INDUCTION AND CHARACTERIZATION OF MICRONUCLEI IN PLANT CELLS

Perspectives for micronucleus-mediated chromosome transfer

INDUCTIE EN KARAKTERISERING VAN MICROKERNEN IN PLANTECELLEN

Perspectieven voor chromosoom overdracht via microkernen

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INDUCTION AND CHARACTERIZATION OF MICRONUCLEI IN PLANT CELLS

Perspectives for micronucleus-mediated chromosome transfer

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 13 juni 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ISN: 272657

STELLINGEN

1

Het feit dat het karyotype van een cybride niet te onderscheiden is van de kerndonor sluit de aanwezigheid van delen van het nucleaire genoom van de organeldonor niet uit.
(Ichikawa et al., 1987) Theor. Appl. Genet. **74:** 746-752.

2

Voor detectie van donor DNA in de recipient na asymmetrische fusie van bestraalde donor-protoplasten met onbestraalde acceptor-protoplasten verdient *in-situ* hybridisatie met soortspecifieke, repeterende probes de voorkeur boven dot-blot hybridisaties.
(Imamura et al., 1987) Theor. Appl. Genet. **74:** 445-450.

3

Oryzaline is de metafase remmer bij uitstek voor het isoleren van metafase chromosomen uit plantecellen. (Dit proefschrift).

4

Om het chromosoom aantal van planten te verdubbelen, kan beter van oryzaline gebruik gemaakt worden dan van colchicine. (Dit proefschrift).

5

De door Hertel waargenomen effecten van oryzaline en trifluralin worden verkeerd geïnterpreteerd.
(Hertel et al., 1981) FEBS Letters **127**: 37-39.

6

Intacte organellen kunnen rechtstreeks vanuit een donor-cel of -protoplast naar een recipiënte plantecel worden overgedragen via microinjectie. (Dit proefschrift).

7

De specifieke drie-dimensionale organisatie van chromosomen in de interfase kern van verschillende neurale celtypen is een aanwijzing voor een algemeen regulerende functie van de drie-dimensionale structuur bij de genexpressie.
(Manuelidis and Borden, 1988) Chromosoma **96:** 397-410.

8

Het veelvuldig voorkomen van soortspecifieke, repeterende DNA sequenties, wijst op een belangrijke functie van deze sequenties bij de soortbepaling.

9

Het feit dat de nog niet uitgestorven diepzee Coelacanth *Latimeria chalumnae* zijn kwastvinnen niet gebruikt om te lopen, is van ondergeschikt belang in de diskussie over een mogelijke verwantschap van kwastvinnigen met land-vertebraten.
(Forey, 1988) Nature **336**: 727-732.

10

De meest waardevolle DNA-sequenties in genetisch gemanipuleerde planten zullen voorlopig de "copyright-sequenties" zijn, die gebruikt zullen gaan worden om genenpiraterij tegen te gaan door genetisch gemanipuleerde planten te beschermen tegen illegaal kopiëren.

11

Introductie van met microprocessors uitgeruste meetapparatuur, waarvan de basis principes en de toegepaste gegevensverwerking niet bekend zijn bij de gebruikers, verhoogt het risiko op "Desk-top Research", waarbij de resultaten op onverantwoorde wijze verwerkt worden.

12

Het geringe succes van "Artificial Intelligence" is vaak te wijten aan onderschatting van de eigen intelligentie door de (potentiële) gebruiker.

13

Het feit dat de meeste sportieve auto's zijn voorzien van opklapbare koplampen, wekt ten onrechte de indruk dat de wetten van de aërodynamica in het donker niet gelden.

14

De succesvolle introductie van de vele "light food" artikelen is een onverteerbare zaak in een wereld, waar minstens 75% van de bevolking honger lijdt.

Stellingen, behorende bij het proefschrift " **Induction and characterization of micronuclei in plant cells: perspectives for micronucleus-mediated chromosome transfer**",
door Harrie A. Verhoeven. Wageningen, 13 juni 1989

The cover shows a three dimensional reconstruction of a micronucleated suspension cell of *N.plumbaginifolia*, visualized by confocal laser scanning microscopy after vital staining with Acridin Orange.

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Contents

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Maart 1989.

CHAPTER 1

GENERAL INTRODUCTION

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Outline

The aim of this study is to investigate the induction, characterization, isolation, and transfer of micronuclei in plant cells and to evaluate the possible application of micronuclei and microprotoplasts for transfer of chromosomes.

Chapter one is an overview of the current knowledge on organelle transfer in both mammalian and plant cells.

Chapter two deals with the induction and characterization of micronuclei in plant cell suspension cultures by the herbicide amiprophos-methyl (APM).

The effects of APM on mitotic divisions of several sources of plant material, and their effects on chromosome numbers and cellular DNA content are presented in chapter three.

To obtain more information on the mechanism which is involved in the formation of micronuclei, the effects of various spindle toxins on metaphase arrest and micronucleus formation are compared (chapter four).

The influence of cell cycle manipulation by DNA-synthesis inhibition on the mitotic index and the formation of micronuclei is the subject of chapter five.

Chapter six describes the isolation and characterization of microprotoplasts from micronucleated suspension cells.

The possibilities for organelle transfer by microinjection are presented in chapter seven.

The results and conclusions are summarized in chapter eight.

PARTIAL GENOME TRANSFER

Introduction

In this chapter an overview is given of the current state of both animal and plant cell biology with respect to partial genome transfer. The aim of this study is the development of methods for partial genome transfer in plants by other means than transfer of cloned genes. The application of molecular techniques for the isolation, cloning and transfer of identified single genes is not considered within the scope of this thesis.

Partial genome transfer can be accomplished by somatic cell hybridization, by microcell-mediated chromosome transfer, by chromosome-mediated gene transfer and by DNA-mediated gene transfer. The complexity of the genetic information is an important factor in deciding which transfer system will be used. In somatic hybridization and microcell-mediated chromosome transfer, where isolated protoplasts (plant cells from which the cell wall has been removed by enzymatic digestion of the celluloses and pectins) with intact plasmamembranes function as carrier, fusion will be applied. For transfer of chromosomes or DNA fragments, microinjection, chemically induced uptake, or electroporation can be used. In this overview, the differences between the carrier-systems of genetic information will be discussed, along with the techniques available for achieving transfer.

Somatic cell hybridization.

By somatic hybridization, induced either by chemical treatments, or by electrofusion, two complete genomes, including the cytoplasmic organelles, are joined in the fusion product. The chromosome number of the resulting somatic hybrid will be the sum of the chromosome numbers of the two parent cells, and the organelles from both parents will be present. During the first divisions of the fusion product, the composition of the cells with regard to chromosome composition, mitochondria and eventually plastids will be determined, and remain stable for the next cell generations. In some cases, when the two genomes are closely related, the original fusion product can persist with minor changes, as is the case in *Solanum tuberosum* (+) *Solanum phureja* fusions (Pijnacker et al., 1989), *Solanum brevidens* (+) *S.tuberosum* fusions (Austin et al., 1985). The plants, regenerated from these fusion products, were fertile, notwithstanding the fact that in several fusion products, one chromosome (e.g. chromosome 2 from *S.phureja*, carrying the NOR region) was eliminated. When the two species are more distantly related, unilateral elimination will occur. In this case the chromosomes from one of the fusion partners will be eliminated preferentially (the "segregant" set), whereas the chromosomes from the other partner will all be retained (the "retained" set, Graves, 1984), as is the case with the human chromosomes in somatic rodent-man

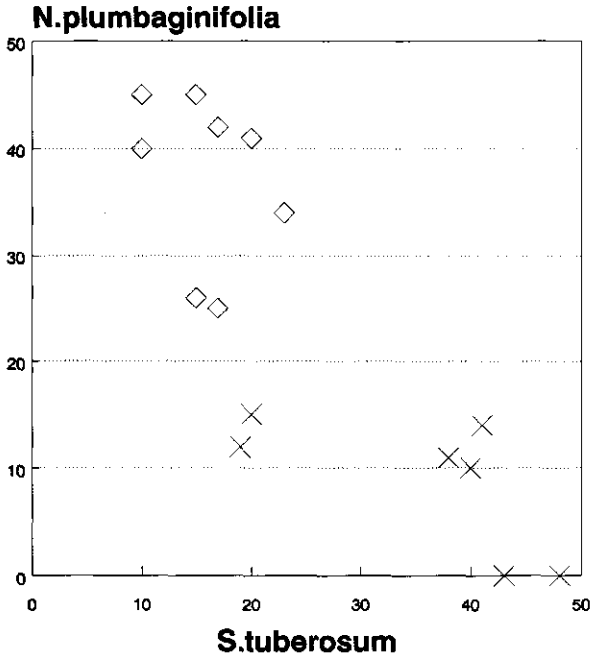

Fig. 1. Diagram, showing the chromosome numbers of somatic hybrid calli, obtained after fusion of diploid *N.plumbaginifolia* (20 chromosomes) with diploid potato (*S.tuberosum*, 24 chromosomes) protoplasts. Chromosome counts were determined from Feulgen stained squash preparations of regenerated calli. (Gilissen et al., 1989).

× Full *S.tuberosum* set

◇ Full *N.plumbaginifolia* set

fusions (Weiss and Green, 1967; Ruddle and Creagan, 1975). In the case of fusions between *S.tuberosum* (+) *Nicotiana plumbaginifolia*, no preferential loss of chromosomes from either species was observed in regenerated plants (de Vries, 1987). Although the mechanism of chromosome elimination is still obscure, a correlation with the direction of chromosome elimination and the ploidy level of the partner genomes was found in a series of mouse-hamster hybrids. Only when the ratio of the mouse:hamster genomes was below 1, the hamster chromosome set could be retained completely, and the mouse chromosomes were eliminated (Graves, 1984). A similar observation has been made recently with somatic hybrids between diploid *N.plumbaginifolia* and dihaploid *S.tuberosum* (Gilissen et al., 1989). Two types of hybrids were found, one with excess *N.plumbaginifolia* chromosomes and with an incomplete set of potato chromosomes; and the other type with excess *S.tuberosum* chromosomes and few *N.plumbaginifolia* chromosomes (Fig. 1).

Recent cytological work on the sexual hybrids between *Hordeum vulgare* and *Zea mays* has shown, that there is a preferential spatial arrangement of two incompatible genomes, which predicts which of the two genomes will be eliminated (Bennett, 1987; 1988). The genes on the chromosomes in the periphery of the interphase nucleus are expressed, but they appear to be more sensitive to chromosome elimination. Species specific chromosome arrangement is also observed in somatic hybrids between *Atropa belladonna* and *Nicotiana chinensis* (Gleba et al., 1983), and could be eliminated only by repeated treatments with colchicine. In this case, no correlation was noted between chromosome orientation and unidirectional chromosome elimination. A similar phenomenon is observed in *Hordeum* X *Secale* hybrids (Schwarzacher-Robinson et al., 1987). A

genetically controlled spatial relationship appears to exist between the centromeres in normal metaphase plates (Heslop-Harrison and Bennett, 1983).

Chromosome elimination of one of the partners can be directed and stimulated by gamma-irradiation. In this way, the chromosomes of the donor will be fragmented, and according to cytological observations, only fragments with functional centromeres are maintained stably during mitosis, while all other fragments appear to be lost. In most cases, only a few additional fragments and even intact-looking chromosomes were observed in the fusion product (Bates, 1987; Gleba et al., 1988). Alternatively, fragments from the irradiated genome incorporate into the recipient genome as has been demonstrated by hybridization with species-specific highly repetitive DNA-probes (Imamura et al., 1987; Piastuch and Bates, 1988).
The karyotypical analysis of the obtained hybrids is complicated, and often impossible, due to chromosome eliminations, rearrangements, deletions and insertions. It has been shown by in-situ hybridization with species-specific, highly repetitive DNA-probes, combined with fluorescent labelling, that large chromosome fragments of the heavily irradiated *N.plumbaginifolia* donor have been integrated into the *N.tabacum* acceptor genome (Piastuch and Bates, 1988). In this case, the metaphase plate did not show any significant aberrations in the karyotype of *N.tabacum*.

Beside the nuclear genes, also transfer of cytoplasmic organelles is accomplished by somatic hybridization (reviewed in Galun and Aviv, 1986). Organelles from the two parents are sorted out in most fusion products during the first divisions after fusion, and chimaeric tissues will be obtained (reviewed in Fluhr, 1983). Although recombination between the two parental mitochondria has been proposed as an explanation of the occurrence of new RFLP bands in some recovered fusion products (Chetrit et al., 1985), recent work has demonstrated that interspecific mitochondrial recombination is a rare event (Kemble, 1988). A possible explanation for the new bands in the mitochondrial RFLP patterns is recombination within the mitochondrial circular genome. This is facilitated by the large numbers of copies of the genome present in one mitochondrion (Morgan and Maliga, 1987). Mitochondrial recombination is also observed after prolonged in-vitro culture (Shirzadegan et al., 1989). Transfer of spontaneously occurring mitochondrial plasmids is proposed as an alternative, explaining the new RFLP bands (Kemble, 1988).

For chloroplasts, sorting out is observed in most cases after somatic hybridization. In some rare cases, both parental chloroplasts coexist for a longer time, giving rise to mosaic chimaeric tissues when they sort out at a later time (Fluhr et al., 1983; reviewed in Fluhr, 1983). The occurrence of such mosaics in sexual crossings of *Oenothera* indicates that two different chloroplasts can persist during many divisions. Alternatively, transfer of *Chlamydomonas* chloroplasts into *Daucus* protoplasts by fusion (Fowke et al., 1979) has shown that in case of excessive incompatibility between organelles and nuclear genes, the introduced chloroplasts degenerate.
Specific transfer of organelles can be obtained by heavy irradiation of the donor species, resulting in a complete fragmentation of the nuclear genome, while only introducing a relatively small number of fragmented organellar genomes (Menczel et al., 1982; Menczel, 1984). Although this procedure seems to be a straighforward way for organelle transfer, and is widely used as such (Aviv et al., 1980; Sidorov et al., 1981; Aviv et al., 1984; Aviv and Galun, 1986; Glimelius and Bonnett, 1986; Ichikawa et al., 1987; Barsby et al., 1987; Menczel et al., 1987), it has the disadvantage of introducing

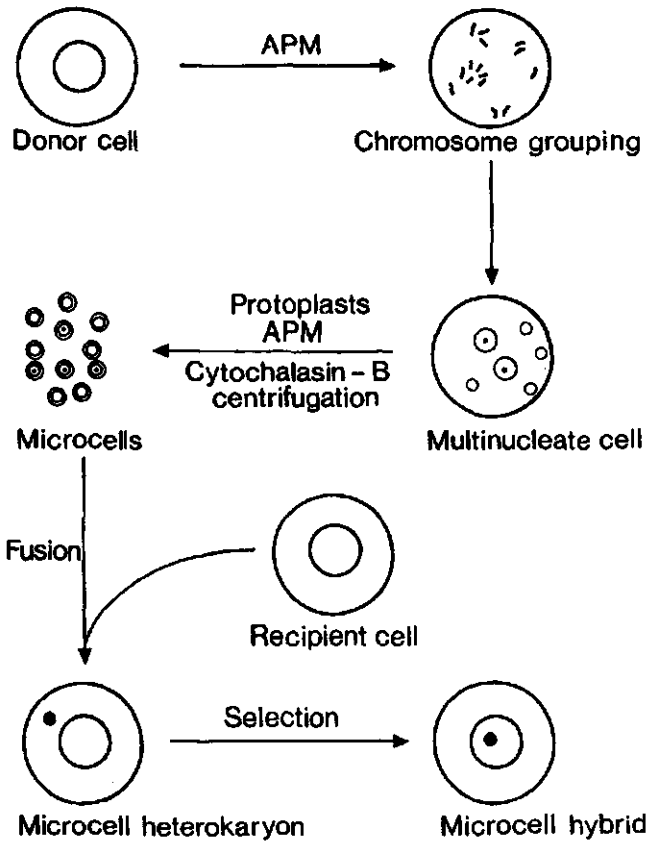

Fig. 2. Schematic diagram, showing the basic steps in microcell fusion experiments. Chromosomes become scattered through the cell by incubation with a spindle toxin (APM in this case), and form micronuclei. Multinucleate protoplasts are isolated from the treated suspension cells, and they are subjected to centrifugation in the presence of cytochalasin-B, and subprotoplasts are formed. Subprotoplasts containing micronuclei are isolated, and fused to recipient protoplasts. The microprotoplast hybrids are selected and regenerated.

nuclear fragments as well (see discussion earlier in this chapter). The use of cytoplasts, which are enucleated cells or protoplasts, as organelle donor avoids this risk (Wallin et al., 1979; Lörz et al., 1981; Maliga et al., 1982). However, cytoplast preparations are often contaminated with miniprotoplasts (evacuolated protoplasts), introducing the need for selection of the desired fusion products afterwards.

Microcell-mediated chromosome transfer.

The number of transferred chromosomes can be limited by using microcells as donor in fusion experiments (Fig. 2). Microcells are prepared by prolonged exposure to metaphase inhibitors, such as colchicine or Colchemid, or by treating mitotic cells with cytochalasin-B (Fournier, 1981). The

metaphase chromosomes become scattered throughout the cell, and decondensate individually or in small groups. A nuclear envelope is formed around the decondensating chromosomes, resulting into micronuclei, with varying numbers of chromosomes per nucleus. By centrifugation of micronucleated cells, microcells can be isolated, with interphase micronuclei containing a subset of the chromosomes. By fusion with recipient cells, karyologically simple hybrids can be constructed, in which the donor chomosomes are maintained mitotically stable (Lugo and Fournier, 1986). When a suitable recipient is used, with a deficiency, which can be complemented by a gene, located on the desired donor chromosome, gene mapping becomes possible. In this way, considerable progress has been made in the localization of genes on human chromosomes by fusion with rodent recipient lines (Dhar et al., 1984; Saxon et al., 1985; Lugo and Fournier, 1986).

Sofar, no such system was available for plant cells, due to the lack of micronuclei-inducing agents, since both colchicine and Colchemid did not induce a significant number of micronuclei in plant cells.

Chromosome-mediated gene transfer (CMGT).

When isolated metaphase chromosomes are transferred to a recipient cell by chemically induced uptake, fragments of the chromosomes become incorporated into the recipient genome (Klobutcher and Ruddle, 1981). In this way, chromosome-specific inserts can be obtained, which still show a more or less syntenic arrangement, i.e. linked genes are found in a linked position in most of the inserts. This opens up the opportunity to select for transformants, expressing a complemented deficiency, in which also linked genes are present. By this procedure, non-selectable properties could be mapped onto specific chromosomes (de Jonge et al., 1985; Sukoyan et al., 1984). The disadvantage of this approach lies in the fact that linkage is not necessarily preserved during chromosome fragmentation and incorporation. In the case, where two independent fragments are cointegrated, a misleading linkage will be the result, whereas in case of intra-fragmental deletions, linkage will not be detected at all. Nevertheless, CMGT has advanced the mapping of the human genome considerably, especially in those cases where important genes were localized in the immediate vicinity of selectable genes (Klobutcher and Ruddle, 1981; Sukoyan et al., 1984; de Jonge et al., 1985; Porteous, 1987). Even though this technique has proved to be of great value for gene mapping in animal cell lines, it has been almost neglected in somatic plant genetics. Sofar, few cases have been reported, in which isolated metaphase chromosomes have been used as donor material (Griesbach et al., 1982; Griesbach, 1987; de Laat and Blaas, 1987; Dudits, 1988). In the first case, lily chromosomes were introduced into recipient protoplasts by PEG-induced uptake, without any selectable marker, and divisions were never observed. Both Griesbach and De Laat employed microinjection as means to introduce the donor chromosomes into the recipient cells. Only Griesbach reported on the resulting putative hybrid lines obtained in this way, and showed that although no cytological evidence for the successfull transfer could be obtained, the hybrids expressed genes of the flavonoid biosynthesis pathway, not previously present in the recipient cells of *Petunia hybrida*. Dudits reported on the transfer of isolated chromosomes from a metothrexate resistant carrot cell line into protoplasts of *Triticum monococcum*, which transferred the resistance, but no carrot chromosomes could be retrieved in most metothrexate resistant colonies, except for one colony, which contained one additional carrot chromosome.

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CHAPTER 2

EFFICIENT INDUCTION BY AMIPROPHOS-METHYL AND FLOW CYTOMETRIC SORTING OF MICRONUCLEI IN NICOTIANA PLUMBAGINIFOLIA.

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EFFICIENT INDUCTION BY AMIPROPHOS-METHYL AND FLOW CYTOMETRIC SORTING OF MICRONUCLEI IN NICOTIANA PLUMBAGINIFOLIA.

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Abstract

Amiprophos-methyl (APM) is a potential herbicide which acts at the level of microtubules. By exposure of suspension cells of *Nicotiana plumbaginifolia* to this agent, a high degree of metaphase arrest was observed and single as well as groups of chromosomes were scattered throughout the cell, offering good prospects for application in cytology and chromosome isolation. After prolonged exposure to APM, the chromosomes decondensed and micronuclei were formed. Based on their DNA content, the micronuclei were sorted by flow cytometry. Prospects for application of isolated micronuclei for partial genome transfer and gene mapping are discussed.

Key words: Amiprophos-methyl Flow cytometry micronucleus (formation, sorting) Microtubule poison *Nicotiana plumbaginifolia.*

Introduction

Both for cytological research and for chromosome isolation in plants, stickiness of chromosomes due to metaphase arrest by spindle toxins is a major problem. In particular, the isolation of individual chromosomes for sorting by flow cytometry (De Laat and Blaas, 1984) and limited genome transfer by transplantation of metaphase chromosomes (Malmberg and Griesbach, 1980; Szabados et al., 1981; De Laat and Blaas, 1987) are largely hindered by the tight association of chromosomes. Micronuclei can be a promising alternative for limited genome transfer by either transplantation of isolated micronuclei containing one or a few chromosomes, or via fusion of microcells with recipient protoplasts. The occurrence of micronucleate cells in mammalian cell cultures after incubation with colchicine or Colcemid is known for several years (Phillips and Phillips, 1969; Ege and Ringertz 1974; Fournier and Ruddle, 1977; Sekiguchi et al., 1978). Several investigators have isolated a small subset of the total genome by applying enucleation procedures to micronucleate cells which resulted in microcells containing varying number of chromosomes (Ege et al., 1977; McBride and Peterson, 1980; Fournier, 1981). By subsequent microcell fusions with recipient cells, the transfer of a limited number of chromosomes was achieved. Amiprophos-methyl (APM) belongs to the class of phosphoric amide herbicides which are considered anti-microtubule drugs in plant cells because of the disappearance of microtubules and of the disruption of microtubule-dependent processes (Sumida and Ueda, 1976; Kiermayer and Fedtke, 1977; Robinson and Herzog, 1977; Quader et al., 1978; Koop and Kiermayer, 1980). Recently, it has been shown that APM is a specific drug which directly poisons microtubule dynamics in plant cells (Morejohn and Fosket 1984). We have observed that APM treatment of cell

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suspensions of *Nicotiana plumbaginifolia* resulted in well scattered metaphase chromosomes allowing the isolation of single chromosomes (Verhoeven et al., 1986; 1987). In this paper we describe the mass induction of micronuclei by prolonged APM treatment, and a procedure for the isolation of micronuclei from multinucleate cells and sorting them by flow cytometry based on DNA content.

Materials and methods

Plant material and culture conditions.

Suspension cells of *Nicotiana plumbaginifolia* ("Doba" line, kindly supplied by Dr. R. Shields, Sittingbourne) were cultured in the dark at 28°C in "Doba" medium (Barfield et al., 1985) on a gyratory shaker (120 rpm). For sustained division activity, sub-culturing was carried out at 3 day-intervals. A fresh solution of amiprophos-methyl (trade name Tokunol M®; O-methyl-O-O- (4-methyl-6-nitrophenyl)-N-isopropyl-phosphoro thioamidate) (Aya et al., 1975; Kiermayer and Fedtke, 1977) obtained from Bayer Nederland B.V., Divisie Agrochemie, was prepared in acetone (20 mg/ml) immediately before use. The chemical solution was applied to cell suspensions one day after subculture. Mitotic index and the number of micronucleate cells were determined by staining the cell samples of suspension cultures directly with orcein dissolved in lactic acid-propionic acid (1:1) (Eriksson, 1966), or in Feulgen after fixing in absolute alcohol: glacial acetic acid (3:1 v/v) for 24 h (Sree Ramulu et al., 1985). The mitotic index was expressed as the percentage of nuclei undergoing mitosis in the total number of nuclei scored in a sample. The number of cells analysed for mitotic index and for scoring multinucleate cells ranged from 300 to 600 per sample. The relative growth rate of cell suspensions treated with APM was determined according to Gilissen et al. (1983). The treated cell suspensions were washed repeatedly (4 times) with culture medium and the packed cell volume was determined at 1-7 days after inoculation.

Protoplast isolation and culture.

Suspension cells treated with APM were washed with culture medium and incubated in 5% Driselase (Kyowa Hakko Kogyo Co., Tokyo, Japan) and 0.5% Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) in 0.4 M mannitol for 2 h at 28°C on a gyratory shaker (30 rpm). Afterwards, protoplasts were isolated by centrifugation at 60 g for 5 min. Purification was carried out as described by repeated washing in 0.4 M mannitol and subsequent floatation on 0.4 M sucrose (De Laat and Blaas, 1987). Protoplasts were cultured at a density of $5x10^4$ protoplasts/ml in "protoplast culture medium 3" used by Kao et al. (1974) containing 0.4 M glucose and 0.8% agarose. After 6 days of culture at 28°C in the dark the plating efficiency (percentage of divided protoplasts) was calculated.

Isolation, flow cytometric analysis and sorting of nuclei and micronuclei.

