application of micronucleus technology for limited chromosome transfer offers a wide range of perspectives in plants, as has been demonstrated in mammalian cells: (a) transfer at cellular level, of specific chromosomes across sexual borders or taxonomic distance, overcoming incongruity or incompatibility barriers, (b) analysis of gene linkage and regulation of gene expression, (c) fine structural mapping of chromosomes (regional or deletion mapping), (d) construction of chromosome-specific DNA libraries for isolation and cloning of genes, using flow-cytometry and molecular biological techniques. Furthermore, transfer of a limited number or even single chromosomes to phylogenetically remote species may provide a new tool for studying genome interactions at the somatic level.

In addition to the above mentioned perspectives, new methods for plant breeding would become feasible. A micronucleus will always contain a multiple of two identical sister chromatids, since the centromeres do not divide during micronucleus formation. This has important implications for the genetic constitution of plants, which will eventually be regenerated from micronucleus hybrid clones. In any case, the number of chromosomes in the hybrid will remain even in number, with at least two copies of the introduced chromosomes. As a consequence, fertility problems in the regenerated hydrids may be lower or absent, compared to asymmetric hybrids with uneven numbers of donor chromosomes. This feature can be essential for the use of hybrids in conventional breeding programmes.

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