Nuclei and micronuclei for analysis and sorting were isolated by the methods previously developed for the isolation of plant metaphase chromosomes (De Laat and Blaas, 1984). For the isolation of multinucleate protoplasts, APM was added to the enzyme and washing solutions as described in the preceding section. Purified protoplasts were incubated for 5 min on ice, in chromosome isolation buffer (Malmberg and Griesbach, 1980), ruptured by the addition of 0.05% Triton-X-100 and repeated passage through a 25 G epidermal needle. Cellular debris was removed by passage through a 80 μm nylon filter. For flow cytometric analysis, the nuclei were stained with the DNA-specific dye ethidium bromide (final concentration 10 μg/ml). The measurements of nuclear DNA content

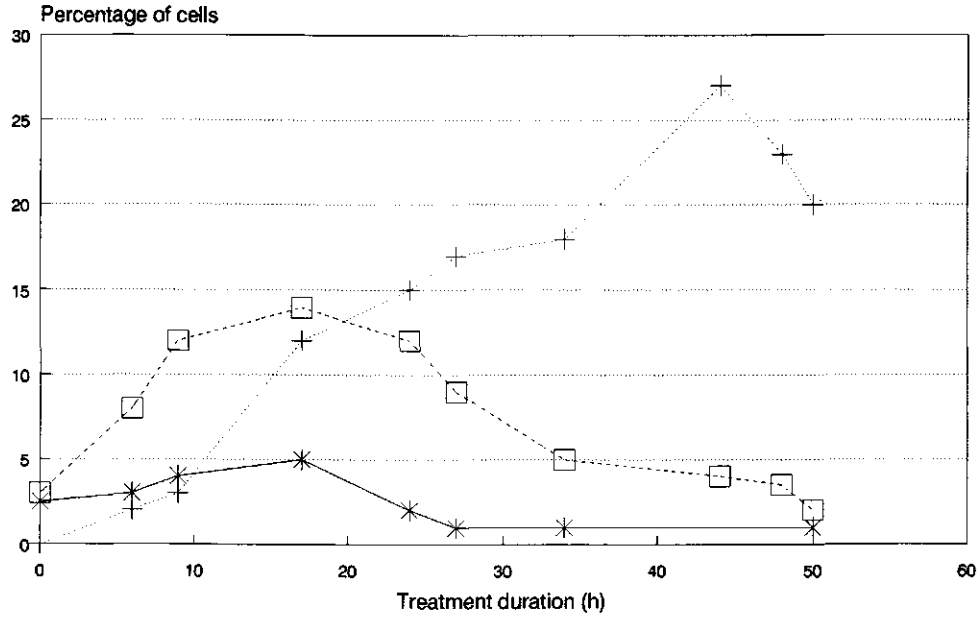

Fig. 1. Mitotic index (MI) and the percentage of micronucleate cells in cell suspensions of *N. plumbaginifolia* treated with APM for various periods. -✳-✳-✳-✳- : MI in control cell suspensions; -□-□-□-□- : MI in APM treated cell suspensions; -+-+-+-+- : Micronucleate cells after APM treatment. Micronucleate cells were absent in the control.

were made with a Fluorescence Activated Cell Sorter (FACS)-IV (Becton Dickinson, Sunnyvale, U.S.A.) equipped with a Spectra Physics argon ion laser, model 164-05 operated at 0.3 W/488 nm with an LP-620 filter in the emission beam. The DNA C-values corresponding to G1 and G2 nuclear phases of the cells were checked as reported previously (Sree Ramulu and Dijkhuis, 1986). The contribution of cellular and nuclear debris in the flow histograms was removed by an interactive computer analysis (van der Linden, 1980). The nuclei were sorted on the basis of DNA content. The sorted nuclei were concentrated on 0.2 μm nucleopore poly-carbonate filters and stained with 4,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342 (20 μg/ml) for microphotography.

Results

Effect of APM on mitotic index and formation of micronuclei.

The frequency of micronucleated cells in cell suspensions of *N. plumbaginifolia* depended on the concentration of APM, lower concentrations (3-18 μM) resulting in less than 10% micronucleate cells and the higher concentration (36 μM) about 27%. We analysed the time course of micronucleation in relation to the mitotic index after treatment with the higher concentration of 36 μM since the induction of large number of micronuclei is necessary for efficient flow-cytometric analysis and sorting. The results presented in Fig. 1 show that in the control, the mitotic index, which was 3.0 at

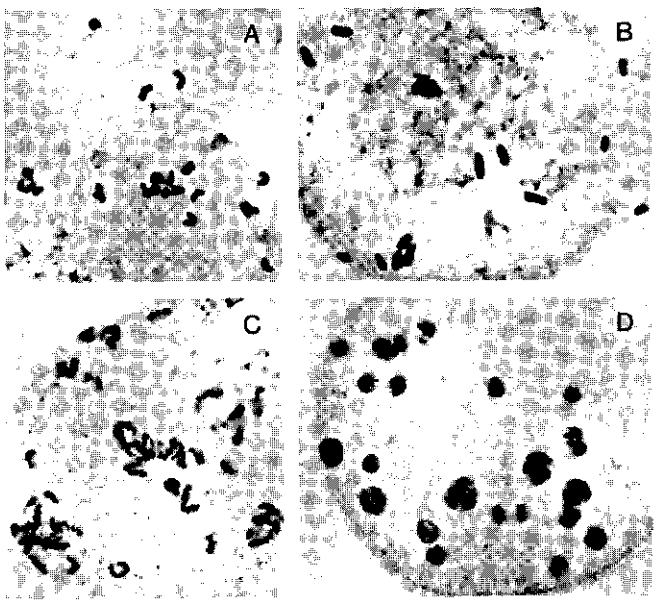

Fig. 2 A-D. Microphotographs illustrating the formation of micronuclei after APM treatment of cell suspensions in *N. plumbaginifolia*. A: Metaphase cell showing scattered chromosomes. B: Metaphase chromosome groups.C: Decondensed chromosomes. D: Micronucleated cell.

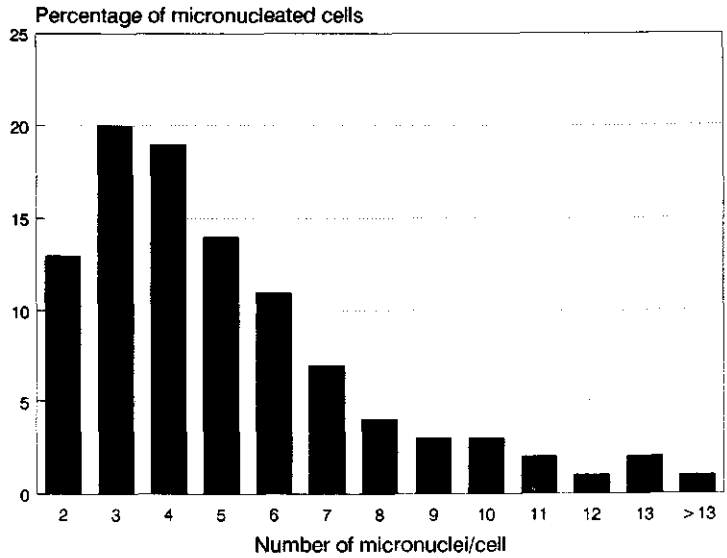

Fig. 3. Frequency distribution of the number of micronuclei in micronucleate cells after treatment of *N. plumbaginifolia* cell suspensions with 18 μM APM during 48 h.

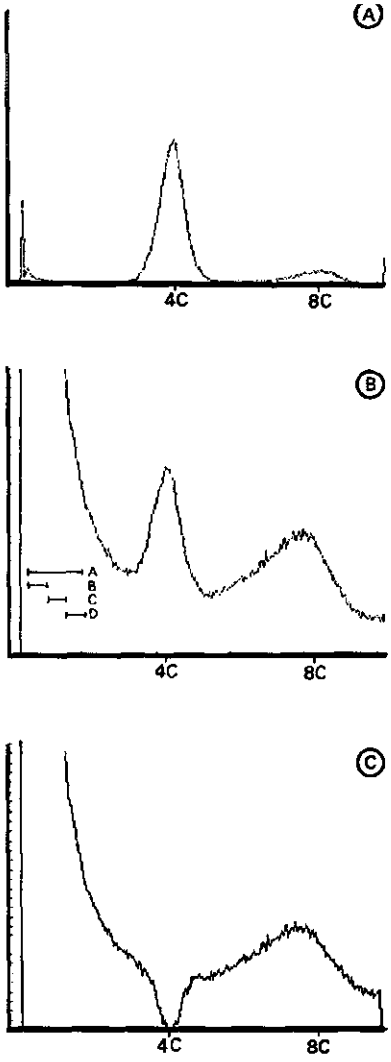

Fig. 4 A-C. Flow cytometric analysis of relative DNA contents of nuclei isolated from control (A) and APM treated cell suspensions (B) of *N. plumbaginifolia*. Subtraction of Fig. A from B gives C, which shows micronuclei and a high frequency of 8C nuclei. In Fig. 4B, A-D refer to the sorting windows used for the separation of entire population of sub-diploid (< 2C) micronuclei (A), and the three different fractions of sub-diploid micronuclei, i.e. the smallest micro-nuclei (B), intermediate micronuclei (C) and the largest micronuclei (D).

the start of the experiment, increased to 5.0 at 17 h and thereafter decreased gradually to 1.2 at 50 h. In the presence of APM, the mitotic index increased significantly within two to four hours, reached a maximum of 13.2 at 17 h and afterwards decreased to 2.0 at 50 h. Micronucleate cells were formed during APM treatment from 6 h onwards. Whereas the mitotic index dropped after approximately 20 h, the frequency of micronucleate cells rised to 27% after 43 h. Examples of microphotographs are presented in Fig. 2, illustrating the effects of APM on mitosis and the formation of micronuclei. Fig. 2A shows the scattering of mitotic chromosomes throughout the cytoplasm. Single as well as groups of chromosomes decondensed (Fig. 2B, C) and subsequently, micronuclei were formed (Fig. 2D). The number of micronuclei per cell varied from 2 to as many as 30 depending on the chromosome number of a cell after treatment with 18 μM or 36 μM APM. Figure 3 presents an example of the frequency distribution of the number of micronuclei per micronucleate cell after treatment with 18 μM APM for 48 h.

Effect of APM on cell growth and plating efficiency of protoplasts.

The effect of APM was analysed after treatments with various concentrations ranging from 1-18 μM for 16-24 h. The results on the relative growth rate of treated cell suspensions showed that it did not differ significantly from that of the control (data not given). Also, the plating efficiency of protoplasts was not affected by APM, except at higher concentration where it decreased slightly.

Flow cytometric analysis and sorting of micronuclei.

Nuclei were isolated from the purified protoplasts prepared from cell suspensions treated with APM (36 μM, 48 h), stained with ethidium bromide and analysed by

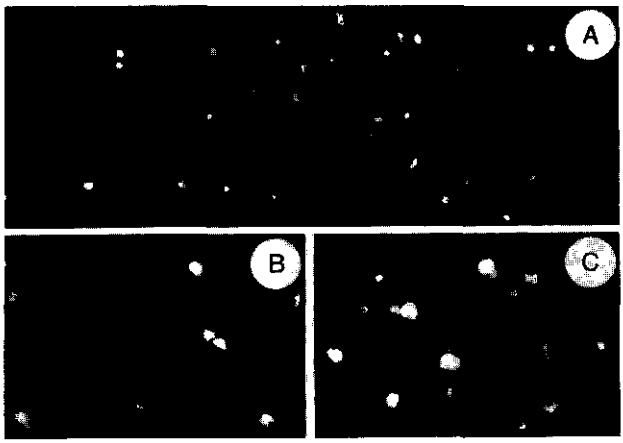

Fig. 5 A-C. Photographs of sub-diploid micronuclei fractions sorted by flowcytometry. A: Smallest micronuclei. B: Intermediate micronuclei. C: Largest micronuclei.

flowcytometry. The results on DNA histograms show that the untreated cell suspensions consisted of 4C and 8C nuclei (Fig. 4A). Chromosome counts in metaphases revealed the presence of predominantly tetraploid (2n = 4x = 40) cells, including some cells hypotetraploid for 1-4 chromosomes. Thus, the nuclei with 4C and 8C correspond to G1 and G2 nuclear phases of the tetraploid cells, respectively. Flow cytometric analysis of nuclei derived from APM treated cells showed that the frequency of 8C nuclei had increased considerably (Fig. 4B). This apparently resulted from the doubling of 4C cells. Moreover, as APM treatment induced about 22% multinucleate cells, a large number of nuclei with varying DNA contents were found (Fig. 4C). As the subdiploid (< 2C) population of micronuclei is relevant for partial genome transfer, three categories of such micronuclei with DNA contents ranging from approximately 0.1C to 2C were sorted. These differed in size, as seen in UV light microscopy (see microphotographs in Figs. 5A-C).

Discussion

The results obtained show that APM is an efficient chemical for high metaphase arrest and for mass production of micronuclei in cell suspensions of *N. plumbaginifolia*. The metaphase cells showed grouping of chromosomes, followed by decondensation leading to the formation of micronuclei. As APM is a drug which specifically poisons microtubule dynamics in plant cells by inhibiting microtubule polymerization (Morejohn and Fosket, 1984), spindle disturbances can lead to irregular

chromosome movement, orientation and grouping of chromosomes (Sree Ramulu et al., 1987). The frequency of micronucleation seems to depend upon the type of cell suspensions treated with APM. The fast dividing cell suspensions of *N. plumbaginifolia* responded with a high frequency of multinucleate cells as compared to the relatively slow-growing cell suspensions of *Haplopappus gracilis*, *Daucus carota* and *Solanum tuberosum* (data not given). With regard to the other effects of APM, the data showed that APM treatments showed no appreciable reduction in cell growth or plating efficiency of protoplasts. Thus, because of low toxicity, APM treatments are compatible with genetic manipulation approaches. Moreover, the recent observations on the mitotic dynamics of micronuclei induced after treatment with APM (36 μM, 48 h) in cell suspensions of *N. plumbaginifolia* revealed that after removal of APM by repeated washing and subculture, cells recovered showing normal functioning of spindle and continuity of mitotic cycles (Sree Ramulu et al., 1987). As demonstrated, flow cytometric sorting can be used to collect large numbers of micronuclei containing one or a few chromosomes. Prior to using micronuclei for gene transfer, it is necessary to remove the DNA intercalator ethidium bromide from the stained micronuclei. To a large extent, this is achieved through dilution (20-fold) with the sheath fluid of the flow system added to the micronuclei suspension during flow sorting. Alternatively, a vital stain, such as Hoechst 33258 can be used for flow sorting of micronuclei. Various methods, such as microinjection or fusion-like uptake (Malmberg and Griesbach, 1980; Szabados et al., 1981; De Laat and Blaas, 1987; Verhoeven and Blaas, 1987) and micro-cell fusion (Fournier and Ruddle, 1977; Fournier et al., 1979) can be used to transfer the desirable genes. In all these methods, availability of markers (phenotypic, genetic/cytological) is a prerequisite for selection and identification of the transferred genetic material. Both the induction of micronuclei and the flow sorting of sub-diploid micronuclei in plant systems, as demonstrated in *N. plumbaginifolia,* open the way for the development of microcell hybrids which constitute valuable tools for partial genome transfer and gene mapping (Ege and Ringertz ,1974; Ege et al., 1977; Fournier, 1982).

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CHAPTER 3

MITOTIC DYNAMICS OF MICRONUCLEI INDUCED BY AMIPROPHOS-METHYL AND PROSPECTS FOR CHROMOSOME-MEDIATED GENE TRANSFER IN PLANTS.

K. Sree Ramulu, H.A. Verhoeven, P. Dijkhuis.

MITOTIC DYNAMICS OF MICRONUCLEI INDUCED BY AMIPROPHOS-METHYL AND PROSPECTS FOR CHROMOSOME-MEDIATED GENE TRANSFER IN PLANTS.

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Summary

Mitotic dynamics and the kinetics of mass induction of micronuclei after treatment of *Nicotiana plumbaginifolia* cell suspensions with the spindle toxin amiprophos-methyl (APM) are reported. The addition of APM to suspension cells resulted in the accumulation of a large number of metaphases. The course of mitosis was strikingly different from normal. Metaphase chromosomes showed neither centromere division nor separation of chromatids. Single chromosomes and groups of two or more chromosomes were scattered over the cytoplasm. After five to six hours of APM treatment, chromosomes decondensed and formed micronuclei. With increasing treatment duration, the frequency of cells with micronuclei as well as those showing lobed micronuclei increased. Similarly, with an increase in APM concentration the frequency of cells with micronuclei increased. After removal of APM, chromosome grouping disappeared, cells showing lobed micronuclei further increased and mitoses with doubled chromosome numbers appeared in the next cell division.

Cytological observations and DNA measurements revealed that several sub-diploid micronuclei containing one or a few chromosomes can be obtained and that flow cytometry can detect and sort out these micronuclei. The applications of micronuclei for genetic manipulation of specific chromosomes and gene mapping are indicated.

Key words: Amiprophos-methyl; mass induction; micronuclei; intact specific chromosomes; gene transfer.

Introduction

Cellular manipulation of specific chromosomes and chromosomal segments in mammals has proven to be valuable in the study of the regulation of gene expression, fine structural mapping of chromosomes, molecular characterization and construction of genomic libraries (Mc Bride and Peterson, 1980; Davies et al., 1981; Lebo, 1982; Fournier, 1982). Many interspecific somatic cell hybrids eliminate chromosomes from one of the parents during culture. This preferential chromosome loss has allowed the assignment of genes to particular chromosomes (Kao, 1983). However, long periods of culture and recloning are often necessary before an accurate assignment can be made. A more direct approach to the production of cell lines possessing a limited amount of genetic material of another species would be to introduce only a single donor chromosome into the recipient cells. Microcell hybridization is the only technique by which the transfer of single, intact chromosomes has been achieved in mammals (Fournier, 1982). Microcell hybridization is conceptionally and operationally distinct from traditional somatic hybridization, in that only a fraction

of the donor genome is introduced into the recipient partner at the time of fusion. Ever since the discovery of the phenomenon that colchicine or Colcemid can induce a high frequency of micronuclei in mammalian cell cultures, several investigators have isolated micronuclei by applying enucleation procedures to micronucleate cells. In this way, microcells are formed, containing micronuclei, and surrounded by a thin layer of cytoplasm and a plasma membrane (Levan, 1954; Phillips and Phillips, 1969; Ege and Ringertz, 1974; Fournier and Ruddle, 1977; Ege et al., 1977; Sekiguchi et al., 1978). By subsequent fusion of microcells with recipient partners, karyotypically simple hybrid clones were constructed which contained only one or a few introduced donor chromosomes (Fournier, 1982). Since the transferred genetic material can often be identified cytogenetically, microcells have been proven to be useful not only for gene mapping, but also for studying the chromosomal sites of integration of foreign DNA (Fournier et al., 1979; Smiley et al., 1978). Microcell fusion can be used to create panels of monochromosomal hybrids in which single, specific chromosomes are maintained by direct selective pressure (Fournier and Ruddle, 1977; Fournier and Frelinger, 1982). Microcell hybrid clones made up of such a monochromosomal hybrid panel have been shown to display simple, stable and homogeneous karyotypes. The genes complementing recessive, conditional-lethal mutations or genes that confer dominant drug resistance phenotypes have been mapped.

In plants such a microcell system has not been available so far, because the mitotic arresting- and chromosome breaking agents induced micronuclei only rarely, the frequency of which is insufficient for fusion purposes (Levan, 1938; 1940; 1954; Hesemann and Fayed, 1982; Marshall and Bianchi, 1983; De Marco et al., 1986). Some chemicals, such as griseofulvin which affects spindle can cause segregation of chromosomes into irregular groups resulting in micronuclei of varying size in cell suspensions of *Medicago sativa* (Lo Schiavo et al., 1980) and 4-epoxyethyl-1,2-epoxy-cyclohexane (VCH-diepoxide) produces micronuclei as a consequence of chromosome breakage in root-tip meristems of *Vicia faba* and *Allium cepa* (Nuti Ronchi et al., 1986 a; b). Moreover, the mass isolation of individual metaphase chromosomes for sorting by flow cytometry or transfer into recipient protoplasts, is greatly hindered by stickyness of chromosomes that often occurs after treatment with spindle toxins (Malmberg and Griesbach, 1980; Szabados et al., 1981; De Laat and Blaas, 1984). Recently, we observed that an organophosphorous herbicide amiprophos-methyl (APM) can cause scattering of metaphase chromosomes and induce micronuclei at high frequency in plant cells (Verhoeven et al., 1986; 1987; De Laat et al., 1987). Amiprophos-methyl belongs to the class of phosphoric amide herbicides which are considered antimicrotubule drugs, because of the disappearance of microtubules after their application, and of the disruption of microtubule-dependent processes (Morejohn and Fosket, 1984). It has been shown that APM directly disturbs microtubule dynamics in plant cells.
This article presents data on the dynamic changes of chromosomes (condensation, decondensation) and the kinetics of mass induction of micronuclei after APM treatment of cell suspensions of *Nicotiana plumbaginifolia*.

Materials and methods

Genotype, cell culture and APM treatment

Nicotiana plumbaginifolia cell suspensions resistant to kanamycine were derived from the "Doba" cell line, kindly supplied by Dr. R. Shields, Unilever Res. lab., Colworth

Home, Sharnbrook, Bedford, UK. These cell suspensions were cultured in the dark at 28°C in "Doba" medium (Barfield et al., 1985) on a gyratory shaker (120 rpm). For sustained division activity, subculturing was carried out at 3 day-intervals.

Amiprophos-methyl was obtained from Bayer Nederland B.V., Divisie Agrochemie, Arnhem, The Netherlands (trade name Tokunol M®; O-methyl-O-O-(4-methyl-6-nitrophenyl)-N-isopropyl-phosphoro thioamidate) (Aya et al., 1975; Kiermayer and Fedtke, 1977). A stock solution of APM in DMSO (20 mg/ml) was prepared and the treatments at concentrations (in medium) ranging from 3.6 μM to 36 μM were given to actively growing log phase cell suspensions, one day after subculturing.

Cytology

The samples of control and APM treated cell suspensions were fixed in ethanol/acetic acid (3:1 v/v) for 24h and examined for mitotic division, chromosome behaviour and micronucleus formation in Feulgen-stained preparations (Sree Ramulu et al. 1985). The mitotic index was expressed as the percentage of nuclei undergoing mitosis among the total number of nuclei scored in a sample. The number of cells analysed per sample ranged from 600-1000. Mitotic arrest or metaphase blocking observed after APM treatment were designated as C-mitosis or C-metaphase in accordance with the general terminology proposed by Levan (1954). Various terms, i.e. "scattered or exploded metaphases, star metaphases and reductional grouping of chromosomes", are according to the definition by Levan (1954).

Flow cytometry

For flow cytometric analysis of nuclei and micronuclei, cell samples were fixed for 24h in buffer solution described by Blumenthal et al. (1979) with some modification as adopted previously for potato cells (Sree Ramulu and Dijkhuis, 1986). Suspension cells were submerged in the buffer for some time, dried with blotting paper and chopped with a sharp razor blade in a petridish. Afterwards, 1.0-1.5 ml buffer solution (pH 7) was added. All these operations have been carried out on ice. The cell materials were pressed by glass rod through an 88 μm nylon filter and then through a 15 μm nylon filter, centrifuged during 1 min at 1000 rpm, and the pellet was resuspended in 0.5 ml buffer, stained with 5 μl ethidium bromide (5 mg/ml) and added to 25 μl Triton X-100 (20 %). The measurements of nuclear DNA content were made with a Fluorescence Activated Cell Sorter (FACS)-IV (Becton Dickinson, Sunnyvale, U.S.A.) equipped with a Spectra Physics argon ion laser, model 164-05 operated at 0.3 W/488 nm with a LP-620 filter in the emission beam. The DNA C-values corresponding to G1 and G2 nuclear phases of the cells were checked using control plants as reported previously (Sree Ramulu and Dijkhuis, 1986). The contribution of cellular and nuclear debris in the flow histograms was removed by an interactive computer analysis (van der Linden, 1980). The micronuclei were sorted on the basis of fluorescence intensity resulting from ethidium bromide staining.

DNA cytophotometry

Feulgen microdensitometry was used to determine the relative DNA content of the sorted fractions of micronuclei. The samples were fixed in absolute alcohol/glacial acetic acid (3:1 v/v) for 24h. The staining and slide preparations were carried out as reported earlier (Sree Ramulu et al., 1984). Hydrolysis of nuclei, spread and dried on a millipore filter (membrane filter SCWP 02500), was carried out in 5 N HCl at room temperature for 55 min. Nuclei were stained with Schiffs reagent. After 3 washes in SO_2 water, 5 min each, the slides were dehydrated and mounted in Canada balsam. Absorption values for the Feulgen-stained nuclei were measured with a Leitz MPV

Fig. 1 A-L. Microphotographs on the sequence of mitotic events leading to the formation of micronuclei after treatment of cell suspensions of *N. plumbaginifolia* with APM, and recovery of cells after removal of APM. A. Scattering of metaphase chromosomes ("exploded metaphases"). B-C. Groups of two, three or more chromosomes (note single chromosomes). D. Decondensation of single and grouped chromosomes. E, F. Micronuclei in different phases.

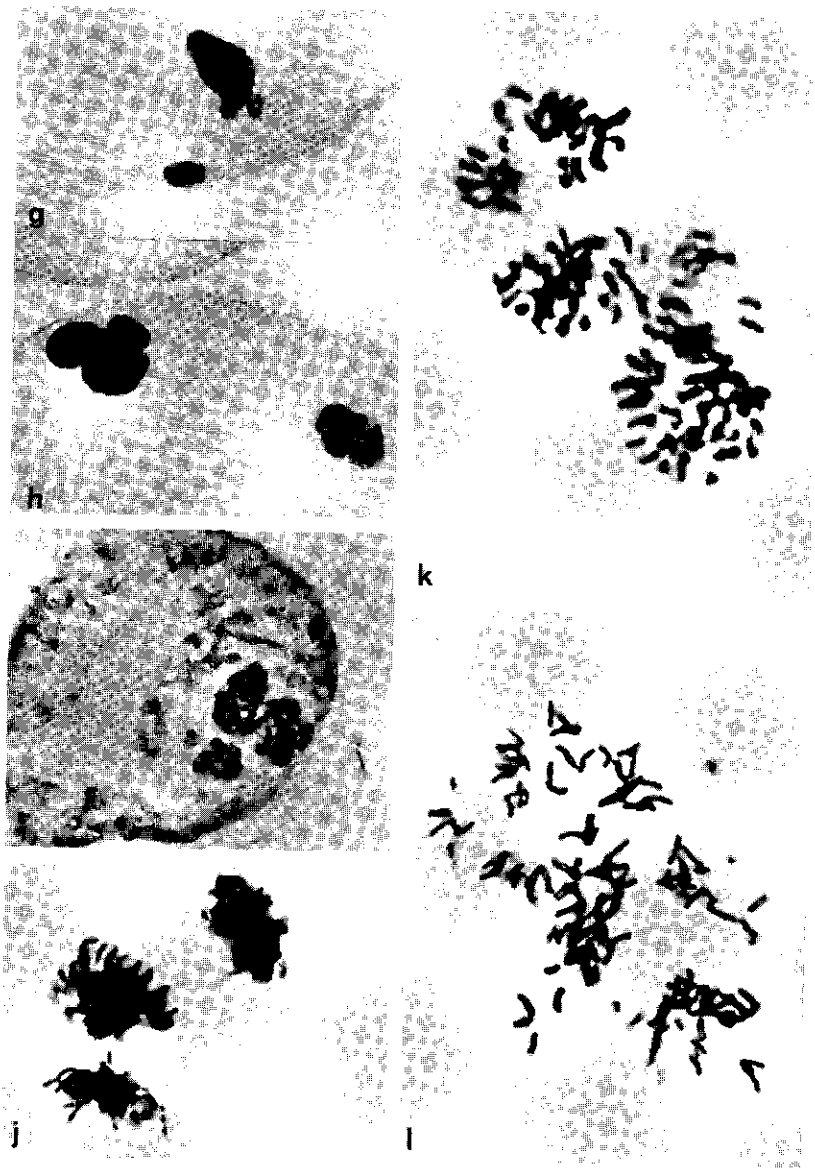


Fig. 1 (contd.) G-I. Lobed micronuclei. J-L. Post C-mitotic cells after removal of APM by washing and subsequent culture (note multipolar metaphases).

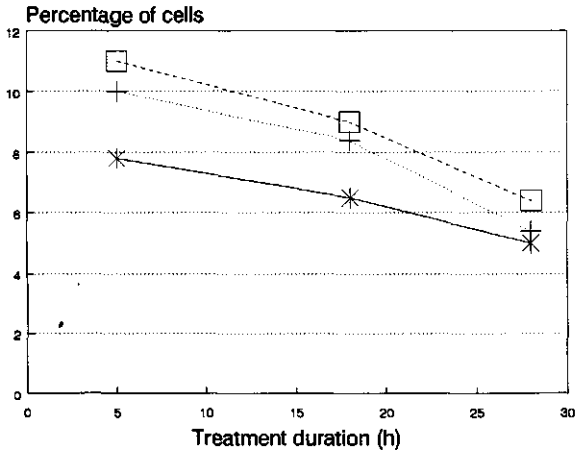
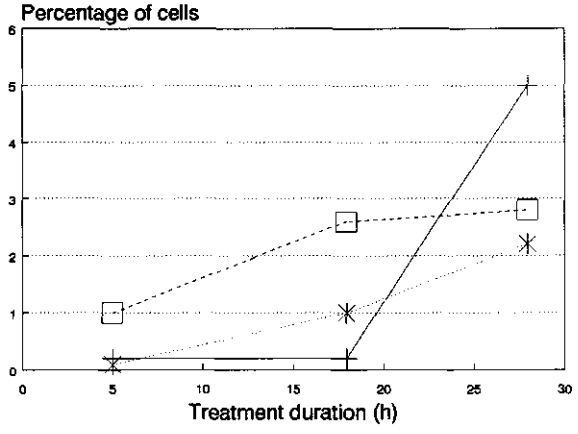


Fig. 2. A: Mitotic index (MI); B: the percentage of cells showing chromosome-groups and C: micronuclei after treatment with various concentrations of APM for different periods. The control suspension cells of *N. plumbaginifolia* showed no chromosome-groups or micronuclei (MI ranged from 1.5-5.0 %).

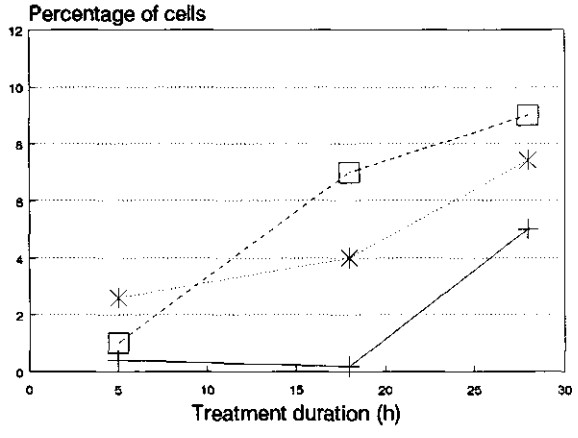
3.6 μM APM
9.0 μM APM
18 μM APM

Fig. 2A.



3.6 μM APM
9.0 μM APM
18 μM APM

Fig. 2B.



3.6 μM APM
9.0 μM APM
18 μM APM

Fig. 2C.

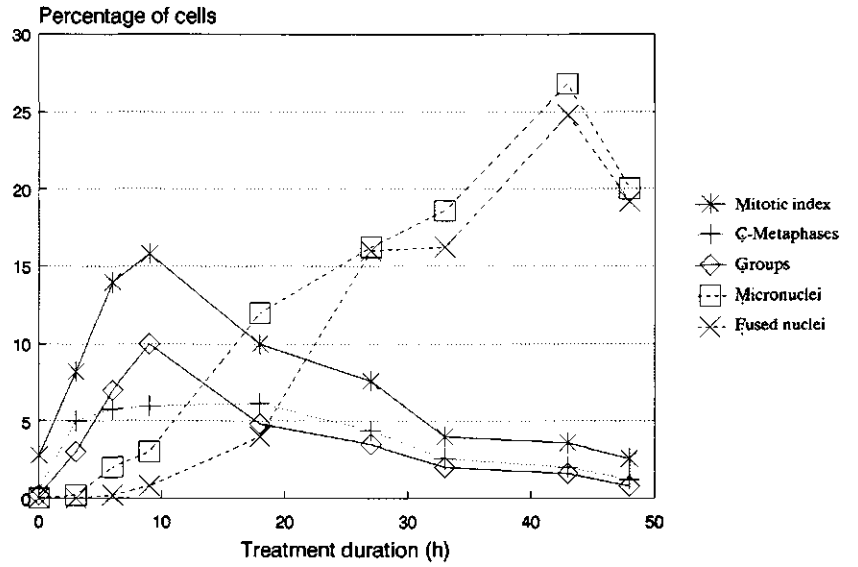


Fig. 3. Time course of inducton of micronuclei in relation to other mitotic events after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM). In this, as in Fig. 7, C-Metaphases: Blocked metaphase cells with well spread or partially clumped chromosomes arranged in "a single group"; Chromosome-groups: Metaphase cells showing separate groups of chromosomes; Micronuclei: Cells showing separate micronuclei; Fused nuclei: Cells with lobed micronuclei.

compact microscope photometer at the wave length of 548 nm and scanning stage using a Hewlett Packard-85 computer. These values were transformed to C-values by taking as standard the DNA content of nuclei isolated from leaf cells of control plants (Sree Ramulu et al., 1984).

Results

Mitotic arrest, chromosome grouping and micronucleation

The cell suspensions of *N. plumbaginifolia* were treated with APM at concentrations ranging from 3.6 to 36 μM in the medium for various periods. Characteristic modifications of mitosis were encountered from one or two hours after initiation of APM treatment. The chromosomes lost their regular arrangement on the spindle and in some cells they gathered into a single group in the centre of the cell. Ball metaphases with all chromosomes in the centre of the cell were also observed. Several cells showed scattering of the chromosomes all over the cytoplasm (Fig. 1 A). In these scattered C-metaphases ("exploded metaphases") the chromosomes were arranged in characteristic groups of 2, 3 or more chromosomes ("reductional grouping"), including separated single chromosomes (Fig. 1 B, C). In some cells several groups of almost radially arranged chromosomes were found, within each group all chromosomes turned their centromere inwards towards one point; in a few cases all chromosomes of one cell are arranged as star. In other cells chromosome-groups were arranged in a wide ring or in a line. Mitosis after APM

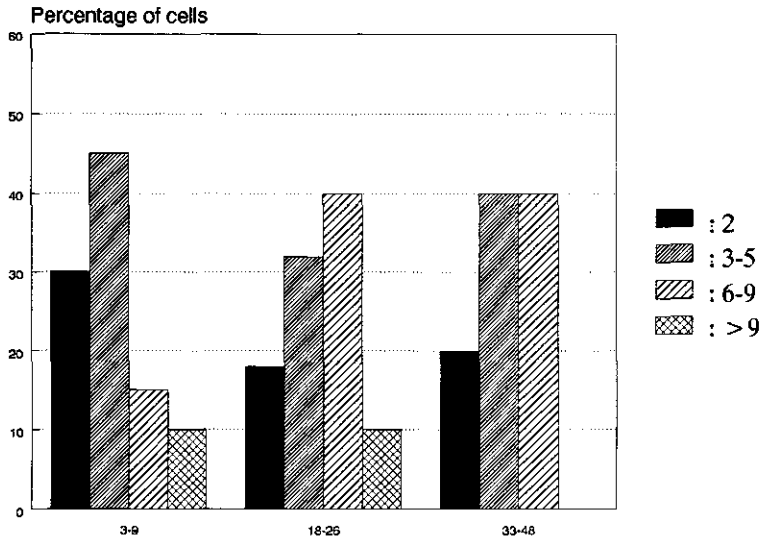


Fig. 4. Relative percentage of cells showing various number of chromosome-groups (i.e. the number of chromosome-groups/cell) after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for various periods. As the data for the periods 3-9 h, 18-26 h and 33-48 h were similar, they were pooled.

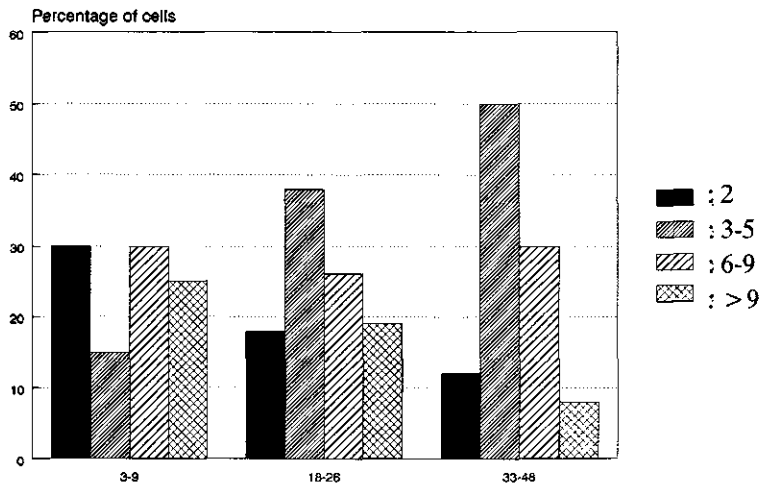


Fig. 5. Relative percentage of multinucleate cells showing various number of micronuclei (i.e. the number of micronuclei/multinucleate cell) after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for various periods.

Table 1. Percentage of groups that contained various number of chromosomes after treatment of cell suspensions of *N. plumbaginifolia* with APM for various periods

Duration of APM (36μM) treatment	Number of chromosome groups groups analyzed (*)	Relative percentage of groups showing various numbers of chromosomes			
		1	2	3	4 or >
3-9 h	139 (38)	50.6	2.	3.7	43.7
18 h	50 (10)	60.0	8.0	4.0	28.0
26 h	113 (16)	69.1	4.4	5.3	21.2
33-48 h	27 (5)	37.2	7.4	7.2	48.2

(*): The number of cells that showed chromosome-groups are given in parantheses.

treatment was devoid of anaphase, metaphase chromosomes entered directly into restitution telophase without division of the centromeres and without separation of chromatids. In the scattered C-mitoses the metaphase to telophase transition could be followed with all its characteristic changes. In some cases, single metaphase chromosomes decondensed developing nuclear membrane and formed telophase-like micronuclei. In other cases, two or more chromosomes grouped together in forming micronuclei (Fig. 1 D). Two examples of cells containing several micronuclei in different phases are presented in Figs. 1 E, F. With an increase in the concentration of APM from 3.6 to 18 μM, there was an increase in mitotic index and in the frequency of cells showing chromosome groups and micronuclei (Fig. 2). As the duration of treatment increased, mitotic index dropped, but the percentage of cells with chromosome groups and micronuclei increased.

Fig. 3 presents more detailed data on the time course of the induction of micronuclei in relation to other mitotic events in cell suspensions treated with 36 μM APM. Up to 9 h, mitotic index, C-metaphases and cells showing chromosome-groups increased with incubation time. Afterwards all these decreased, except the frequency of C-metaphases, which did not decrease before 18h. With increasing duration of APM treatment from 3 to 26 h, the relative percentage of cells showing 6-9 chromosome groups increased, whereas those with 2 or 3-5 chromosome groups decreased (Fig. 4). With further increase in the incubation period (33-48 h), the frequency of cells containing 2 chromosome-groups increased and that of cells with >9 groups decreased.

Each of these groups contained varying numbers of chromosomes. The data presented in Table 1 show that the relative percentage of groups containing 1, 4 and above 4 chromosomes was higher than those with 2 or 3 chromosomes after treatment with APM for various periods.

Micronuclei were formed from about 5-6 h onwards, and the frequency of cells with micronuclei increased gradually, with a corresponding decrease in mitotic index. The maximum frequency of cells with micronuclei was about 28 % at 48 h after APM treatment (36 μM) (Fig. 3). With prolonged incubation of cells in the presence of APM under certain conditions 70 % of the cells showed micronucleation (data not given).

The number of micronuclei per cell generally varied from 2 to 13. In a few cells >20 micronuclei were also observed. The proportion of cells containing 2 or >9 micronuclei decreased with an increase in the duration of APM treatment (Fig. 5). On the other hand, the percentage of cells showing 3-5 micronuclei increased and those with 6-9 remained unchanged.

Lobed nuclei often resulting from fusion of two or more micronuclei, were observed

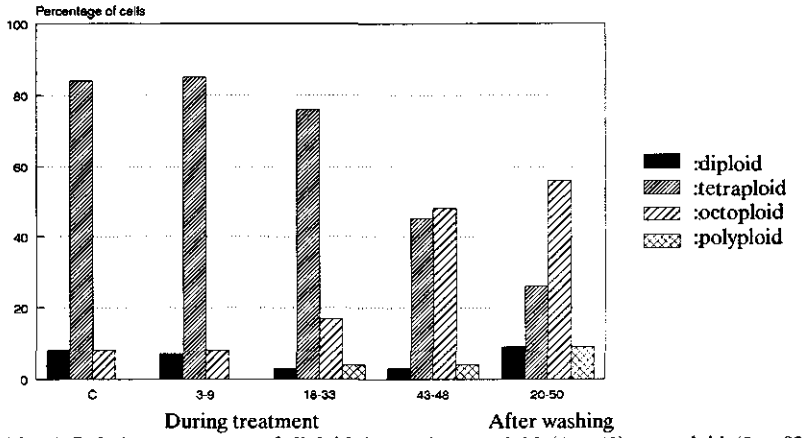


Fig. 6. Relative percentage of diploid (2x = 20), tetraploid (4x = 40), octoploid (8x = 80) and higher ploid (16x = 160) cells after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for various periods, after washing out APM and subculturing. Chromosome numbers generally varied by 2 for diploid (i.e. inclusive of hypodiploid) and 4 for tetraploid (inclusive of hypotetraploid) cells and 5 for octoploid or poly ploid cells.

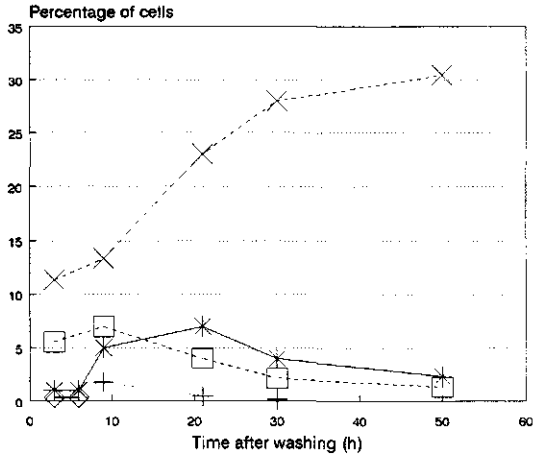


Fig. 7. Mitotic index and frequency of cells showing C-metaphases, chromosome groups, micronuclei and fused (lobed) nuclei after washing out APM (36 μM) and subculturing of cells.

from 9 h onwards (Figs. 1 G-I). With increasing duration of APM treatment, the proportion of cells showing lobed nuclei also increased (Fig. 3).

Recovery after C-mitosis

The control cell suspensions contained predominantly tetraploid cells and some diploid and octoploid cells (Fig. 6). The analysis of chromosome numbers after various periods of APM treatment (36 μM) revealed that the cells underwent one or more divisions with a consequent doubling or quadrupling of the chromosomes complement. The proportion of octoploid cells gradually increased with corresponding decrease of diploid and tetraploid cells. When APM was removed by washing the frequency of octoploid and higher ploid cells further increased. Thus, about 65 % of the cells underwent doubling or quadrupling of the chromosome number (Fig. 6). It was further observed that after washing out

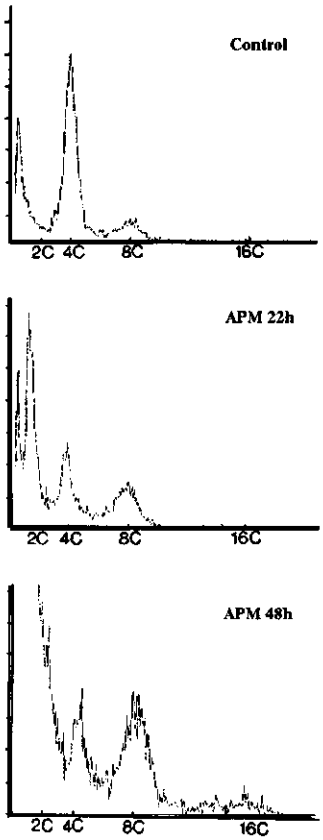


Fig. 8. Flow cytometric analysis of relative DNA contents of nuclei isolated from control and APM (36 μM: 22 h, 48 h) treated cell suspensions of *N. plumbaginifolia*.

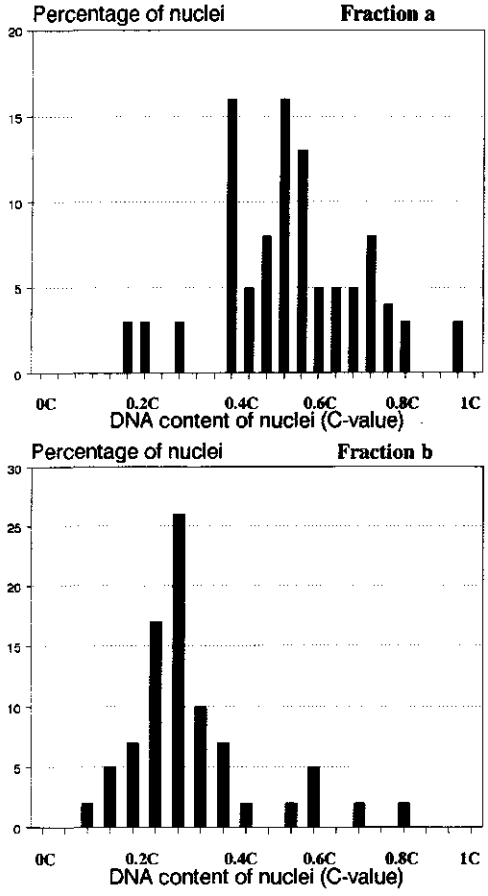


Fig. 9. Feulgen microdensitometric measurement of DNA contents of fraction-a and -b of subdiploid (<2C) micronuclei sorted by flow cytometry. The C-values were calibrated by the 2C level of control nuclei, isolated from leaves of diploid (2n = 2x = 20) plants of *N. plumbaginifolia*

APM and subculturing for 50 h, chromosome grouping almost completely disappeared and C-metaphases and cells with micronuclei decreased gradually (Fig. 7). By contrast, the frequency of cells showing lobed nuclei greatly increased. Mitotic index was similar to that of the control. Several cells showed normal bipolar mitoses, and some multipolar mitoses. Figs. 1 J-L show some examples of multinucleate cells progressed into post C-mitotic stages.

DNA content of micronuclei

Flow cytometry was used not only to monitor mass induction of micronuclei after APM treatment, but also for sorting and characterization of the micronuclei containing one or a few chromosomes. Fig. 8 shows the flow DNA histograms of the control and APM (36 μM) treated cell

suspensions. The control cell suspensions consisted of nuclei with 4C and 8C DNA contents. Chromosome counts in metaphases of control squash preparations revealed the presence of predominantly hypotetraploid to tetraploid cells ($2n = 4x = 36$-40). Therefore, the nuclei with 4C and 8C DNA values correspond to G1 and G2 nuclear phases of the hypotetraploid to tetraploid cells.

After APM treatment, sub-diploid (< 2C) micronuclei were produced, which increased in frequency with increasing duration of APM treatment. In addition, the proportion of nuclei showing 8C DNA content also increased and nuclei containing 16C DNA content appeared, due to chromosome doubling of cells (Fig. 8; APM 48h). Fig. 9 gives data on Feulgen microdensitometric measurements of DNA contents of the two fractions (fraction-a and -b) of sub-diploid micronuclei sorted by flow cytometry. Eighty micronuclei from each of the fraction-a and -b were individually measured for DNA content and compared with control nuclei ($n = 80$) isolated from leaves of diploid ($2n = 2x = 20$) *N. plumbaginifolia* plants. The results suggest that smaller micronuclei (fraction-b) contain one or two chromosomes as can be seen from the position of the peak relative to the 2C value, i.e. G1 nuclei of the diploid control plants. The fraction-a showed a broader distribution of micronuclei with a peak around 2-4 chromosomes.

Discussion

The results obtained in the present study show that APM is a highly efficient mitotis arresting agent which leads to the accumulation of a large number of metaphases and to the formation of high frequencies of micronuclei. To our knowledge, mass induction of micronuclei by spindle toxins or chromosome-breaking agents has not yet been reported in plant cells. Amiprophos-methyl has been discovered as early as 1975 (cf. Aya et al., 1975). Since then, this phosphoric amide herbicide has been investigated in several in vitro and in vivo experiments dealing with physiological or biochemical aspects and microtubule polymerization (see Morejohn and Fosket, 1984), but so far not on the induction of micronuclei.

As compared to colchicine blocked metaphase cells, which mostly showed chromosome clumping, APM treatment resulted in well scattered chromosomes allowing the isolation of single chromosomes (Verhoeven et al., 1987). The isolation of a large number of individual metaphase chromosomes is of considerable importance for genetic manipulation of specific chromosomes by means of flow cytometric sorting, karyotyping and genome cloning (Mc Bride and Peterson, 1980; Davies et al., 1981; Lebo, 1982).

Strikingly, the course of mitosis after APM treatment was different from that of normal mitosis or C-mitosis induced by colchicine in most plants. In normal mitosis the daughter chromosomes separate during anaphase and become surrounded by nuclear membranes, directly as a group or at first as single chromosomes. This is followed by decondensation of the chromosomes to form the interphase nuclei. Colchicine inactivates the spindle leading to disturbed chromatid distribution in anaphase, delayed centromere division and the formation of restitution nuclei (Levan, 1954).

By contrast, after APM treatment two additional features were observed. First, the chromosomes showed no centromere division or chromatid separation. Second, single chromosomes as well as grouped chromosomes remained scattered and decondensed, developed nuclear membranes and formed micronuclei. These occurred irrespective of the APM concentration, or the plant species (Sree Ramulu et al. unpublished results). The change of metaphase chromosomes into micronuclei after APM treatment was

accompanied by structural and functional changes in chromosomes resembling the progression of a telophase nucleus in the normal cell cycle. This process has been previously reported in mammalian cells treated with colchicine or Colcemid (Levan, 1954; Obara et al., 1974), but is rare in plants (cf. Levan, 1954).
In cell suspensions of *Medicago sativa* griseofulvin, which affects the spindle, induces metaphase arrest and polyploidization. After separation of the chromatids (during the recovery period) chromosomes segregate into irregular groups resulting in micronuclei of varying size (Lo Schiavo et al., 1980). The spontaneous occurrence of spindle abnormalities in a cell suspension line of *Daucus carota* also seems to result in metaphase arrest, chromosome scattering and multinucleate condition (Nuti Ronchi, Personal Communication).

In Colcemid treated Chinese hamster cells, lowering of Ca^{2+} promoted "telophasing", i.e. the formation of the nuclear membrane around the chromosomes and increased the frequency of micronuclei (Matsui et al., 1982). Several studies emphasized the importance of cellular Ca^{2+} in cell cycle progression, spindle functioning and in the reformation of nuclear membrane. From previous investigations on the effects of APM, it is known that the chemical can deregulate Ca^{2+} level and can inhibit Ca^{2+} uptake by mitochondria, the function of which is important for maintaining a normal spatial relationship of the nuclear membrane to the chromatin (review in Matsui et al., 1982). Therefore, it is likely that these effects of APM on cellular Ca^{2+} play a role in the formation of micronuclei.
Recovery of cells from the "APM-metaphase block" can be seen as the concentration decreases below the C-mitotic threshold with prolonged incubation. After washing out APM and subculturing, recovery was more pronounced. Many multinucleate cells showed lobed micronuclei. The lobed condition might be due to incomplete separation of chromosome-groups i.e. the groups which were not aggregated together at the time of nuclear membrane formation or when passing into the resting stage. Alternatively, it is also possible that two or more micronuclei fuse together forming lobed nuclei. Light microscopic observations also suggest that lobed nuclei often result from nuclear fusion. Previously, some authors working on the induction of micronuclei by Colcemid in mammalian cells have reported fusion of micronuclei, vital functioning of micronuclei and continued proliferation of micronucleate cells (Brues and Jackson, 1937; Klein et al.,1952; Levan, 1954; Stubblefield, 1964; Phillips and Phillips, 1969; Ege et al.,1977). As the cells did not undergo centromere division, chromatid separation or anaphase movement in the present study, the chromosome number doubled in the next cell division and they continued growth normally.

Cytological observations and DNA measurements showed that several sub-diploid micronuclei containing one or a few chromosomes can be obtained after APM treatment. These micronuclei can be detected and sorted in extremely large numbers by flow cytometry. This opens the way to fusion and generation of microcell hybrids suitable for genetic manipulation of specific chromosomes and gene mapping in plants.

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CHAPTER 4

COMPARISON OF THE EFFECTS OF VARIOUS SPINDLE TOXINS ON METAPHASE ARREST AND ON FORMATION OF MICRONUCLEI IN CELL SUSPENSION CULTURES OF NICOTIANA PLUMBAGINIFOLIA.

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COMPARISON OF THE EFFECTS OF VARIOUS SPINDLE TOXINS ON METAPHASE ARREST AND ON FORMATION OF MICRONUCLEI IN CELL SUSPENSION CULTURES OF NICOTIANA PLUMBAGINIFOLIA.

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Summary

The effect of the anti-microtubular drugs colchicine, amiprophos-methyl (APM) and oryzalin on mitotic index, chromosome scattering and on the induction and yield of micronuclei were compared. Results from cytological analysis and flowcytometry show that oryzalin is most suitable for metaphase arrest, and consequently for isolation of metaphase chromosomes, whereas APM is most effective in induction of micronuclei. A hypothesis is presented, explaining the difference of action of APM and oryzalin and the mechanism of micronucleus induction by APM.

Key words: metaphase arrest, micronucleus, spindle toxin, colchicine, herbicides, *Nicotiana*.

Introduction

Genetic manipulation of plants can be achieved by many techniques, ranging from the introduction of single, cloned genes, to the addition of the complete genomic information of a donor species by somatic hybridization. In between these extremes, transfer of chromosomes has already been available for some time, both in mammalian cell systems and in plants (Klobutcher and Ruddle, 1981; Griesbach et al., 1982a). Since transfer of isolated metaphase chromosomes results in the fragmentation of the chromosomes, and subsequent incorporation of the fragments into the recipient genome, microcell fusion was developed as a tool to transfer intact chromosomes into mammalian cells (Lugo and Fournier, 1986). With this technique, micronuclei are induced by prolonged exposure to Colcemid, and the micronucleated cells are centrifuged, to isolate subcells with a few micronuclei containing a small number of chromosomes.

Both chromosome- and microcell-mediated gene transfer require efficient procedures of chromosome isolation and mass induction of micronuclei in plants. A high mitotic index is necessary for the efficient isolation of metaphase chromosomes from plant cells. This can be achieved by utilizing the naturally occurring synchrony of gametophytic tissues, such as anthers or meiocytes (Malmberg and Griesbach, 1980), or by metaphase arrest in other tissues through spindle toxins, such as colchicine (Malmberg and Griesbach, 1980; Szabados et al., 1981; Hadlackzy et al., 1981; 1983; Matthews, 1983; de Laat and Blaas, 1984; Guri et al., 1984; de Laat and Schel, 1986; Griesbach, 1987; Conia et al., 1987a; 1987b).

Colchicine has been shown to bind with high affinity to tubulins in mammalian cells, and induces metaphase arrest at concentrations

of 10^{-7} M and higher (Dustin, 1984). The concentration required for metaphase blocking in plant cells is a thousand-fold higher than that in animal cells. This has been attributed to lower affinity of colchicine to plant tubulin heterodimers (Dustin, 1984). Podophyllotoxin has the same mode of action as colchicine, and binds to the same site on the tubulin hetero-dimer, as was demonstrated by competition studies (Dustin, 1984). The colchicine binding site is poorly conserved during evolution, as can be concluded from the observation that colchicine binds with a lower affinity to the binding site on plant tubulins than to the binding site on mammalian tubulins.

Some herbicides act on plant tubulin polymerization, and prevent cell cycle progression through mitosis (reviewed in Dustin, 1984). The dinitroanilines 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 and Fosket, 1984; Morejohn et al., 1987).

On the basis of the finding that prolonged APM treatments induce micronucleation in plant cells (Verhoeven et al., 1986; 1987; de Laat et al., 1987), it was suggested that the disturbance of the mitochondrial calcium uptake by APM (Hertel et al., 1980; 1981) was responsible for micronucleus formation (Sree Ramulu et al., 1988a; this thesis, chapter three). The deregulation of the calcium metabolism by these herbicides may play an important role in the induction of micronuclei.

In this paper, the effects of various spindle-toxins on the accumulation of mitotic cells and the induction of micronuclei are studied in the context of genetic manipulation using isolated metaphase chromosomes, micronuclei and microprotoplasts.

Materials and methods

Cell culture

A kanamycin resistant suspension cell culture of *N. plumbaginifolia*, obtained after transformation of a haploid cell suspension provided by Dr. Shields, Unilever Res. Lab., Colworth Home, Sharnbrook, Bedford, UK was cultured in the dark at 28°C in "Doba" medium (Barfield et al., 1985) on a gyratory shaker (120 rpm), and transferred to fresh medium every week. All experiments were performed with cells, grown for a week to plateau phase. The cell suspensions from several flasks were mixed, and subcultured by a fourfold dilution with fresh "Doba" medium.

Chemical treatments

Amiprophos-methyl (Tokunol M®; O-methyl-O-O-(4-methyl-6-nitrophenyl)-N-isopropyl-phosphorothioamidate) (Kiermayer and Fedtke, 1977) was a gift from Bayer Nederland B.V., Divisie Agrochemie, Arnhem, The Netherlands. Oryzalin was a gift from Eli-Lilly Co. Colchicine was obtained from Jansen Pharmaceuticals, Beerse, Belgium. Stock solutions of APM and oryzalin were prepared at 20 mg/ml in water-free DMSO, and could be stored for at least 6 months at -20°C, without loss of activity. Concentrated aqueous solutions of 100 mg/ml colchicine were prepared freshly for each experiment. All treatments were started 24 h after subculture of a one week- old cell suspension culture.

Cytology

Cells were fixed in 3:1 ethanol : acetic acid and prepared for cytology as described earlier (Sree Ramulu et al., 1985). Mitotic index, micronucleus index (percentage of micronucleated cells) and number of micronuclei per cell were determined from

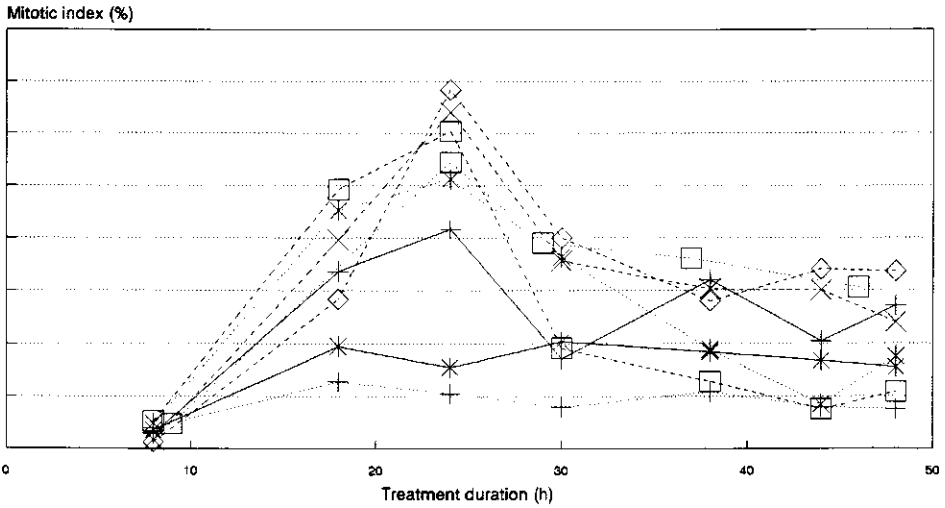

Fig. 1. Mitotic index of *N.plumbaginifolia* cell suspensions, treated with: 0.5 mM colchicine (—+—+—); 5mM colchicine (—✳—✳—); 3 μM oryzalin (—◇—◇—); 15 μM oryzalin (—×—×—); 30 μM oryzalin(—□—□—); 3 μM APM (—✳—✳—); 32 μM APM (—□—□—) and untreated control (—+—+—). Chemicals were applied at t = 0, i.e. 24 h after subculture.

five random counts of 100 cells each per sample for a given treatment period in each experiment. Data from the relevant treatments of two experiments were pooled.

Flow cytometry

For flow cytometric determination of the nuclear DNA content, protoplasts were isolated from small samples of the cell suspension. About 400 μl of an enzyme mixture, containing 25% Cellulase "Onozuka" R10 (Yakult, Honska Co, Tokyo, Japan), 5 % Macerozyme R10 and 1.2 M of sucrose, was added to 800 μl of cell suspension. After 30 min incubation at 28°C in a 2.5 cm petridish on a gyratory shaker at 30 rpm, the enzyme mixture was centrifuged for 5 min at 60 g. Floating protoplasts were transferred to nuclear isolation buffer, containing 10 mM Tris-HCl, pH 7, with 10 mM spermine-tetrahydrochloride, 2.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI), 10 mM NaCl, 200 mM hexyleneglycol and 0.025% Triton-X100. Samples were analyzed on a Partec PAS-II flowcytometer, with the UG5 excitation filter, TK420 and TK520 dichroic mirrors, and GG435 longpass filter. Channel analysis was performed with the standard software of the PAS-II.

Chromosome isolation.

Chromosomes were isolated from cell suspensions as described before (de Laat and Blaas, 1984).

Results

Mitotic index

Subculture of a cell suspension, grown to plateau phase, results in a sudden increase of the mitotic index up to 6%, occurring after 18-24 h of treatment (42-48 h of subculture) (Fig. 1). Addition of spindle toxins results in accumulation of mitotic cells in metaphase (metaphase arrest: MA), and an increase of

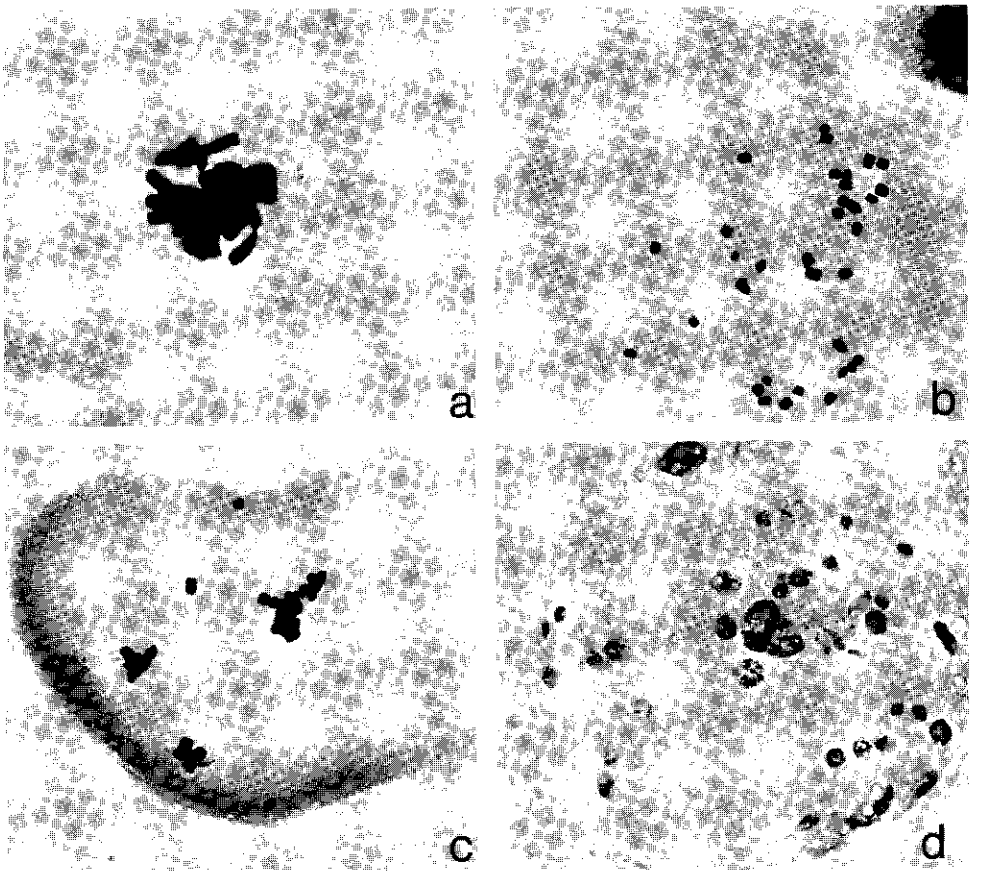

Fig. 2. Photomicrographs of a: cell with ball metaphase; b: cell with scattered chromosomes; c: cell with chromosome grouping; d: cell with micronuclei.

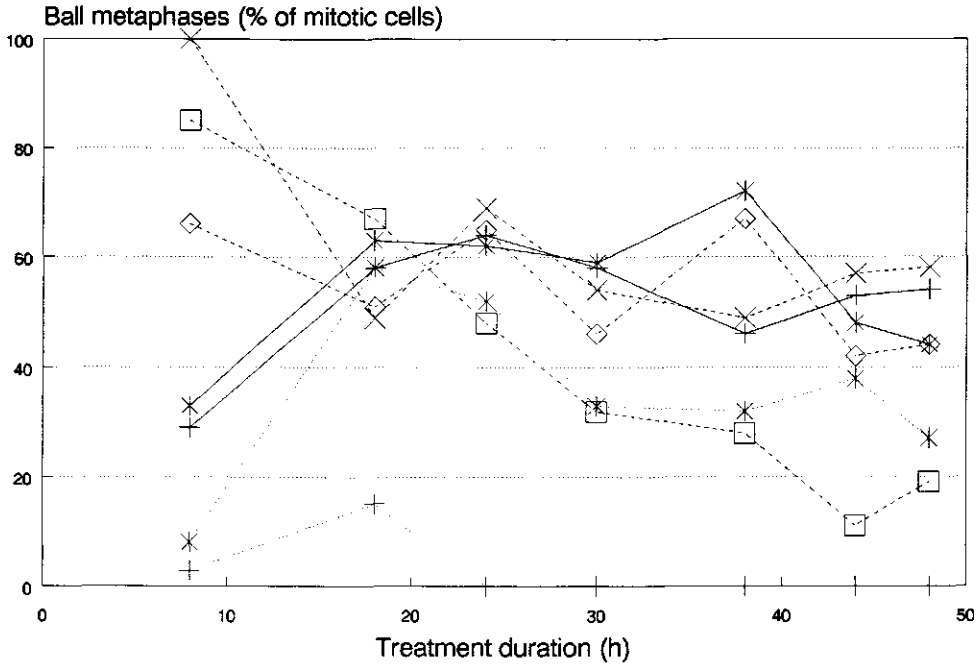

Fig. 3. Frequency of ball metaphases (cells with clustered chromosomes), expressed as percentage of the total number of mitotic cells, after treatment with: 0.5 mM colchicine (—+—); 5mM colchicine (—✳—); 3 μM oryzalin (--◇--); 15 μM oryzalin (✳--✳); 30 μM oryzalin(---□---); 3 μM APM (·✳·✳·); 32 μM APM (□·□) and untreated control (+—+). Chemicals were applied at t = 0, i.e. 24 h after subculture.

the mitotic index (Fig. 1). The minimal concentration required for MA varies considerably between the different inhibitors. Colchicine at 0.5 mM induced 20% MA while higher concentrations (e.g. 5 mM, Fig. 1) were less effective, resulting in 10% MA. APM and oryzalin induced up to 34% MA at concentrations as low as 3 μM and a tenfold higher concentration had no adverse effect on mitotic index. Maximum levels of the mitotic indices were obtained after APM and oryzalin treatments, resulting in mitotic indices of 25 to 27% for 3-32 μM APM and 30 to 35 % for 3-30 μM oryzalin which are significantly higher than the 20% obtained with the best colchicine treatment (0.5 mM).

Apart from the mitotic index, the type of metaphases also differed after treatments with colchicine, APM and oryzalin. Metaphases were classified into two types: ball metaphases, (cells with clustered chromosomes (Fig. 2a)) and scattered metaphases (cells with scattered chromosomes or chromosome groups (Figs. 2b, 2c)). Treatments with APM at all concentrations and oryzalin at the lowest concentration induced ball metaphases in less than 40% of the total number of mitotic cells after 30 hours. All concentrations of colchicine and higher concentrations of oryzalin (15 and 30 μM) induced more than 50% ball metaphases (Fig. 3). Flow cytometric data on nuclear DNA content of isolated nuclei (Fig. 4) showed an efficient doubling of the nuclear DNA content (progression to G2/M) during the 18 h following the addition of the inhibitors, in all

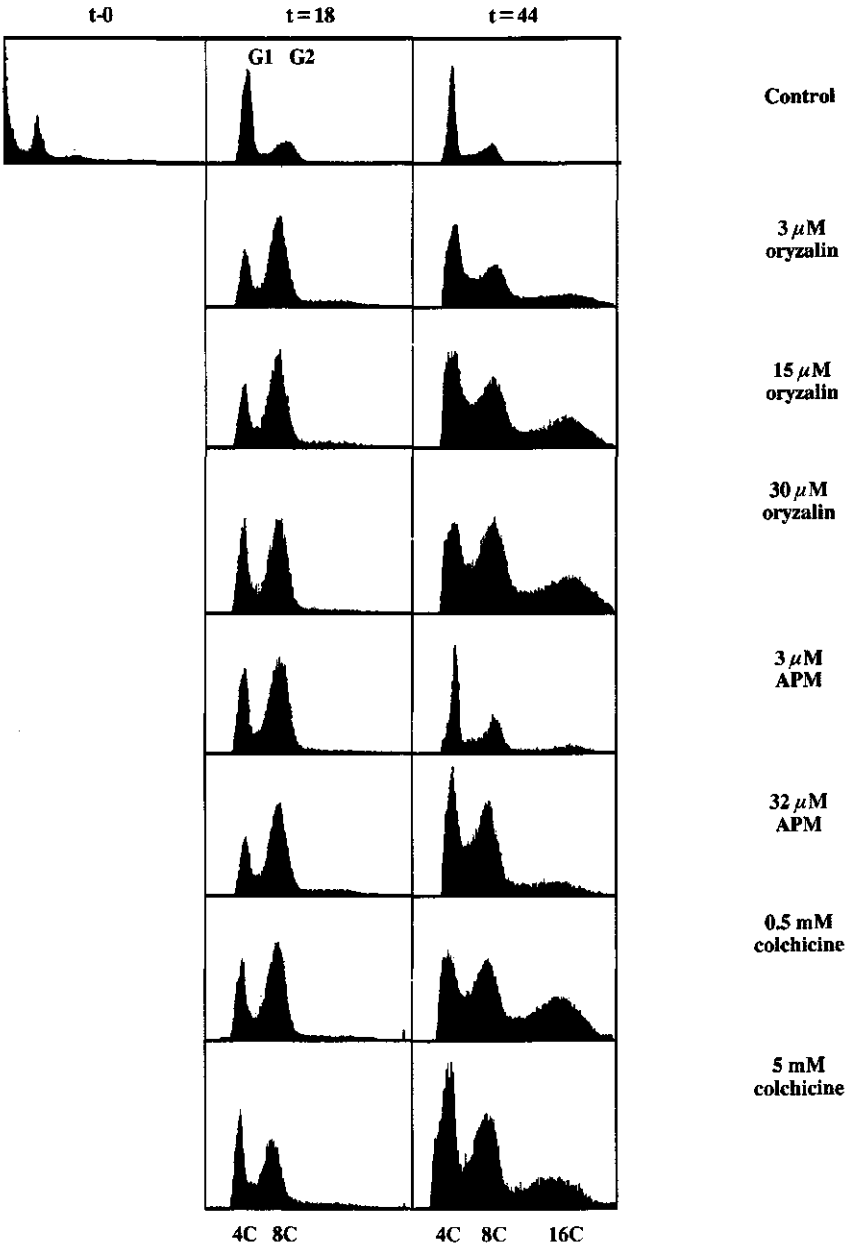

Fig. 4. Flow-cytometric histograms of nuclear DNA content of the control (upper row, t = 0, 18, 44 h), and the treated cell suspensions (t = 18, 44 h). On the horizontal axis is the DNA content expressed in C-value, in which 1 C is the DNA content of a haploid *N.plumbaginifolia*; on the vertical axis is the number of particles .

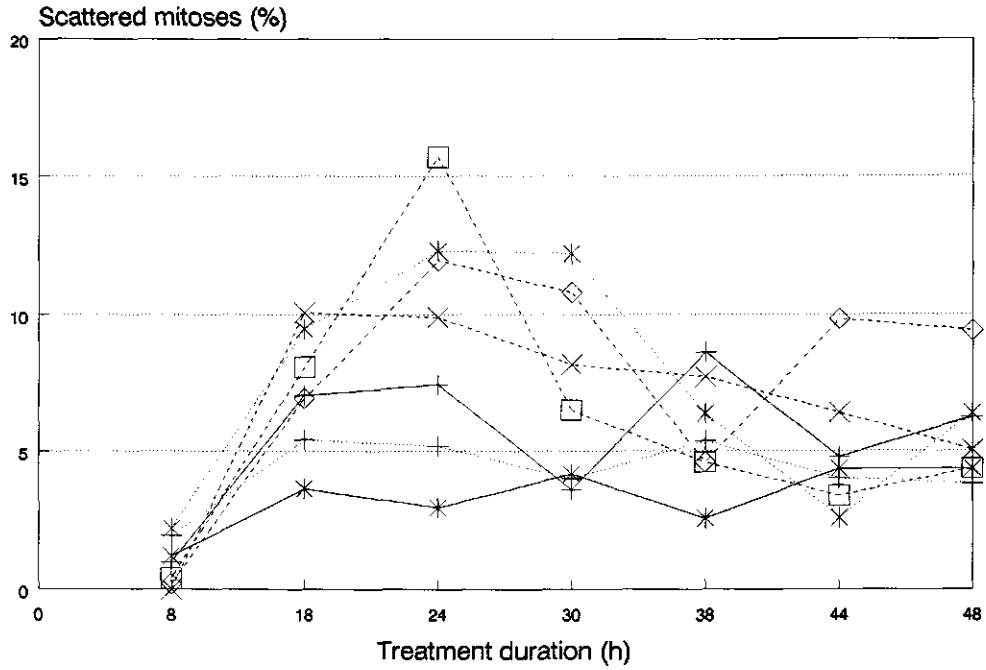

Fig. 5. Percentage of scattered mitoses (cells with scattered chromosomes and chromosome groups) after treatments with: 0.5 mM colchicine (-+-+-); 5mM colchicine (-✳-✳-); 3 μM oryzalin (--◇--); 15 μM oryzalin (-·✳·-); 30 μM oryzalin(-·-□-·-); 3 μM APM (···✳···); 32 μM APM (□···□) and untreated control (+···+). Chemicals were applied at t = 0, i.e. 24 h after subculture.

preparations. This can be concluded from the shift of the DNA level to the 8C-level at t = 18 and a further shift to the 16-C level at t = 44. The only exception at t = 18 is the preparation treated with 5 mM colchicine. At t = 44, the 0.5 mM colchicine treatment, and the 15 and 30 μM oryzalin treatments show a high degree of DNA doubling, whereas 3 and 32 μM APM and 3 μM oryzalin show less efficient doubling at t = 44, due to the scattering of chromosomes, and the induction of micronuclei. The lower number of cells with doubled DNA in the preparations treated with 3 and 32 μM APM or 3 μM oryzalin, coincides with a low number of ball metaphases (< 40%). The results on scattered metaphases (Fig. 5) show that 5 mM colchicine treatment even prevents scattering of the chromosomes, reducing the number of cells with scattered chromosomes (4%) to below that of the untreated control (5%). Treatment with 3 μM APM or 3 μM oryzalin, however, resulted in 12 and 16% cells with scattered chromosomes.

Chromosome isolation

It appeared to be impossible to resolve ball metaphases into separate chromosomes by shearing forces, resulting in a poor yield of isolated metaphase chromosomes after colchicine treatments (data not shown). Due to the scattering of chromosomes (Fig. 2b, 2c), isolation of individual chromosomes was markedly improved by the APM

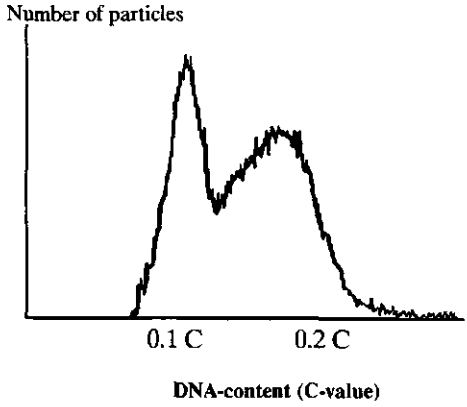

Fig. 6. Flowcytometric analysis of isolated chromosomes showing sister- chromatids (left peak) and metaphase chromosomes (right peak). On the horizontal axis is the DNA content expressed in C-value, in which 1 C is the DNA content of a haploid *N.plumbaginifolia*; on the vertical axis is the number of particles.

treatment. During chromosome isolation an increased release of separate sister chromatids was obtained as a consequence of the infuence of the spindle toxins on the stability of the centromeres. This resulted in a large number of sister chromatids in the flow histogram (Fig. 6, left peak), together with the metaphase chromosomes (Fig. 6, right peak).

Micronucleation

When the micronucleus index was determined after the different treatments, striking differences were observed. Treatments with colchicine resulted in levels of micronucleation up to 4%, whereas treatments with APM rendered micronucleus indices up to 12% (Fig. 7). Although metaphase arrest was hardly affected by the APM or oryzalin concentration, the micronucleus index appeared to be influenced by the concentration of oryzalin. At a concentration of 3 μM oryzalin, the induction of micronuclei is more efficient than at higher concentrations, which are more effective for ploidy doubling. The optimal induction of micronuclei was obtained by treatments with 32 μM APM, which resulted in up to 480 micronuclei per 500 cells, with an average number of 8 micronuclei per micronucleated cell (Fig. 8).

Discussion

The results presented in this paper, show that accumulation of metaphases can be increased significantly by using the anti-microtubular herbicides APM or oryzalin as compared to colchicine treatments. Furthermore, APM treatment results in a reduction of the frequency of cells showing chromosome clumping (ball metaphases) to less than 30% of the frequency of ball metaphases after 0.5 mM colchicine treatment. The same effect was observed after incubation with low concentrations of oryzalin, which is in agreement with reports of the effects of oryzalin on mitotic endosperm cells of *Haemantus katherinae* (Morejohn et al., 1987).

The accumulation of metaphases increases from 30 to 35% with increasing concentrations of oryzalin, however, relatively more ball metaphases are induced as well, resulting in poor chromosome scattering. The inhibition of spindle

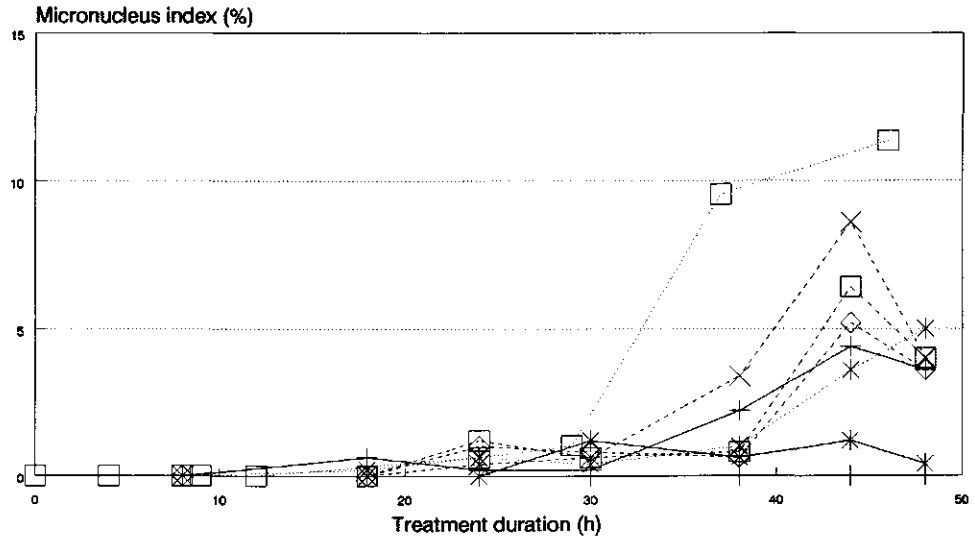

Fig. 7. Micronucleus index (percentage micronucleated cells) after treatments with: 0.5 mM colchicine ( ); 5mM colchicine ( ); 3 μM oryzalin ( ); 15 μM oryzalin ( ); 30 μM oryzalin( ); 3 μM APM ( ); 32 μM APM ( ) and untreated control ( ). Chemicals were applied at t = 0, i.e. 24 h after subculture.

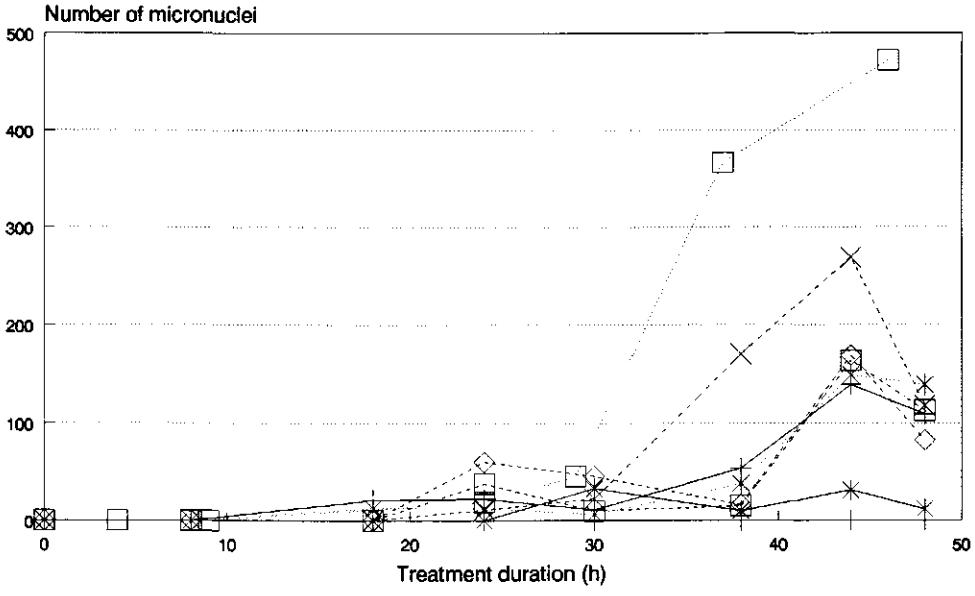

Fig. 8. Total number of micronuclei per 500 cells after treatments with: 0.5 mM colchicine ( ); 5mM colchicine ( ); 3 μM oryzalin ( ); 15 μM oryzalin ( ); 30 μM oryzalin( ); 3 μM APM ( ); 32 μM APM ( ) and untreated control ( ). Chemicals were applied at t = 0, i.e. 24 h after subculture.

formation is much stronger with APM or oryzalin than with colchicine, resulting in a more efficient accumulation of metaphases. Disruption of the calcium storage in the mitochondria has been proposed as the cause of the destruction of tubulins by APM and oryzalin, as has been shown in the green alga *Chlamydomonas* (Quader and Filner, 1980). However, for deregulation of the calcium metabolism, 10- to 100-fold higher concentrations of the inhibitor were needed, than for disturbance of mitotic activities (Morejohn and Fosket, 1986). Recently, APM and oryzalin have been shown to affect chromosome behaviour directly by binding to the tubulin hetero-dimers (Morejohn and Fosket, 1984; Morejohn et al., 1987).

The toxic effects of high concentrations of colchicine reduce the number of cycling cells, as can be concluded from the flow-cytometry data. Since this does not occur when APM and oryzalin are used, high mitotic indices can be induced, even with high concentrations of these herbicides. This is partly due to their very low solubility in water (7 μM for oryzalin, and 225 μM for APM). The observation that concentrations higher than 7 μM oryzalin have additional effects may be due to the lipophilic nature of oryzalin, which causes an accumulation of the chemical in the membranes (Morejohn et al., 1987). Thus, the additional effects of higher concentrations of oryzalin may be attributed to membrane effects, and most likely the disruption of the accumulation of calcium in the mitochondria.

Chromosome scattering is the first stage in the induction of micronuclei. As described earlier, chromosomes group together, and a nuclear membrane is formed around each single chromosome and around grouped chromosomes in various species (Sree Ramulu et al., 1988a; b). After prolonged treatments with APM, micronuclei were induced at high frequency (Verhoeven et al., 1987; de Laat et al., 1987). Micronuclei fuse together forming a restitution nucleus (Sree Ramulu et al., 1988a; b; this thesis, chapter three). The micronucleus index is dependent on the chemical used for metaphase arrest, and there seems to be a relation between the maximum level of mitotic index, and the level of the micronucleus index. APM is the most effective inducer, followed by oryzalin, whereas colchicine is the least effective (Figs. 7, Fig. 8). Another interesting observation is that micronuclei induced by oryzalin persist for only a few hours before forming a restitution nucleus by fusing together. Micronuclei, induced by APM, persist longer, as can be deduced from Fig. 7, which shows that the micronucleus indices for the 3 and 32 μM APM treatment do not decrease for at least 10 hours.
The difference in influence on calcium uptake and accumulation might explain this phenomenon, since the action of both chemicals on the spindle is comparable for the tested concentrations. The effective concentrations for inhibition of microtubule assembly are in the submicromolar range for both APM and oryzalin. Disruption of calcium storage in the mitochondria starts at about 5 μM for oryzalin and APM, which makes it possible to manipulate the two actions more or less independently. There is a large difference in the effectiviness of the herbicides compared to colchicine. Colchicine has hardly any influence, even at concentrations as high as 10 mM, whereas APM reduces mitochondrial calcium uptake to 50% at 15 μM (Hertel et al., 1980). Oryzalin is slightly less effective than APM in inhibiting the calcium uptake by the mitochondria, and reduces the active excretion of calcium by the plasma membrane to less than 60% of the untreated control, whereas APM leaves this activity almost unchanged at 90% (Hertel et al., 1980). As a result, intracellular cytoplasmic calcium level will be higher for oryzalin than for APM treatments, resulting in a lower tendency to form nuclear membranes around single or grouped chromosomes after oryzalin treatments. High cytoplasmic

calcium might also explain why micronuclei disappear sooner in oryzalin treatments than in APM treated cells, since high calcium concentrations promote the fusion of membranes. Changes in the intracellular calcium concentrations are known to have effects on the formation of nuclear membranes, as has been shown by the effects on the transition from metaphase to anaphase in stamen hair cells of *Tradescantia* (Wolniak et al., 1983; Wolniak and Bart, 1985). Compartmentalization of calcium ions by the smooth endoplasmatic reticulum plays a role in the regulation of mitosis in barley cells (Hepler, 1980). Lowering of the cytoplasmic calcium concentration induces telophasing and leads to the formation of nuclear membranes around individual chromosomes in cultured mammalian cells (Matsui et al., 1982). A comparison on the effects of APM and oryzalin on the accumulation of calcium in the mitochondria and their influence on active excretion by the plasma membrane might elucidate the mechanism involved in the induction of micronuclei in plants.

The data obtained in this study indicate that oryzalin at 3 to 15 μM is the most suitable chemical to be used for accumulation of metaphase chromosomes, whereas 3 to 32 μM APM treatments are more efficient for the induction of micronuclei. Large numbers of cells with scattered metaphase chromosomes, which can be obtained by oryzalin treatments, are useful for flow cytometric sorting (de Laat and Blaas, 1984; Conia et al., 1987a; 1987b) and chromosome-mediated gene transfer (Griesbach, 1987). Micronucleated cells can be used to isolate microprotoplasts, consisting of one or a few micronuclei, surrounded by a small amount of cytoplasm and an intact plasma membrane. Thus, a system analogous to the microcell-mediated gene transfer in animal cells (Fournier, 1982) would become feasible in plants.

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CHAPTER 5

IMPROVED ACCUMULATION OF MITOTIC AND MICRONUCLEATED SUSPENSION CELLS OF NICOTIANA PLUMBAGINIFOLIA BY AMIPROPHOS-METHYL AFTER PRETREATMENT WITH DNA-SYNTHESIS INHIBITORS

H.A. Verhoeven and H.C.P.M. van der Valk

IMPROVED ACCUMULATION OF MITOTIC AND MICRONUCLEATED SUSPENSION CELLS OF NICOTIANA PLUMBAGINIFOLIA BY AMIPROPHOS-METHYL AFTER PRETREATMENT WITH DNA-SYNTHESIS INHIBITORS

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Summary

The accumulation of suspension cells of *N.plumbaginifolia* at metaphase and the induction of micronuclei was optimized by inhibition of DNA synthesis, preceding the amiprophos-methyl (APM) treatment. The effects of a starvation pretreatment and varying concentrations of hydroxyurea (HU) or aphidicolin (APH) on cellular DNA content, mitotic index (MI) and micronucleus index (MNI) were investigated, resulting in a procedure for accumulation of up to 50% for both metaphases and micronuclei.

Key words: DNA synthesis inhibition, hydroxyurea, aphidicolin, *Nicotiana*, mitotic index, micronucleus.

Introduction

Accumulation of plant cells in a specific phase of the cell cycle is an important tool in many biological investigations. Simultaneous division is important especially in the case of mass isolation of metaphase chromosomes (Griesbach et al., 1982; Hadlaczky et al., 1983, Matthews, 1983; de Laat and Blaas, 1984), and for the induction of micronuclei (Verhoeven et al., 1986; 1987; de Laat et al., 1987; Sree Ramulu et al., 1988a).

Passage through the cell cycle can be prevented either by starvation procedures or chemical inhibitors, acting on different stages of the DNA synthesis.

For inhibition by starvation, suspension cells are grown to plateau phase, and partial synchrony is induced by adding fresh medium (reviewed in King, 1980; Gould, 1984). Starvation results in a drastic reduction of the mitotic index. After replacement of the medium, the mitotic index rises sharply. During the starvation period, growth can be retarded by carbohydrate, phosphate or nitrogen limitation. Starvation is a simple procedure, however, it seldom yields a satisfactorily high accumulation of cells in a particular phase of the cell cycle. Cells are arrested at the G1/S boundary or in G2 phase, and not at one specific point, resulting in poor synchrony.

A similar approach is based on the observation that isolated protoplasts show synchronous division after removal of the cell wall-degrading enzymes (Weber and Schweiger, 1985).

Most chemical treatments for cell cycle arrest interfere with DNA synthesis during S-phase. Two different mechanisms are known for inhibition of DNA synthesis.

Firstly, the enzymes involved in the production of the nucleotides can be inhibited by substrate analogues, such as

hydroxyurea, 5-aminouracil and fluorodeoxyuridine. In this way, the endogenous pool of intermediates has to be exhausted, before the inhibition becomes effective. As a consequence, also DNA repair is inhibited.

Secondly, the DNA α-polymerase enzyme itself can be inhibited. Once all enzyme molecules have bound the inhibitor, DNA synthesis is blocked completely, but the DNA repair mechanisms remain operational. The nucleotides, necessary for DNA repair, are still available because nucleotide synthesis is unaffected by the inhibitor. Since DNA repair is achieved with a different DNA polymerase, for which the substrates are still available, no DNA damage will be accumulated during G2-phase.

Hydroxyurea is an inhibitor, belonging to the first class, acting on the ribonucleotide reductase enzyme (Kihlman et al., 1966; Keil and Chaleff, 1983). During HU treatments, abberations are induced in the nuclear DNA. The generation of abberations by hydroxyurea seems to occur especially during G2 phase, by inhibition of DNA repair mechanisms owing to lack of precursors (Anderson, 1983). It has also been shown that the action of HU can be reversed when the duration of the treatments is 24 h or less (Coyle and Strauss, 1970). Besides, work in our laboratory has shown, that damage to the chromosomes of *Happlopappus gracilis* due to 24 h hydroxyurea treatment, could not be estimated by electron microcoscopic evaluation, or by alkaline gradient centrifugation (de Laat and Schel, 1986). Prolonged exposure (> 24 h) of both animal and plant cells to hydroxyurea induces chromosome breaks, and leads to the formation of sister chromatid exchanges (Kihlman et al., 1966; Coyle and Strauss, 1970; Andersson, 1983), which are irreversible.

Aphidicolin (APH) belongs to the second group of DNA synthesis inhibitors (Galli and Sala, 1982). APH inhibits DNA α-polymerase directly and reversibly (Sala et al., 1980). In contrast to other DNA synthesis inhibitors, aphidicolin does not block DNA repair mechanisms (Sala et al., 1982). By using aphidicolin, followed by a colchicine treatment, high mitotic indices, up to 65 %, were obtained in *Petunia* cell suspensions (Guri et al., 1984).

In this paper, a study is presented about the effects of starvation combined with different concentrations of HU and APH on mitotic index (MI) and micronucleus index (MNI). For this purpose, the division activity was evaluated cytologically and by flow cytometric DNA analysis. Besides, the synchronizing treatments were combined with amiprophos-methyl blockage (Verhoeven et al., 1986) to accumulate cells in metaphase and study the process of induction of micronuclei (de Laat et al., 1987).

Materials and methods

Cell culture

A suspension cell culture of *N. plumbaginifolia*, kindly provided by Dr. Shields, Unilever Res. Lab., Colworth Home, Sharnbrook, Bedford, UK was cultured in the dark at 28°C in "Doba" medium (Barfield et al., 1985) on a gyratory shaker (120 rpm). From this cell suspension, a kanamycin resistant line was derived by transformation with *Agrobacterium tumefaciens* as described before (de Laat and Blaas, 1987). For optimal growth, the suspension was subcultured twice a week, with a fourfold dilution with fresh medium. Starvation experiments were performed with cells, grown for a week to plateau phase. The material from several flasks was pooled, mixed, and subcultured by fourfold dilution with fresh "Doba" medium.

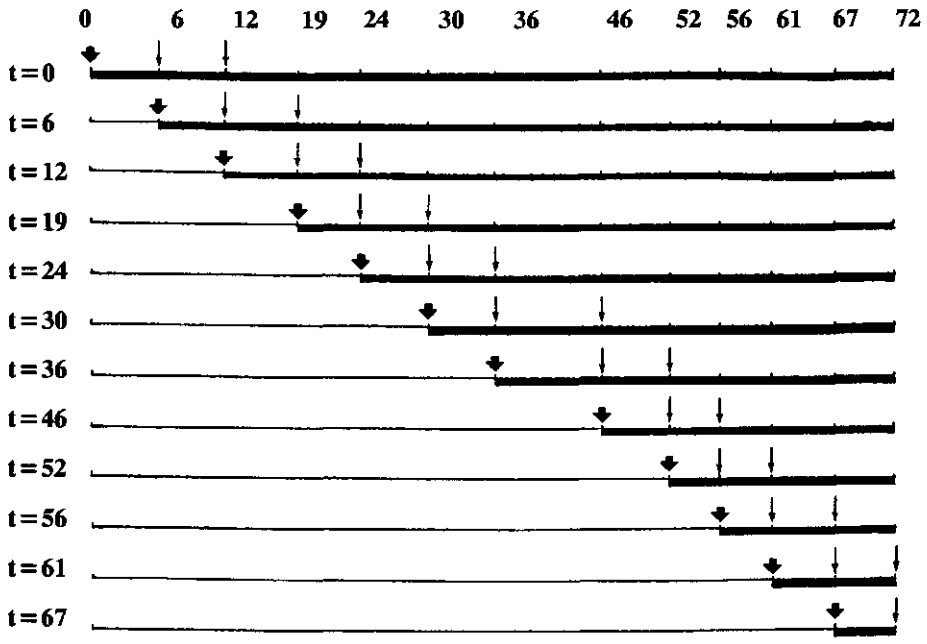


Fig. 1. Time schedule for the determination of the percentage of cells, which go into division during the indicated intervals. APM is added to identically treated cultures, at the times indicated by the big arrows. Samples were taken at the times indicated by the small arrows, and mitotic indices were determined. The increase in mitotic index following the addition of APM was considered to be the number of divided cells in the test period.

Chemical treatments.

DNA synthesis was blocked by various concentrations of hydroxyurea (HU) or aphidicoline (APH)(both supplied by Sigma). HU was added as a concentrated, freshly prepared solution in medium (40 mg/ml). APH was added from a stock solution (20 mg/ml) in dimethylsulphoxide (DMSO). Amiprophos-methyl (Tokunol M®; O-methyl-O-O-(4-methyl-6-nitro phenyl)-N-isopropyl-phosphoro thioamidate, Kiermayer and Fedtke, 1977) was a gift from Bayer Nederland B.V., Divisie Agrochemie, Arnhem, The Netherlands. Stock solutions of APM were prepared at 20 mg/ml in waterfree DMSO, and could be stored for at least 6 months at -20°C, without loss of activity.

Cytology

Squash slides were prepared by staining a small amount of dried suspension cells with a solution of 1% orceine in 1:1 lactic acid:propionic acid, followed by heating of the slides to about 60°C (Eriksson, 1966) and gentle squashing. Mitotic index was determined by counting between 300 and 600 cells. All stages from prophase up to and including telophase were counted as mitotic cells.

Flow cytometry

For flow cytometric determinations of the nuclear DNA content, protoplasts were isolated from small samples of the cell suspension. To 800 μl of cell suspension, 400μl of an enzyme mixture, containing 25%

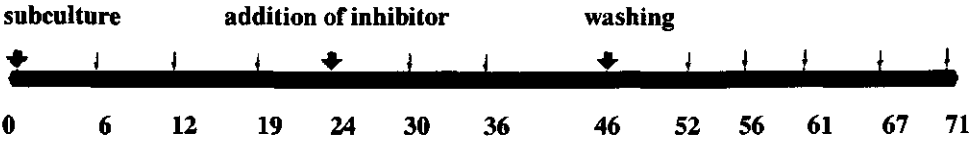


Fig. 2. Time schedule for experiments on the influence of DNA-synthesis inhibitors. Samples for FCM and MI were taken at the times, indicated by arrows. At 24 h after subculture, chemicals were added, and removed at 46 h after subculture by washing with excess medium (5 volumes).

Cellulase "Onozuka" R10 (Yakult, Honska Co, Tokyo, Japan), 5 % Macerozyme R10 and 1.2 M of sucrose, was added. After 30 min incubation in a 2.5 cm petridish, at 28°C, on a gyratory shaker at 30 rpm, the enzyme mixture was centrifuged for 5 min at 60 g. Floating protoplasts were transferred to nucleus isolation buffer, containing 10 mM Tris-HCl, pH 7, with 10 mM spermine-tetrahydrochloride, 2.5μg/ml 4,6-diamidino-2-phenylindole (DAPI), 10 mM NaCl, 200 mM hexyleneglycol and 0.025% Triton-X100. Samples were analyzed on a Partec PAS-II flowcytometer, with the UG5 excitation filter, TK420 and TK520 dichroic mirrors, and GG435 longpass filter. Channel analysis was performed with the standard software package of the PAS-II.

Time schedule

The number of cells, going into division during the various stages of the treatment, was determined by adding APM to a final concentration of 16μM to different flasks with cells. Mitotic index and DNA content were determined at regular intervals (Fig. 1). The increase in mitotic index during each incubation period was determined, and the average was calculated from this value with the value for the corresponding interval from the previous treatment.
For the evaluation of the effects of the inhibitors on mitotic index, samples were taken for cytological analysis according to the scheme of Fig. 2. After the chemical inhibition of DNA synthesis, cells were washed with 5 volumes of culture medium, and half of the cell mass was subcultured in fresh medium with 32 μM APM.

Results

Starvation

The effect of the starvation period is represented in Fig. 3. Control cell suspension cultures were subcultured as usual on the third day of culture. The 3 day old culture showed a mitotic index of 4% at the time of subculture (t = 0) and recovered rapidly as revealed by the rapid increase of MI to 7%. Mitotic index was increased already 4 h after subculture, and reached a maximum during log-phase close to 7% (8-24 h after subculture). In the starved, 7 day old culture, mitotic index remained below 2% until 20 h after subculture. From this moment on the mitotic index gradually increased, reaching a value of 8% at 52h after subculture.

Division activity

Fig. 4 represents the percentage of mitotic cells (the sum of cells in mitosis or with micronuclei) after subculture, determined by accumulating all mitotic cells during APM treatment. The treatments were started according to the scheme in Fig. 1, and the average of the increase during two subsequent intervals was graphically represented in Fig. 4. This graph shows that recovery from starvation treatment lasts at least 20 h. Then a sudden rise in the division

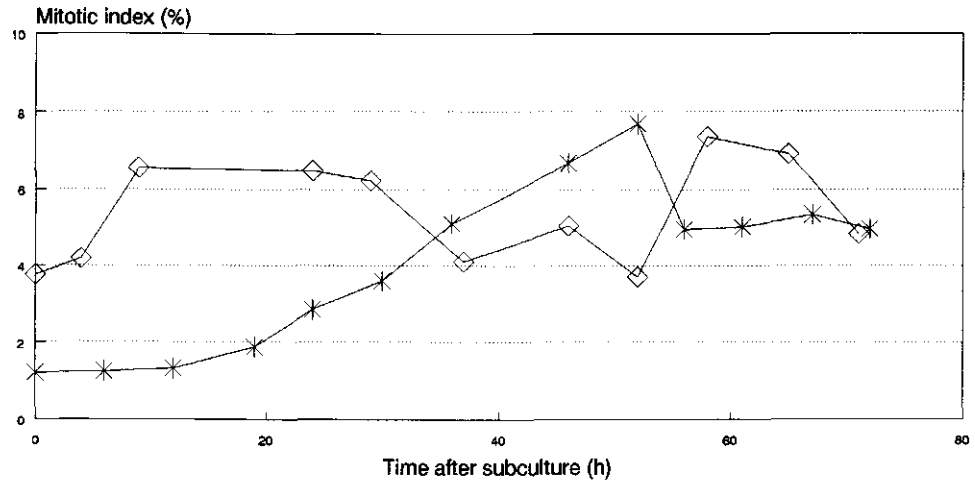


Fig. 3. Effect of starvation of a cell suspension culture on the mitotic index after subculture. (-◇—◇-): 3-day old culture; (-✳—✳-): starved, 7-day old culture.

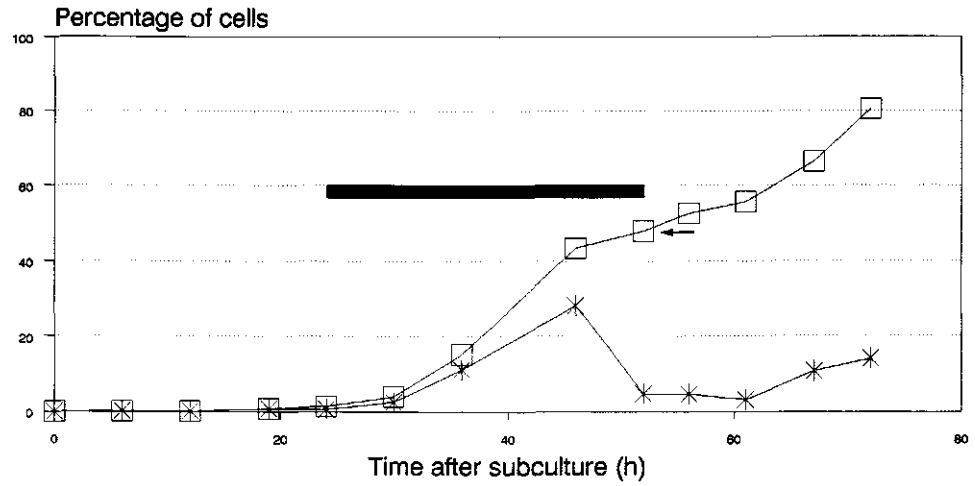


Fig. 4. Division activity (-✳—✳-) of a starved (7-day old) cell suspension culture at different times after subculture, and the total number of cells which have undergone cell division at that time (-□—□-). Bar indicates the duration of DNA-synthesis inhibition, and the arrow the percentage of divided cells at the end of the treatment.

activity is observed, which is less pronounced in the mitotic index of the control without APM treatment (Fig. 3). The burst of 25% of divided cells is followed by a divison rate of 5-17%. The graph shows that more than 50% of the cells would have undergone division during the HU and APH treatments which took place from 24 to 46 h (indicated by the bar in Fig. 4).

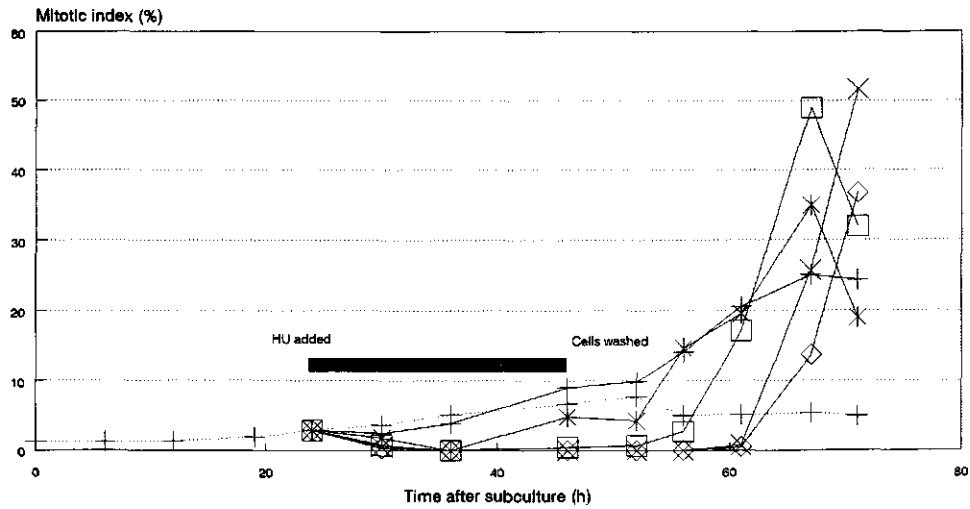


Fig. 5. Mitotic index of a starved cell suspension at different times after subculture, and the influence of different concentrations of HU, combined with APM treatment after washing (—+—): untreated control; (—+—+—): 1 mM HU; (—✳—✳—): 2 mM HU; (—□—□—): 5 mM HU; (—✕—✕—): 10 mM HU; (—◇—◇—): 20 mM HU. Bar indicates period of HU treatments.

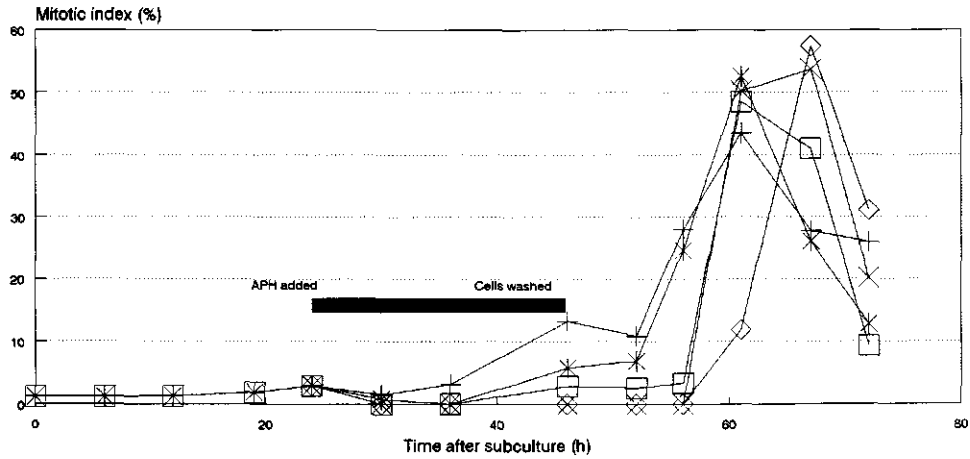


Fig. 6. As Fig. 5, with APH instead of HU. (—+—+—): 3 μM APH; (—✳—✳—): 6 μM APH; (—□—□—): 15 μM APH; (—✕—✕—): 30 μM APH; (—◇—◇—): 60 μM APH. Bar indicates period of APH treatments.

Chemical inhibition

The effects of the addition of different concentrations of HU and APH on mitotic index are represented in Figs. 5 and 6, respectively. For this experiment, a suspension culture was used, which was grown to plateau phase at the time of subculture (t = 0). A concentration dependent inhibition is observed, which

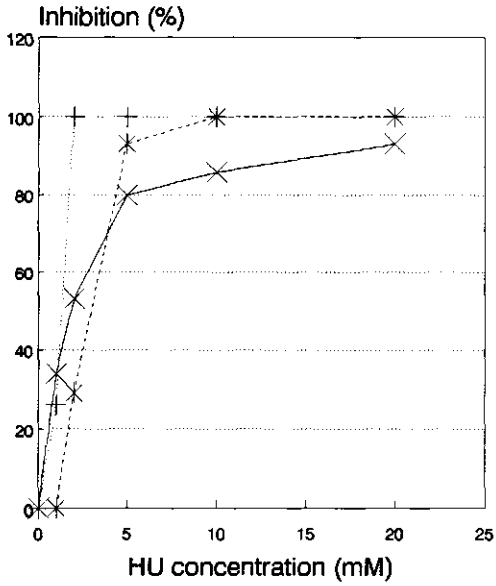


Fig. 7. Inhibition of division as a consequence of DNA synthesis inhibition by various concentrations of HU, determined at various times after start of the treatment. 100% inhibition causes a MI of 0%, and 0% inhibition results in a MI identical to the untreated control.

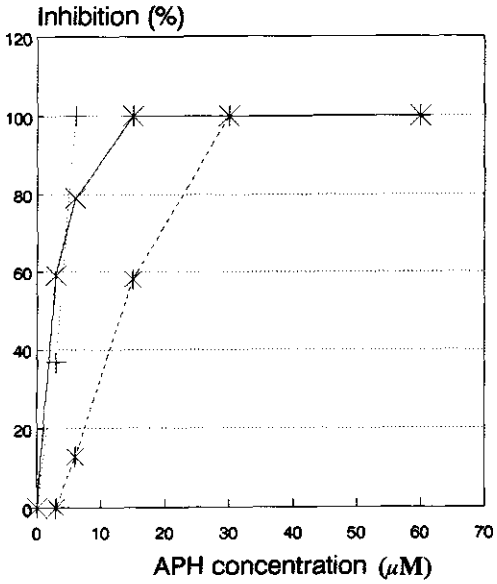
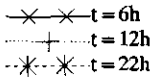


Fig. 8. As Fig. 7, with APH instead of HU.



shows some characteristic differences between HU and APH. The inhibition was calculated by dividing the mitotic index of the treated cells by the mitotic index of the control cell suspension. The result was expressed as percentage and subtracted from 100%. The resulting values are presented in Figs. 7 and 8, at different times of treatment (6, 12 and 22h of treatment).

Treatment with HU results in a concentration dependent reduction of the mitotic index, which was apparent after six hours of treatment (t = 30 h after subculture). Higher concentrations showed a stronger inhibition. After 12 h of treatment (t = 36 h after subculture), all concentrations tested, except 1mM, resulted in 100% inhibition of mitosis. The effect of the lower concentrations (1 mM, 2 mM and 5 mM) is reduced after 22 h of treatment (t = 46 after subculture), and in case of 1 mM HU an increase of the MI to 136% of the untreated control was observed (see Fig. 5). The effects of APH treatment are comparable to the HU treatment, with two differences. Inhibition is already 100% at six hour after the start of the treatment for concentrations of 15μM and higher. The samples of 12 h of treatment show a pattern similar to the lower HU treatments, but the 22 h samples show a strong decrease of the inhibition, resulting at 3 μM in doubling of the mitotic index as compared to the control (see Fig. 6).

After washing, the mitotic index remains at a low level for all concentrations of the DNA-synthesis inhibitors, which were still active at the time of washing (10, 20 mM HU and 15, 30 and 60μM APH). This effect lasts at least until t = 57 h after subculture (11h

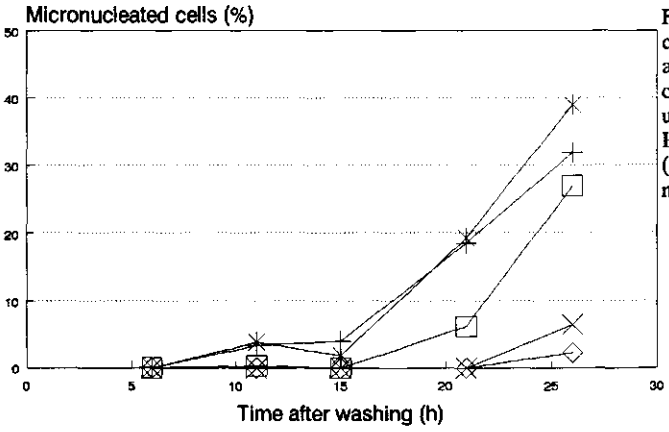


Fig. 9. Micronucleus index (MNI) caused by 32 μM APM treatment, after pretreatment with varying concentrations of HU. (···+···): untreated control; (+——+): 1 mM HU; (-✳—✳-): 2 mM HU; (□—□): 5 mM HU; (×—×): 10 mM HU; (◇—◇): 20 mM HU.

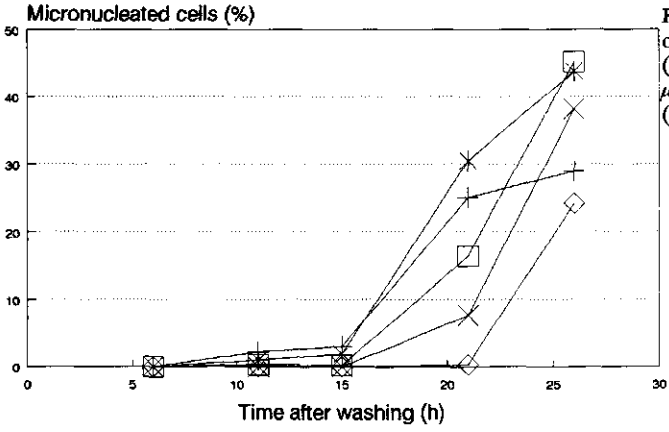


Fig. 10. As Fig. 9, with APH instead of HU. (-+——+-): 3 μM APH; (✳—✳): 6 μM APH; (□—□): 15 μM APH; (-×—×-): 30 μM APH; (◇—◇): 60 μM APH.

Time after washing (h)	HU Concentration (mM)									
	1		2		5		10		20	
	MI	MNI	MI	MNI	MI	MNI	MI	MNI	MI	MNI
0	9.1	0	4.7	0	0.4	0	0	0	0	0
6	10.0	0	4.2	0	0.6	0	0	0	0	0
11	14.2	3.4	14.7	3.9	2.7	0.3	0	0	0	0
15	20.6	3.4	19.6	1.9	17.1	0	0.7	0	0.4	0
21	25.1	18.5	34.9	19.3	48.9	6.1	25.7	0	3.7	0
26	24.4	31.8	18.9	39.0	31.9	26.9	51.6	6.4	36.8	2.2
	APH Concentration (μM)									
	3		6		15		30		60	
	MI	MNI	MI	MNI	MI	MNI	MI	MNI	MI	MNI
0	13.3	0	5.8	0	2.8	0	0	0	0	0
6	11.0	0	6.9	0	2.6	0	0	0	0	0
11	27.9	2.2	24.5	1.0	3.3	0.3	0	0	0	0
15	43.6	2.9	52.6	1.8	48.5	0.2	50.2	0	12.0	0
21	27.7	25.0	26.0	30.5	41.0	16.3	53.7	7.6	57.4	0.3
26	25.9	29.0	12.8	43.8	9.4	45.2	20.1	38.1	31.1	24.2

Table 1. Relation between MI and MNI at various times after washing the suspension cell cultures free from HU or APH. The concentrations of HU and APH are indicated.

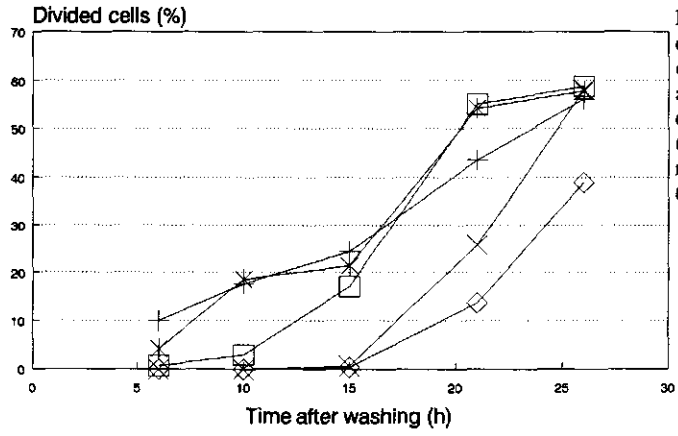


Fig. 11. The percentage of divided cells following HU treatment, determined by the sum of the MI and the MNI. (—+—): untreated control; (-+—+-): 1 mM HU; (—✳—✳—): 2 mM HU; (—⊟—⊟—): 5 mM HU; (—✕—✕—): 10 mM HU; (—◇—◇—): 20 mM HU.

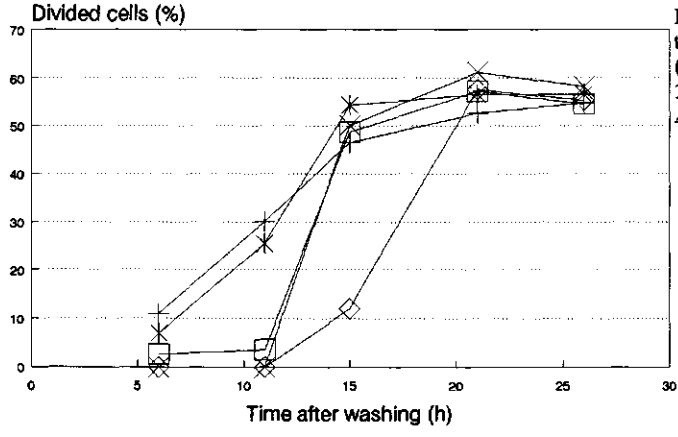


Fig. 12. As Fig. 11, after APH treatment. (-+—+-): 3 μM APH; (—✳—✳—): 6 μM APH; (—⊟—⊟—): 15 μM APH; (—✕—✕—): 30 μM APH; (—◇—◇—): 60 μM APH.

after washing), and the mitotic index rises sharply at t = 61 h after subculture for the 5 mM HU and the 15, 30 and 60μM APH treatments. A significantly longer period is necessary to recover from the inhibition after 10 and 20 mM HU treatments. Maximum mitotic index (about 50%) is reached at 67 h after subculture (21 h after washing) for the HU treatments, whereas all APH treatments exhibit the maximum mitotic index (53%) from t = 61 to t = 67, and show a sharp decrease at t = 72 after subculture (26 h after washing).

Remarkable is the rapid decrease of the mitotic index due to formation of micronuclei (Figs. 9 and 10). HU and APH treatments result in comparable levels of mitotic index, but with HU the increase is slower as compared to APH treatments, resulting in a less efficient accumulation of metaphases and an insufficiently separated occurrence of micronuclei and metaphase chromosomes. The relation between MI and MNI is represented in Table 1. The table shows, that a high MI is a prerequisite for a high MNI, and that there is a delay of 12 h between the rise in MI and the rise in MNI (see also Figs. 9 and 10). When the MI rises slowly, as after 10 and 20 mM HU treatments, the appearance of metaphases and micronuclei is insufficiently separated in time. In both treatments, a high MI (40%) occurs simultaneously with a high MNI

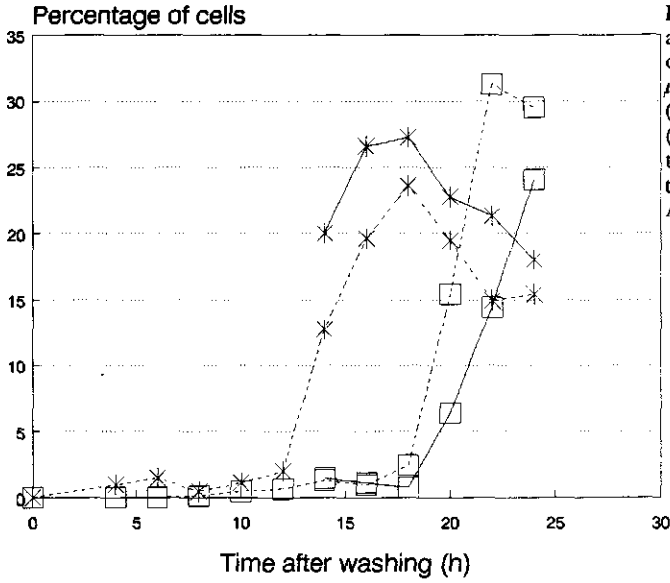


Fig. 13. Influence of the delayed addition (6 h after washing, instead of immediately after washing) of 32 μM APM on the MI and MNI. (-✳--✳-): MI, APM added at t = 0; (-□--□-): MNI, APM added at t = 0; (—✳—✳—): MI, APM added at t = 6 after washing; (—□—□—): MNI, APM added at t = 6 after washing.

(6%). Treatment with 15 μM APH, however, shows a very sharp transition from metaphase arrest to accumulation of micronuclei between t = 15 and t = 26 h after washing (MNI rises from 0% to 45%, while mitotic index decreases from 48% to 9%). In all cases a good yield of micronuclei is achieved when mitotic index shows a synchronous increase, whereas a poor micronucleation is obtained after a slow increase of mitotic index. From Figs. 9 and 10 can be concluded, that micronucleus induction requires an exposure time of mitotic cells to APM for at least 11 h, before a significant transition from mitotic cells to micronucleated cells takes place.

The total number of mitotic cells and micronucleated cells (MI and MNI added) is represented in Figs. 11 and 12, for HU and APH respectively. The graphs show that for all of the treatments except for 20 mM HU, the total number of mitotic cells has reached a value of about 55%, which is in good agreement with the total number of dividing cells, determined earlier. The lower value of mitotic cells obtained with 20mM HU treatment, is due to incomplete recovery from inhibition. A similar effect can be observed with 60 μM APH, which reaches the 55% level later than all other APH treatments.

Fig. 13 shows the effect of addition of APM at 6 h after washing, compared to addition of APM directly after washing, on the mitotic index and the occurrence of micronuclei. The graph shows, that it is possible to obtain a cell suspension with a high mitotic index and a low micronucleus index by adding APM at 6 hours after washing, without affecting the level of the mitotic index.

Flow cytometric analysis revealed the strong inhibition of cellular DNA synthesis by 10 and 20 mM concentrations of HU and 30 and 60 μM APH at the time of washing (Figs. 14 and 15, respectively). In all treatments the G2 phase is completely absent, which is in agreement with the data on mitotic index. Lower concentrations did not inhibit DNA synthesis completely, as is demonstrated by the presence of a G2 peak in the DNA histograms. At the time of flow cytometric

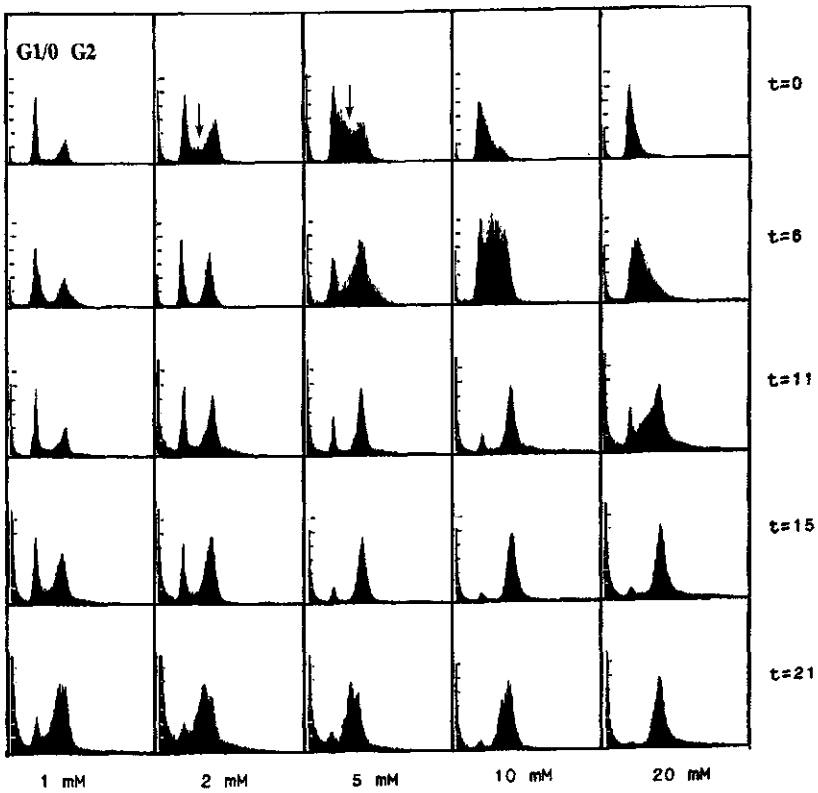


Fig. 14. DNA histograms of the cellular DNA content at the time of washing (t = 0) and later, for all concentrations of HU tested. The DNA content is put on the horizontal axis, and the number of cells on the vertical axis. The position of the G1/G0 level is indicated, as well as the position of the G2 level.

analysis, DNA synthesis had already restarted during the very short duration of protoplast isolation procedure in the 2 and 5 mM HU treatments, and in the 15, 30 and 60 μM APH treatments, as can be seen from the number of cells passing through S-phase at t = 0 (arrows in t = 0 row). The number of cycling cells is determined by the difference in the number of G1/G0 cells in the 60 μM APH treatment sample at t = 21 h after washing with the number of G1/G0 cells in the same treatment, at t = 0 after washing, which is 80%. This number is in good agreement with the number indicated in Fig. 4 at t = 72 h after subculture (26 h after washing).

The duration of S and G2-phase can be deduced from the histograms of the 30 μM APH treatment. Since inhibition was still 100% with this treatment at the time of washing, and most of the cells were already passing through S-phase at the time of analysis, this represents the least affected situation in this experiment. Six hours after washing, all cycling cells (> 80%) had already reached the G2-phase (Arrows in t = 6 row). This indicates that S-phase lasts 6

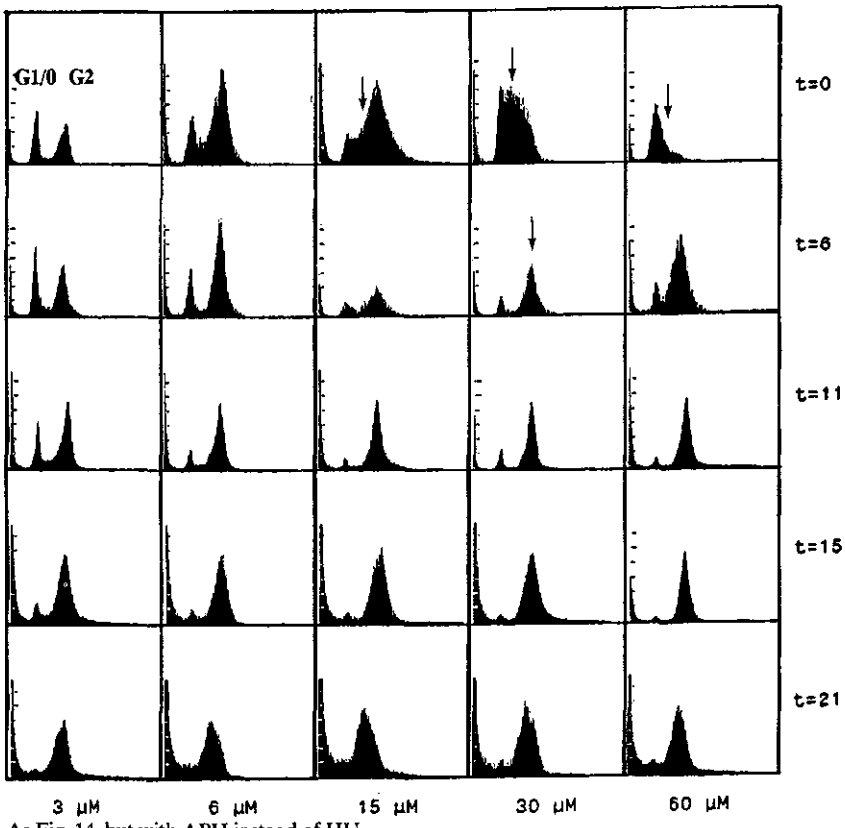


Fig. 15. As Fig. 14, but with APH instead of HU.

h or less. The same time was required for the passage through S-phase with the 60 μM APH treatment. It took less than 11 hours before the MI started to increase, indicating the occurrence of the first divisions. The G2-phase must therefore be somewhat less than 11 hours. The total time necessary for the cells to pass through S- and G2-phase was about 17 h. Only the higher HU concentrations (10 and 20 mM) required a longer time for recovery, and from the DNA histograms can be concluded, that especially S-phase was prolonged: in case of 20 mM HU even with more than 6 hours.

Discussion

Most protocols for accumulation and isolation of metaphase chromosomes, do not provide information on the characteristics of the cell material used, such as the number of cycling cells and the duration of the relevant stages of the cell cycle (Griesbach et al., 1982; Hadlaczky et al., 1983; Szabados et al., 1981; Matthews, 1983). To obtain an optimal induction of synchronous division without knowledge of these parameters is almost impossible without performing many

laborious empirical experiments. The aim of our experiments was to establish efficient procedures for the induction of simultaneous mitosis, necessary for the mass isolation of metaphase chromosomes, and induction and isolation of micronuclei. Both approaches require levels of mitotic cells and micronucleated cells, as high as possible. Cell cycle synchronization implicates that the cell material used is of a homogeneous composition with regard to the length of the cell cycle. Only cells with the same duration of the cell cycle can be synchronized (Gould 1984).
However, a truly synchronous passage through the cell cycle is not required for isolation of metaphase chromosomes and micronuclei, since for this purpose a double inhibition can be applied, using both DNA synthesis blockers and metaphase arresting agents.

Firstly, the number of actually cycling cells during the period of accumulation was determined, since this number limits the maximum accumulation of cells at a given stage of the cell cycle. For this purpose, cell division activity was determined by APM addition at different times after subculture, to otherwise identically treated cultures. By counting accumulated metaphases during treatment, and later also micronucleated cells, an accurate picture was obtained from division activity at various times during the experiment. It turned out that the cells needed 30 h before they had recovered from the starvation treatment, resulting in a sudden increase in mitotic activity between $t = 30$ and $t = 46$. This period coincided with DNA synthesis inhibition, and resulted in accumulation of more than 50% of the cells in or at the beginning of S-phase, when inhibition was sufficiently strong. The cells going into division after $t = 46$, were only partially accumulated: only the cells which would have been in S-phase during the inhibition would have accumulated. Since S-phase lasts for about 6 hours, this will result in a further increase with 5% to 55% of the inhibited cells. With cell cultures which were not grown to plateau phase, a less synchronous division was observed, resulting in a lower number of cells which can be arrested during a 24 h treatment with DNA-sythesis inhibitors. As a consequence, the history of the culture together with its growth characteristics are important parameters, determining the maximum level of the mitotic index.

Secondly, the inhibition of DNA-synthesis was examined by cytological and flow cytometric DNA analysis. Flow cytometry has the advantage, that large numbers of cells can be analysed with a high degree of accuracy in a very short time (Crissman and Tobey, 1974;Galbraith et al., 1983; Bergounioux et al., 1988). Especially the effects of DNA-synthesis inhibitors can be analysed readily (Fox et al., 1987). From the results, presented in this paper, conclusions can be drawn on the number of cycling cells ($> 80\%$), together with their cell cycle parameters (S-phase 6 h; G2-phase 11 h), without the need of radioactive treatments in labelling studies (Wang et al., 1986). The information obtained is also relevant for a more efficient way to establish procedures for accumulation of mitotic cells. Since the number of cycling cells can be estimated, either cytologically or by flow cytometry during any period, the best period for DNA-synthesis inhibition can be defined. Furthermore, the effect and the duration of the treatment can be analysed easily, allowing the assay of many chemicals and their concentrations.

The results from the present analysis show that both HU and APH are efficient DNA synthesis blockers, but at completely different concentrations.
The effects of both HU and APH treatment are fully comparable. At low concentrations, the inhibition lasts only a short time, due to degradation of the inhibitor in the case of APH (Sala et al., 1983). A degradation mechanism for HU is not known, but our

data suggest that such a mechanism exists in the cell supension of *N.plumbaginifolia*. As a consequence of the decreased inhibition, the simultaneous division of the treated cell suspension is impaired.
The difference in time, necessary for maximum inhibition may reflect the difference in polarity of both compounds. HU is highly polar, dissolves easily in water, and penetrates membranes only slowly, whereas APH is highly apolar, dissolves only as a trace in water, and crosses membranes easily. With APH, a higher mitotic index can be obtained than with HU, and synchronization is also better, resulting in a better control of the appearance of micronuclei. This is due to the fact that HU does not arrest cells at the beginning of S-phase, but somewhere in S-phase, resulting in poor synchrony after release of the HU block (Walters et al., 1976). APH is the inhibitor of choice for the cell line tested, because of its low cytotoxicity and the fact that DNA repair is not affected (Sala et al., 1982).

With the procedures described, suspension cultures of *Nicotiana plumbaginifolia* and other plant species (Sree Ramulu et al., 1988b) can be analyzed rapidly, resulting in a protocol for the induction of simultaneous division. The pretreatment with DNA synthesis inhibitors (10mM HU or 15 μM APH during 24 h), followed by washing and the addition of APM, results in a synchronous increase of MI up to 50%. Twelve hours after its maximum, MI decreases and the MNI rises to 40%. By varying the moment of addition of APM, the time at which metaphase chromosomes or micronuclei appear can be controlled accurately. In this way, metaphase chromosomes can be isolated with low contamination by micronuclei, and vice versa. Furthermore, both the number of micronucleated cells and the number of micronuclei per cell are significantly improved after cell cycle arrest. This may open up the way for a more efficient isolation of metaphase chromosomes, micronuclei and microprotoplasts (Verhoeven et al., 1988).

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CHAPTER 6

ISOLATION AND CHARACTERIZATION OF MICROPROTOPLASTS FROM APM-TREATED SUSPENSION CELLS OF NICOTIANA PLUMBAGINIFOLIA.

H.A.Verhoeven and K. Sree Ramulu

ISOLATION AND CHARACTERIZATION OF MICROPROTOPLASTS FROM APM-TREATED SUSPENSION CELLS OF NICOTIANA PLUMBAGINIFOLIA.

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Summary

A procedure is described for the isolation of subprotoplasts with a DNA content of less than the G1 level, so called micro-protoplasts. Microprotoplasts consist of one or more micronuclei surrounded by a thin rim of cytoplasm and an intact plasma membrane. Micronuclei are induced after treatment of a suspension culture of *N.plumbaginifolia* with amiprophos-methyl (APM). Protoplasts are fractionated on a continuous iso-osmotic gradient of Percoll, resulting in several visible bands. The bands were analyzed, and data on cytology, DNA content of individual (sub)protoplasts, vitality and membrane integrity are presented. The results reveal the presence of up to 40% microprotoplasts in the fraction, just below the major visible band. These microprotoplasts have DNA content less than G1 level, some showing an amount equivalent to that of one or a few chromosomes. The application of microprotoplasts for chromosome mediated gene transfer in plants is discussed.

Key words: Cellular manipulation, Micronucleus, Microprotoplast, Evacuolation, Chromosome-mediated gene transfer.

Introduction

Recent progress in the field of plant genetic manipulation has resulted in a wide range of techniques for the introduction of foreign genes. Protoplast fusion can be used when polygenically determined complex characters are of interest, or when the molecular basis of a particular trait is not yet known. However, since the entire donor genome is added to the recipient, the resulting hybrid constitutes a complex genetic system, which can not be analyzed properly. Therefore, X-ray irradiation is often applied to induce chromosome loss from the donor (Bates et al., 1987). Recent work has shown however, that a large proportion of the fragmented genome incorporates as small fragments into the recipient genome (Imamura et al., 1987).

Mammalian somatic cell genetics employed two additional techniques for gene mapping: chromosome mediated gene transfer (Klobutcher and Ruddle, 1981) and microcell-mediated chromosome transfer (Fournier, 1982). So far, only microcell-mediated chromosome transfer has resulted into stable maintenance of donor chromosomes, while still transferring only a small fraction of the donor genome, even single chromosomes (Lugo and

Fournier, 1986). The procedure involves the induction of micronucleation in cell lines carrying selectable markers, through modified C-mitosis after colchicine or Colcemid treatment. Single or groups of chromosomes undergo decondensation and form micronuclei. Subsequently, microcells are prepared by either treatment with cytochalasin-B, or by centrifugation in the presence of cytochalasin-B, and fused with the recipient cells (Ege et al., 1977; Fournier, 1981).

Until recently, no treatment or agent (physical or chemical) was known for the efficient induction of micronuclei in plants, which prevented the development of a microprotoplast system, analogous to the microcell system in mammals. Since the finding, that the herbicide APM induces micronuclei at high frequency in cell suspension cultures of *N.plumbaginifolia* (Verhoeven et al., 1986, 1987; de Laat et al., 1987), the application of microprotoplasts for limited genome transfer became feasible. APM is efficient in mass induction of micronucleation and induction of high number of micronuclei per micronucleated cell. It interferes with the polymerization of plant cortical and spindle microtubules (Morejohn and Fosket, 1984; Falconer and Seagull, 1987). Normal mitosis and growth were resumed after removal of APM by washing out and subculturing of cells, demonstrating that its inhibitory effect on the mitotic spindle is reversible (Sree Ramulu et al., 1988a). The induction of micronuclei by APM was observed in a wide range of genotypes and species (Sree Ramulu et al., 1988b).

In this paper, we report on the isolation and characterization of microprotoplasts from APM treated suspension cultures of *N. plumbaginifolia*. Some preliminary results have been communicated before (Verhoeven et al., 1988).

Materials and methods

Cell culture

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

APM treatments

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

Micronucleation

Micronuclei were induced by the addition of APM at 32 μM to cell suspensions on the second day after subculture. For optimal synchronization, this treatment was preceded by a 24 h incubation with 10 mM HU. After that, cells were washed free from HU and APM was added to the fresh culture medium.

Protoplast isolation

Protoplasts were isolated by treating 1 g of suspension cells with an enzyme solution, containing 5% Cellulase R10 and 1%

Macerozyme R10, and 0.4 M mannitol as osmoticum. To prevent the fusion of micronuclei during protoplast isolation, APM (32 μM) was added to the enzyme mixture. Cytochalasin-B (10mg/l) was added to disrupt microfilaments during protoplast isolation. Protoplasts were purified as described before (de Laat et al., 1987).

Centrifugation

Continuous iso osmotic gradients of Percoll were prepared by adding 7.2% (w/v) of mannitol to Percoll solution (Pharmacia), and centrifuged during 30 min in a Christ Omega ultra centrifuge (170,000 g), using the 6X5 ml swing-out rotor. For calibration purposes, density marker beads (Pharmacia, code 17-0459-01) were used. The top layer of 2-5 mm was removed from the preformed gradient, and the protoplast suspension containing 10 mg/l cytochalasin-B was layered on top. Cell fractionation was achieved by centrifugation during 1 h at 170,000 g at a temperature of 25 °C. The gradient was collected in small samples using a peristaltic pump. For cytological analysis of subprotoplasts, 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma chemical co.) was added to the gradient.

Microdensitometry

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

Flowcytometry

All measurements were made with a Partec PAS II flowcytometer, equipped with a HBO-100 mercury lamp. Protoplast membrane integrity was assessed by incubation of protoplasts with 10 μg/ml fluorescein-diacetate (FDA) prior to centrifugation. Samples were collected from the gradient and analysed for the fluorescein content of the fractionated protoplasts. For this analysis, the FITC filter-set (BP 450, dichroic mirrors TK500 and TK590 with BP520 as barrier filter) was used.

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

Subprotoplast culture

To determine viability, about 500 subprotoplasts, collected from several fractions of the gradient were cultured at very low densities (1000 protoplasts per ml) in Millicell-CM Culture plate Inserts (Millipore, Cat. No. PICM 01250). The Inserts were put in a 5 cm petridish, containing 3 ml of K3 culture medium (Kao et al., 1974) with 10^5 protoplasts/ml of *N.plumbaginifolia* as feeder.

Results

Isolation of micronucleated protoplasts

Prolonged exposure of suspension cells of *N.plumbaginifolia* to APM resulted in a high frequency of micronucleation. With APM only, up to 15 % of the cells became micronucleated. After HU treatment,

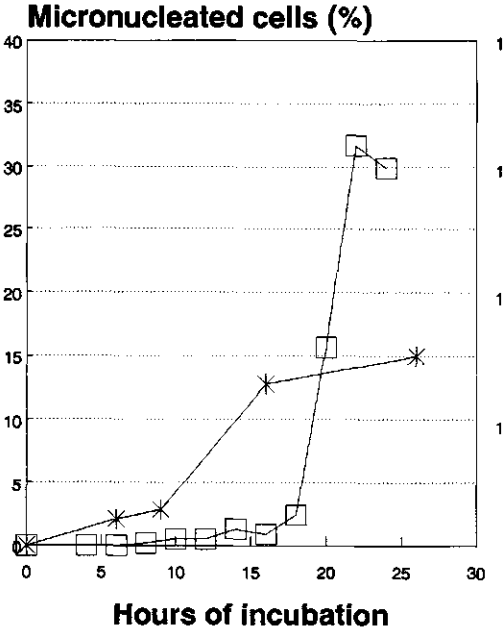

Fig. 1. Percentage of cells with micronuclei after treatment with APM (32 μM) only(-✳-✳-) or after treatment with HU (10 mM during 24 h), followed by washing and APM (32 μM) treatment (-□-□-) in cell suspension cultures of kanamycin resistant *N.plumbaginifolia*. The 0 h point refers to the starting of incubation time after 24 h HU treatment and washing.

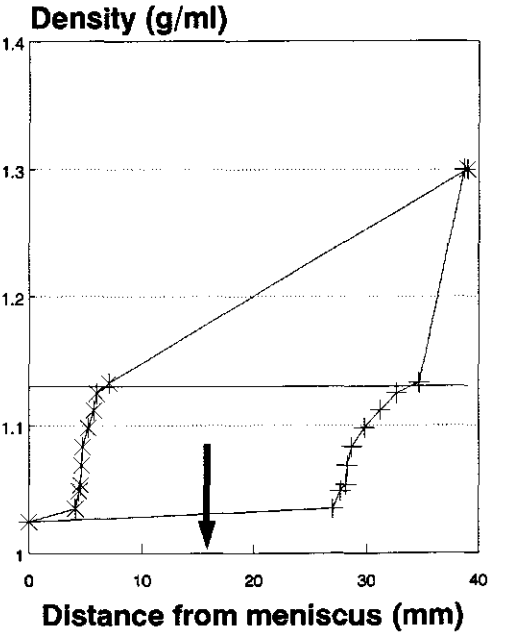

Fig. 2. Density profiles of Percoll solution before centrifugation (———), after 30 min. preformation at 170,000 g (-✳-✳-) and after protoplast fractionation (90 min. at 170,000 g) (-+-+-). The arrow indicates the position of the main band of subprotoplasts after protoplast fractionation.

followed by the addition of APM, this value was more than doubled (Fig. 1), and micronucleation was more efficient in terms of number of nuclei per cell (data not shown). During protoplast isolation, the addition of APM (32 μM) to the enzyme mixture resulted in the maintenance of the number of micronucleated cells at the same level as in the treated suspension cells. In the absence of APM, micronuclei in the protoplasts tend to fuse together, forming multilobed nuclei. The addition of cytochalasin-B induced a slight but significant increase in the number of micronucleated protoplasts (data not shown).

Fractionation of protoplasts

After a short incubation in 0.4 M mannitol with 10 μg/ml cytochalasine-B, the protoplasts were layered on top of the preformed Percoll gradient. The preformed gradient was analyzed, using the Density Marker Beads. After centrifugation, the pattern of the beads was again recorded (Fig. 2). In this way, each band was related to the specific density of the gradient at the position of the band. Fig. 3 gives the relation

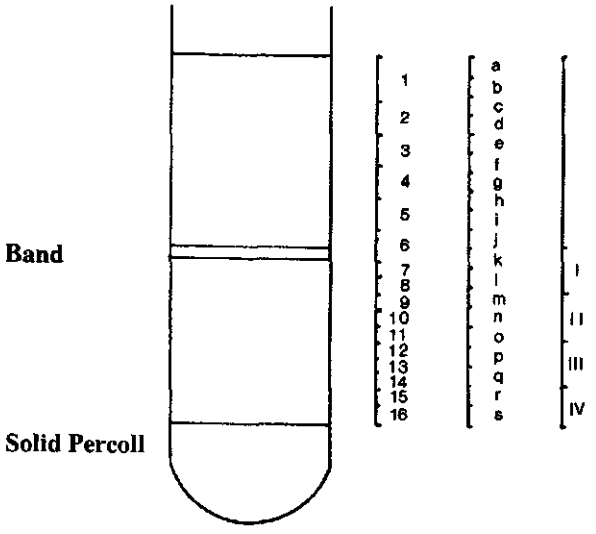

Fig. 3. Diagram of the position of the band in a Percoll gradient after protoplast fractionation. The bars illustrate the position of the fractions and the codes used in the text.

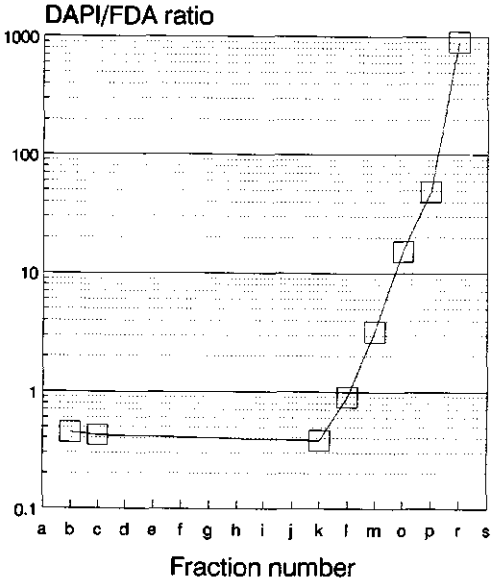

Fig. 4b. Ratio of the number of DAPI-positive subprotoplasts and the number of FDA-positive subprotoplasts. DAPI only stains nuclei, and FDA only stains subprotoplasts with an intact plasmamembrane. The numbers were derived from the diagrams in fig 4a.

between the fraction number and position in the gradient. Fractionation of protoplasts was carried out in four different experiments. For microdensitometric and cytological analyses, the lower fractions of the gradient (**I-IV**) were used. For more detailed analysis, the whole gradient was separated into 16 fractions (**1-16**). Flow cytometric analysis was performed with another gradient separated into 19 fractions (**a-s**).

Flow cytometric analysis

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

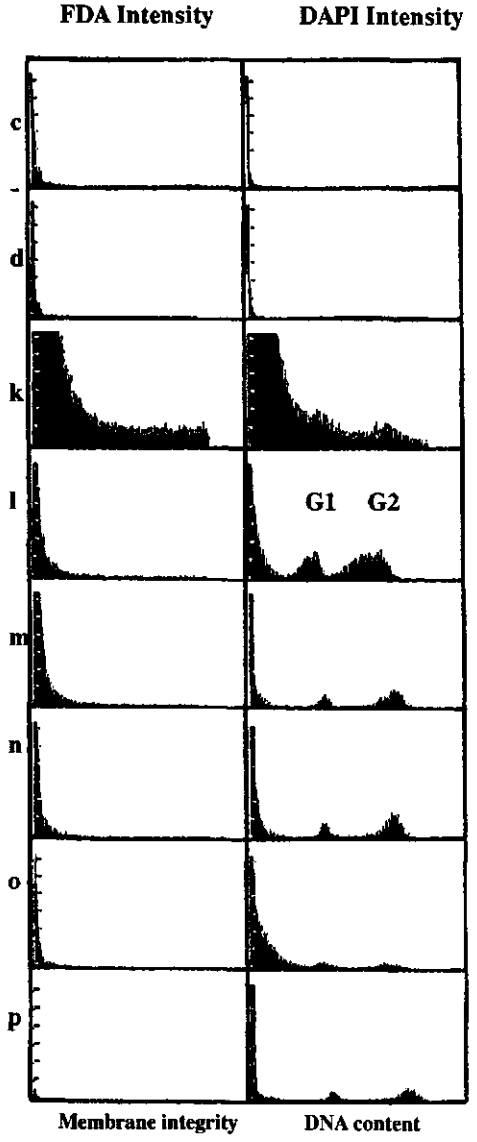

Fig. 4a. Intact subprotoplasts, determined by FDA-staining of subprotoplasts (left row), and DNA analysis of subprotoplasts after lysis and DAPI-staining (right row). Intact subprotoplasts show large FDA signals, whereas lysed ones show hardly any fluorescence. Indicated are the position of the G1 and the G2 level in the DAPI intensity histograms.

contents (Fig. 4a). This band contained the highest number of subprotoplasts. The nuclear DNA content of the subprotoplasts was determined by DAPI staining, in combination with Triton-X100, to make the membranes permeable to DAPI, which cannot cross an intact plasma membrane. The number of particles still containing fluorescein after centrifugation, is higher than the DAPI positive particles in fractions a to k (fraction **k** contains the major band), while these numbers are equal in fraction l (Fig. 4a). Down from fraction **l**, less FDA positive structures were observed compared to the number of DAPI positives. The ratio of the number of DAPI positive particles and the number of FDA-positive particles is represented in Fig. 4b. This graph shows, that all subprotoplasts in the main band, and in the fractions just below the main band (from fraction **a** down to and including fraction l), still possess an intact plasma membrane (Ratio < 1). Fractions **m-r** contain increasing numbers of damaged subprotoplasts or isolated nuclei.

Microdensitometry

Microdensitometric analysis revealed the presence of relatively high numbers of microprotoplasts in or just below the dense band, depending on the size of the fraction. Fig. 5 shows the cumulative distribution of the DNA content of intact looking subprotoplasts of the band (fraction **I**) and the three lower regions (fractions **II-IV**), and Fig. 6 shows this area divided over 9 samples (fractions **6-16**). These data show that just below the main band (fraction **6**) a high proportion (upto 40%) of intact microprotoplasts with less than G1 DNA content was observed in fraction **7** (Fig. 6). In Fig. 5 a lower frequency (20%) of microprotoplasts with $<$ G1 DNA level was found due to the simultaneous presence of the major band in fraction **I**. The other fractions also contained microprotoplasts showing less than G1 DNA content, but at lower frequency (20%) (Fig. 6).

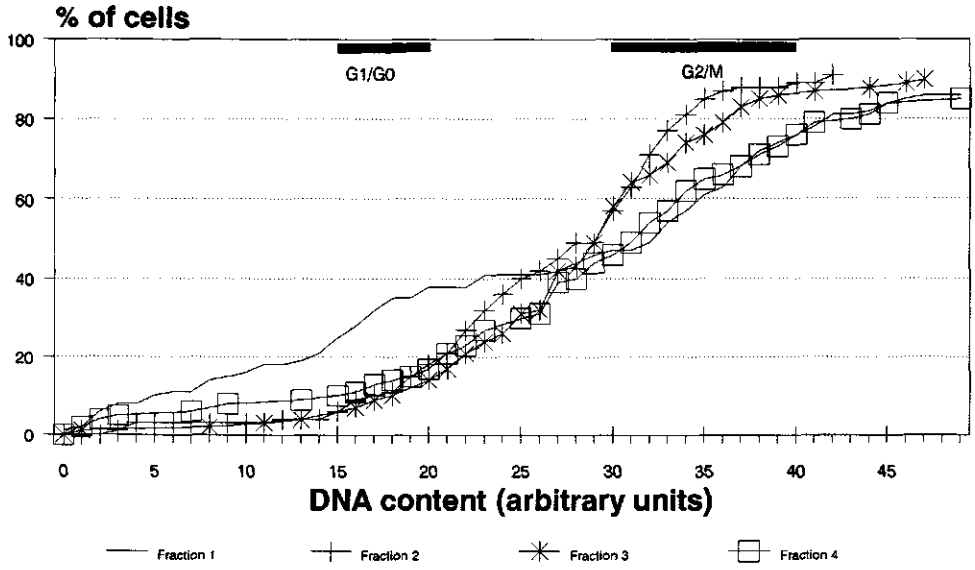

Fig. 5. Cumulative distribution of nuclear DNA content of subprotoplast isolated from gradient fractions I-IV. The frequency (%) of each value of DNA content was added to the preceding value to obtain cumulative DNA distribution. The bars indicate the G1 and G2 DNA levels, as calculated from the DNA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

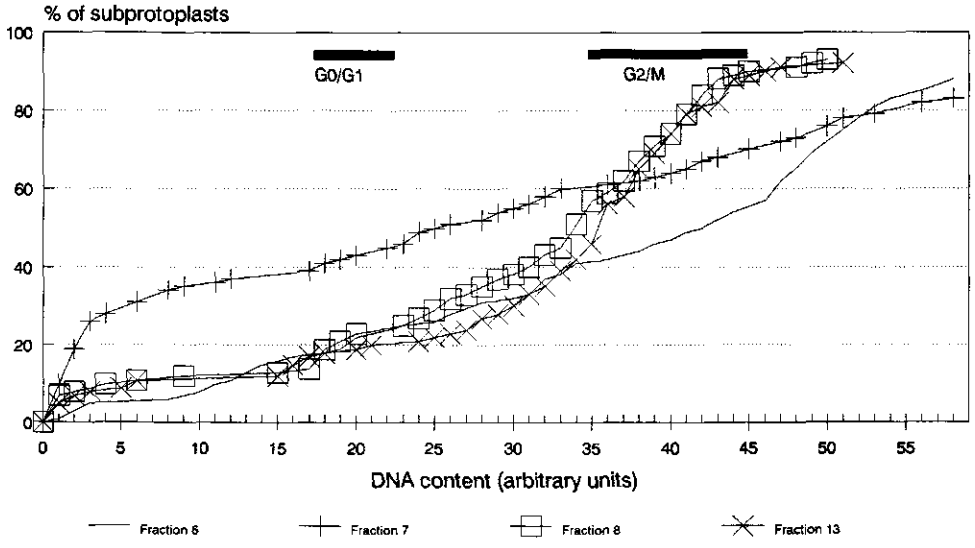

Fig. 6. Cumulative distribution of nuclear DNA content of subprotoplasts isolated from gradient fractions 6-16. Fractions 13-16 gave similar patters of DNA distribution and therefore only the data on fraction 13 are presented, as typical example. Bars indicate the DNA content of the G1 and G2 level.

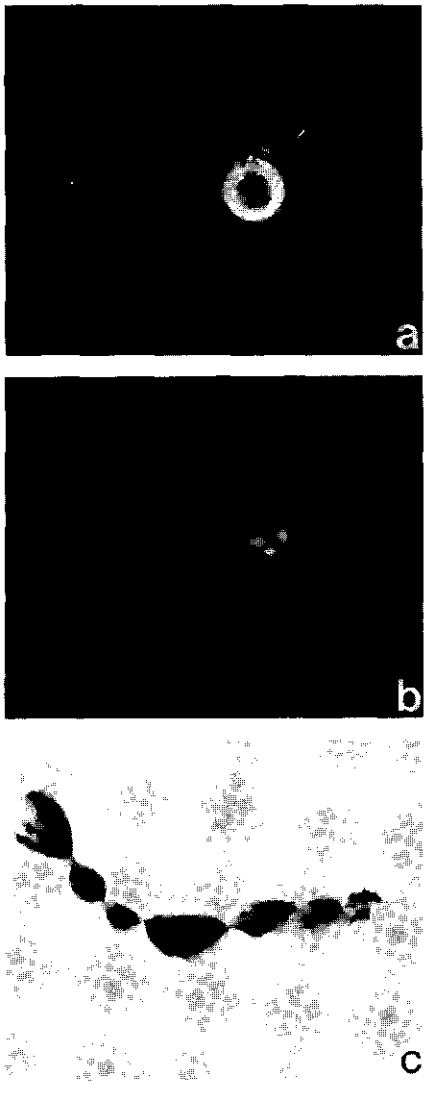

Fig. 7. Photomicrograph of a microprotoplast after centrifugation. a: phase contrast image; b: fluorescence image, showing the DAPI-stained micronuclei; c: incompletely fractionated microprotoplast, with the nuclei visible on the strands of cytoplasm.

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

	percentage distribution of various types of subprotoplasts in gradient fraction			
types of sub-protoplasts	Fractions I	II	III	IV
Cytoplasts	7	9	6	9
Evacuolated protoplasts	70	84	86	78
Micro pps < G2	2	3	4	4
Micro pps < G1	21	4	4	9
Number of microcalli				
Microcalli	XX	90	49	35

XX: Lost due to contamination.

Cytology

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

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

Subprotoplast culture

From the fractions **I**, **II**, **III** and **IV**, respectively 1000, 1000, 200 and 35 FDA positive subprotoplasts were isolated, and cultured in Millicell-CM Plate Inserts, using

N.plumbaginifolia cell suspension derived protoplasts as feeder in the petri-dish. In this way, microcalli were obtained from each fraction tested, except fraction **l**, which was lost due to contamination (Table 1). The microcalli were transferred to solid MS-medium with 1 % sucrose when they were 0.5-1 mm in diameter.

Discussion

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

The main difference between the procedures employed by the previous authors, and the one presented in this paper, is the use of cytoskeleton disrupting agents, namely APM for the microtubuli and cytochalasin-B for the microfilaments. The negative effects of cytochalasin-B on the regeneration of subprotoplasts, reported by Lörz et al.(1981), are compensated by the higher yield of evacuolated, intact subprotoplasts and better fractionation under the present experimental conditions. The main band appeared to contain 100% of evacuolated, intact (sub)protoplasts, as can be deduced from the data on flow cytometry. The upper band, with a very low percentage of nuclei contains only vacuoplasts. This is evident from the fact that only vacuoles surrounded by an intact protoplast membrane, retain FDA efficiently (Lesney et al., 1986). From the main band downwards, the amount of FDA-positive structures decreases strikingly. In fraction **l**, the ratio FDA-positive: DAPI-positive particles is about one, and decreases in fraction **m** and lower. Also, particles with a lower DNA content than the G1 level, are still present in fraction **l**, whereas they completely disappear from the lower fractions. This pattern is confirmed by the cytological data, which show a high proportion of subprotoplasts containing micronuclei in fraction **l**. This finding is also consistent with the results of two different microdensitometric analyses of the gradients: the highest frequency of subprotoplasts with DNA content less than G1 level is found in or just below the main band, depending on the size of the sample.
The formation of microcalli from subprotoplasts demonstrates their viability. Microprotoplast culture might have a valuable application in the production of cell lines with partial genomes and in studies of genome function in cell differentiation.
With the procedure for the isolation of microprotoplasts, described in this paper, a system for transfer of chromosomes by microprotoplast fusion, becomes feasible. Up to 40% of the subprotoplasts, isolated from the fraction just below the main band, has a DNA content less than the G1 level, some showing amounts equivalent to that of one or a few chromosomes. This means that 60 % of the protoplasts still contains at least the normal DNA content. Consequently, it is desirable to fuse and culture the microprotoplasts in a controllable way. The

fusion of individual protoplasts, such as developed by Koop et al. (1983), provides an elegant system for fusion of microprotoplasts with recipient protoplasts. This would open up the possibility to transfer single or a few chromosomes for gene mapping and expression studies in plants, and for the direct construction of addition lines important for use in plant breeding.

Acknowledgements

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CHAPTER 7

DIRECT CELL TO CELL TRANSFER OF ORGANELLES BY MICROINJECTION: METHODOLOGY AND FIRST RESULTS.

H. A. Verhoeven and J. Blaas.

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Summary

A novel technique for transfer of organelles between plant cells is presented. Organelles are removed from evacuolated donor protoplasts by micromanipulation, and microinjected directly into acceptor cells. First results obtained with this technique for transfer of chloroplasts and fluorescently labelled mitochondria are presented.

Key words: Microinjection, Micro manipulation, Organelle transfer, Complementation.

Introduction

The exchange of organelles between two plant species or varieties is a complicated procedure in conventional plant breeding. Transfer by means of protoplast fusion is now available for some years (reviewed in Galun and Aviv, 1986), but the undesirable nuclear genes from one of the partners have to be eliminated, e.g. by irradiation. One way to limit the number of possible combinations is to use cytoplasts as the organelle donor (Maliga et al., 1982; Lörz et al., 1981). Also uptake of isolated organelles by means of fusion-like processes has been reported, but no evidence for the functionality of the introduced organelles was presented (Giles, 1978). In animal cells, microinjection has been applied successfully to the transplantation of organelles, and even entire nuclei (Willadsen, 1986). Although the application of microinjection of DNA fragments into plant cells and protoplasts has received considerable attention (Crossway et al., 1986; Reich et al., 1986; Spangenberg et al., 1986; Morikawa and Yamada, 1985), the transfer of organelles has been limited to the injection of mass isolated chromosomes (Griesbach, 1987; de Laat et al., 1987). The major problems using microinjection for plant organelle transfer are the rigid cell wall of plant cells, the fragile nature of protoplasts, and the presence of large vacuoles in most plant cells, which interfere with the demand for large bore capillary needles. Although some microinjection systems are suitable for transferring mass isolated organelles (Griesbach, 1987; De Laat and Blaas, 1987), no system has been described by which organelles and chromosomes can be taken out of a donor protoplast and transferred into a recipient cell. Using this approach, damage to the transferred organelles might be minimized.

In this paper we present an injection system which allows the direct cell to cell transfer of organelles. Data are presented which show that protoplasts and cells survive injections with cytoplasm, and go into division. First results demonstrating the successful transfer of chloroplasts, leading to the complementation of a plastome deficient albino tobacco cell line are presented, and the prospects for this microinjection technique are discussed. Part of this work has been communicated earlier (Verhoeven and Blaas, 1988; Verhoeven et al., 1988).

Materials and methods

Plant material

For the cell to cell transfer of cytoplasm, a cell suspension of transformed *Nicotiana plumbaginifolia*, was used as donor and recipient. The original supension was kindly provided by Dr. R.Shields, Sittingbourne, UK.

In chloroplast complementation experiments, the VBW albino line of *N.tabacum* was used as acceptor. This cell line contains deficient chloroplasts, and can be complemented by the green chloroplasts from the donor tobacco line 92, as was demonstrated by fusion experiments (Aviv et al., 1984). The donor cell line 92 contains chloroplasts, conferring streptomycin and spectinomycin resistance, and mitochondria, both originating from *N. undulata*, in a *N.tabacum* nuclear background. Both cell lines were kindly provided by Dr. D. Aviv and Dr. E. Galun, Rehovot, Israel.

Cell culture

Suspensions from *N. plumbaginifolia*, were cultured in liquid medium (Barfield et al., 1985) and subcultured with 3-day intervals. The VBW tobacco line was cultured as aseptically grown plantlets on solid MS-medium in glass containers. The line 92 donor was either cultured in the same way as plantlets, as a source of mature chloroplasts, or as a cell suspension in MS-medium with 1 week subculture intervals for the transfer of proplastids.

Protoplast isolation

Protoplasts were prepared from suspension cells of *N. plumbaginifolia* by overnight incubation in enzyme mixture, containing 2% Cellulase R10 and 0.4% Macerozyme R10 and 0.4 M mannitol. The VBW acceptor protoplasts and the line 92 donor protoplasts were isolated after overnight incubation of leaves in an enzyme mixture, containing 1% Cellulase R10, 0.2% Macerozyme R10 and 0.4 M mannitol. Protoplasts were purified as described before (de Laat and Blaas, 1987), and resuspended in a small amount of K3 (Kao et al., 1974) medium.

Protoplast evacuolation

The microfilaments of the donor protoplasts were disrupted by a 15 min. 10 μg/ml cytochalasin-B (Sigma) pretreatment. Before staining, pretreated protoplasts were evacuolated by centrifugation on a preformed, isoosmotic Percoll gradient (0.4 M mannitol in Percoll (Pharmacia)) during 60 min at 25 °C at 170,000 g, in a 6X5 swing-out rotor.

Protoplast staining

To visualize the transfer of mitochondria, donor protoplasts were treated with 20 μg/ml of di(hexyl)-oxocarbocyanine-iodine (DIODC, Serva), which results in specific staining of the mitochondria (Matzke and Matzke, 1986). After 10 min of staining, excess free stain was removed by washing twice with culture medium.

Protoplast immobilization

Protoplasts were immobilized in specially prepared petridishes as described before (De Laat and Blaas, 1987). The donor protoplasts were immobilized in the same petridish with the acceptor protoplasts. After the acceptor had been cultured for a few days, the liquid medium was removed, and the petridish was put on ice. The stained, freshly isolated and evacuolated donor protoplasts, suspended in 20 μl K3 medium with 0.8% LMT agarose (FMC Corporation, Rockland, USA), were spread out on the cold surface of the Petridish. After gelling, fresh K3 medium was again added to the petridish.

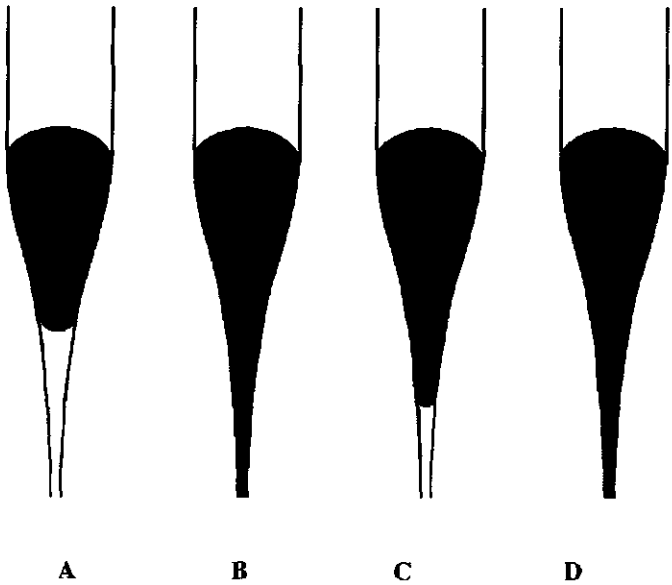

Fig. 1 Illustration of the injection system, operating at different pressures during an injection cyclus. a: no over pressure; b: mercury forced into the tip by 4 bar overpressure; c: uptake of cytoplasm by reduction of the overpressure to 3 bar; d: injection of the cellular material by increasing the overpressure to 4 bar again.

Microinjection system

The basics of the microinjection system have been described elsewhere (De Laat and Blaas, 1987). For precise control of suction and injection with large-diameter bevelled capillaries, the system was modified according to the same principles described by Hiramoto (1962) for the introduction of sperm cells into sea urchin eggs. A small droplet of mercury was put in the capillaries, near the tip of the needle (Fig. 1). This droplet could be moved by controlling the pressure from about 0.5 bar to 4.0 bar in the pneumatic injection system, which was adapted to these pressures. The compensation pressure forced the mercury into the tip of the capillary, and was adjusted until the mercury was close to the opening; suction was achieved by reducing the pressure. For injections, the pressure was again increased (Fig. 1).

Results

Methodology

The use of mercury in combination with an air pressure injection system allows the precise regulation of suction and injection over a very large range, from femto-liter up to micro-liter volumes. Also liquids with high viscosities, or liquids containing particles, can be accurately handled. The use of sharp, bevelled glass needles minimizes damage to the regenerating protoplasts, resulting in up to 80% survival in a typical experiment, after injection with a needle of 5 μm diameter.

Several days after the isolation of the acceptor protoplasts, the freshly isolated donor protoplasts were immobilized in the same petridish. This short period of preculture of the acceptor was necessary for

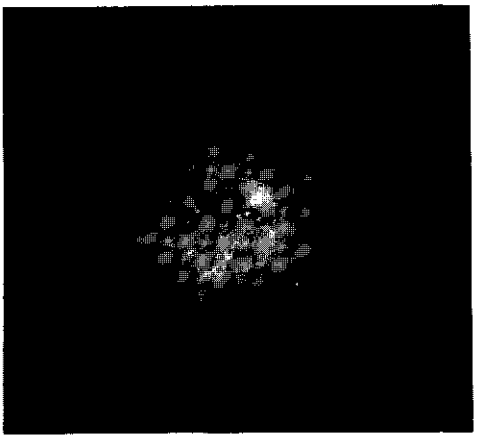

Fig. 2 Fluorescence photomicrograph of a DIODC-stained protoplast of *N.plumbaginifolia*.

partial regeneration of the cell wall. It appeared almost impossible to inject cytoplasm into freshly isolated protoplasts, and remove the needle without destruction of the membrane. A partially regenerated cell wall protected the membranes sufficiently. Visual preselection of well regenerating protoplasts appeared to be a successful approach, and made it possible to apply microinjection to protoplasts with a low plating efficiency.

To visualize the transfer of mitochondria, the first experiments were performed with DIODC-stained donor mitochondria from a cell suspension of *N.plumbaginifolia*, and unstained protoplasts of the same species as acceptor. Albino VBW was used as acceptor and tobacco line 92 as donor to demonstrate chloroplast transfer.

Initially, normal, freshly isolated protoplasts were used as organelle donor. However, it was difficult to extract a sufficiently large amount of cytoplasm without damaging the vacuole.

To reduce the risk of vacuole punctures, the donor protoplasts were evacuolated (Griesbach and Sink, 1983). Pretreatment of the donor protoplasts with cytochalasin-B improved the yield of evacuolated significantly. An additional effect of this microfilament disrupting treatment appeared to be the reduction of viscosity of the cytoplasm, facilitating handling of the organelles during extraction and injection (data not shown). However, all of the cytoplasm could not always be removed from the needle after each injection. Therefore, the needles could be used for an average of five injections. With these modifications, up to 50% of the cytoplasm from an evacuolated protoplast could be removed from the donor, and injected into the recipient.

Organelle transfer

The transfer of mitochondria was demonstrated by using stained donor mitochondria with the mitochondrial membrane specific stain DIODC (Matzke and Matzke, 1986) (Fig. 2).
Rapid redistribution of the fluorescent dye from the injected mitochondria to the unstained mitochondria of the acceptor was used as an indicator of a true cytoplasmic injection: when the fluorescent cytoplasm was deposited against the outer surface of the recipient, no staining of the acceptor was observed. Both regenerating protoplasts (Fig. 3 a-d) and already divided cells could be used as acceptor cell (Fig. 3 e-h), and subsequent divisions were observed (Fig. 3d, 3h). Occasionally, green chloroplasts were seen to leave the injected clump by cytoplasmic streaming (data not shown). After some time, divisions could be observed in injected cells, finally leading to the formation of microcalli, which were collected by micromanipulation and transferred onto callus growth medium.

Since the cell sytem used for the demonstration of the transfer of

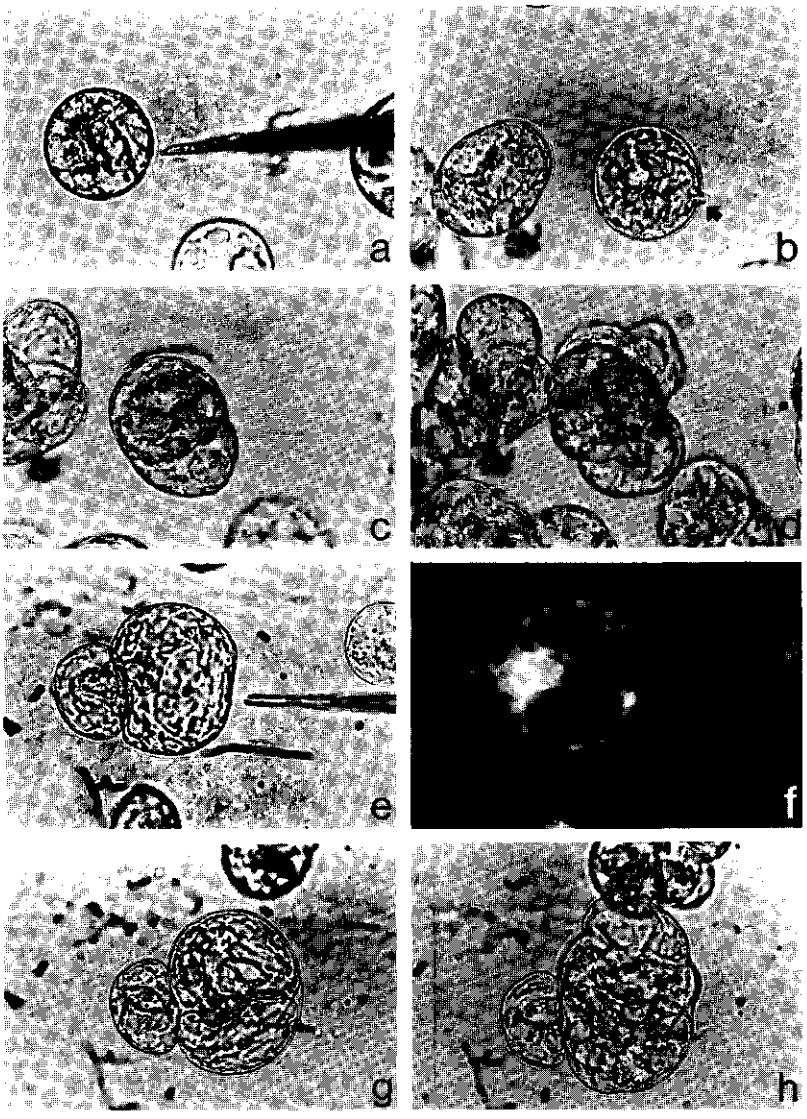

Fig. 3: Injection of mitochondria into a regenerating protoplast (a-d), and into an already divided regenerating protoplast (e-h). a: protoplast prior to injection, after 1 day of culture; b: mitochondria injected into the protoplast (arrow); c: after 3 days of culture (two days after injection); d: after seven days of culture; e: divided cell prior to injection; f: fluorescence micrograph of the injected cell, showing the fluorescently labelled mitochondria (arrow); g: one day after injection; h: after 10 days of culture.

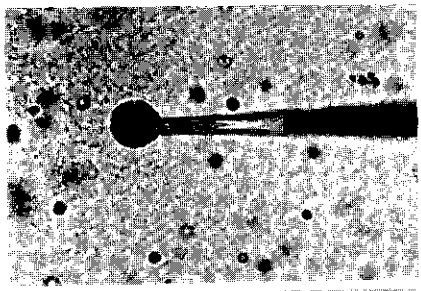

Fig. 4 Uptake of cytoplasm from an evacuolated mesophyll protoplast, containing mature green chloroplasts.

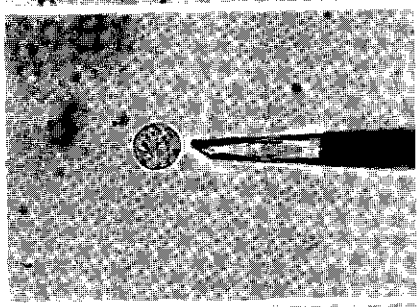

Fig. 5 Uptake of proplastids and mitochondria from an evacuolated suspension donor protoplast.

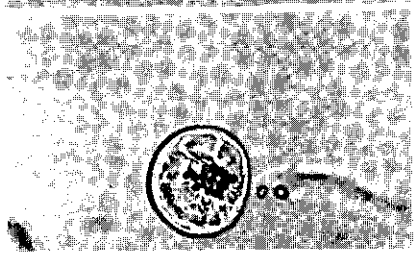

Fig. 6 Green mesophyll chloroplasts injected into an albino recipient protoplast.

mitochondria did not contain any useful genetic marker, the albino VBW and the tobacco line 92 were used to investigate the fate of the injected organelles. Evacuolated mesophyll leaf protoplasts and suspension cell protoplasts of line 92 were used as organelle donor, to transfer mature chloroplasts and proplastids together (Fig. 4), and proplastids only (Fig. 5), respectively. In both cases, also transfer of mitochondria is likely to occur.

The injected green chloroplasts from the mesophyll donor could be discriminated from the albino chloroplasts during several days after the injection (Fig. 6). Some of the chloroplasts left the injected clump and were transported in the cell by cytoplasmic streaming. It was possible to inject up to 10 mature chloroplasts, whereas the number of mitochondria and proplastids could not be estimated visually.

From an albino VBW acceptor protoplast, injected with cytoplasm from the evacuolated mesophyll donor protoplasts, one microcallus was obtained which showed sorting out of the red fluorescence of chlorophyll (data not shown). The number of injected green chloroplasts was far below the number of red fluorescing chloroplasts in the microcallus. Hence at least part of the red fluorescence originated from new chloroplasts, derived from the injected

Fig. 7a Photo micrograph of a microcallus, regenerated from an albino protoplast, four weeks after the injection of suspension cell donor cytoplasm.

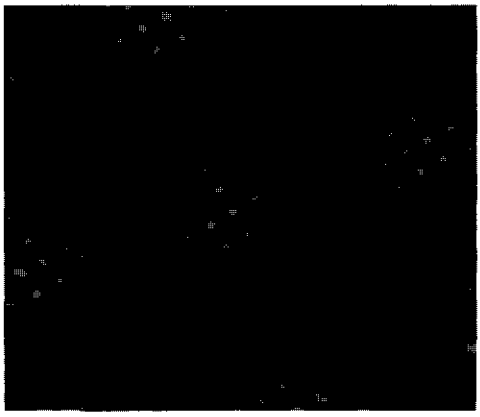

Fig. 7b Fluorescence photo micrograph of the microcallus of fig. 7a, showing the sectors with red fluorescence due to the newly synthesized chlorophyll.

plastids. Sorting out was also observed in the case where donor cytoplasm from the cell suspension of line 92, which does not contain any mature, green chloroplasts, was injected into the albino, indicating the complete de novo synthesis of chlorophyll (Fig. 7 a,b). Out of fifty injections, five microcalli were obtained showing the red fluorescence: two from injections with mesophyll protoplast cytoplasm, and three from injections with suspension cell protoplast cytoplasm. With mock injected albino protoplasts, this red fluorescence was never observed.

Discussion

Transfer of cytoplasm and organelles (nuclei, microchondria) by microinjection has already been established in animal research and protozoan biology. Nuclei from sheep egg cells have been removed by micromanipulation techniques, followed by microinjection to introduce new genetic material (Willadsen, 1986). Transfer of cytoplasm has been developed for research in *Paramecium* using similar techniques as described in this paper (Koizumi,1974; Knowles 1974). However, *Paramecium* cells are much larger than plant protoplasts, impeding the application of this technique with plant cells. The only reports on organelle transfer to plant cells by microinjection employ mass isolated chromosomes (Griesbach, 1987; De Laat and Blaas, 1987). This approach carries the risk of organelle damage during the isolation procedure. The risk is largely absent in the direct cell to cell transfer. Furthermore, the precise control over the injection flow and the possibility to use very large orifices, reduce the damage due to shearing forces during the injection of fragile organelles (Griesbach, 1987). Although polyethyleneglycol induced uptake of isolated chloroplasts has been reported (Giles, 1978), no evidence for the functionality of the introduced organelles has been presented sofar.

This is the first report on complementation of a cytoplasmic albino trait by other means than fusion. The albino was successfully complemented by injection with mesophyll protoplast organelles, and by injection of suspension protoplast cytoplasm as well. No

evidence could be obtained for a preferential donor, since both mesophyll and suspension cell plastids were efficient in complementation. The green chloroplasts from the mesophyll donor gradually bleached and could no longer be distinguished after about one week following the injection. Nevertheless, complementation was observed in the case of injected mature chloroplasts. This indicates that complementation is accomplished by proliferating proplastids which were present in the injected cytoplasm, or by dedifferentiated mature chloroplasts, since mature chloroplasts are presumed to have lost their division activity. A similar phenomenon is often observed after fusion of mesophyll and suspension cell protoplasts.

Out of a total of fifty injections, five microcalli were obtained which showed red fluorescence. Even with the low number of injections, which can be done on one day (up to five surviving protoplasts), this is a useful frequency for achieving transfer of cytoplasmic traits.

Protoplast isolation is no longer a prerequisite for genetic manipulation, since the cell wall of plant tissues e.g. embryos can be penetrated by microinjection (Neuhaus et al., 1987). This reduces the problems with the regeneration of protoplasts from difficult species, like monocots, and circumvents the concomitant somaclonal variation (Sree Ramulu et al., 1985). In this way, new approaches to the manipulation of the organelle composition of a given plant species or variety may become feasible. The directly visible segregation patterns, along with the possibility to control the amount of donor cytoplasm, creates new possibilities to study the interaction between the nucleus and the cytoplasmic organelles.

Additionally, the application of this microinjection technique for plant cell research opens up new ways for plant genetic manipulation. Preliminary results, obtained in our laboratory, demonstrate the possibility to extract metaphase chromosomes and micronuclei (Verhoeven et al., 1986; 1987; de Laat et al., 1987) from stained, immobilized protoplasts (data not shown). This may be a valuable new approach for the aimed introduction of donor chromosomes, useful for chromosome mapping and genome interaction studies.

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CHAPTER 8

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

In this thesis, micronucleation in plant cells has been investigated and systems for isolation and transfer of organelles have been established.

The discovery, described in chapter two, that the phosphoric amide herbicide amiprophos-methyl induced micronuclei at a high frequency in cell suspensions of *N.plumbaginifolia*, has opened the possibility to develop a microcell-mediated chromosome transfer system analogous to that in mammalian cell lines. In mammalian cells, micronuclei are induced by prolonged exposure of cells to spindle toxins (colchicine, Colcemid), resulting in up to 60% micronucleated cells (Matsui et al., 1982). Micronucleated cells are isolated by the "shake-off" method, and subjected to high speed centrifugation, which results in fractionation of the cells into microcells, containing micronulei with one or a few chromosomes. Subsequently, microcells are fused to the recipient cells. The transferred chromosomes were found to remain intact and mitotically stable (Fournier, 1982). This technique has hitherto not been available for plant cells or protoplasts, due to the lack of efficient procedures to induce micronuclei. Gamma-irradiation is now often used in the construction of monochromosomal addition lines by somatic hybridization (Bates et al., 1987), to induce chromosome damage which promotes chromosome elimination from one of the fusion partners. As has been pointed out in the introduction (chapter one), ionizing radiation induces chromosome rearrage ments, deletions and insertions (Menczel et al., 1982). From research on mammalian cells, it is known that these phenomena occur with a lower frequency after microcell-mediated chromosome transfer (Fournier, 1981). If microprotoplasts would become available for plant genetic manipulation, transfer of a limited number of chromosomes by microprotoplast fusion would offer an alternative to the use of gamma-irradiation. With the finding that APM induces micronuclei at high frequency in plants, transfer of low numbers of chromosomes after micronucleation can now be tested for use in plant genetic manipulation. The APM treatment was found to be reversible, as was demonstrated by washing the cell suspension cultures free from APM. After washing, normal growth and cell division were soon resumed, with some abnormal, multipolar spindles in the first division after washing. This observation is in good agreement with the the reversible inhibition of microtubule polymerization by APM (Falconer and Seagull, 1987). This low cytotoxicity makes APM a useful tool in the induction of micronuclei in plants.

The flow cytometric analysis of the nuclear DNA content of APM-treated cel suspension cultures of *N.plumbaginifolia* revealed the presence of many micronuclei with a DNA content equivalent to one metaphase chromosome (which consists of both sister chromatids). Similar observations have been made in micronucleated rat kangaroo cells after treatment with Colcemid (Sekiguchi et al., 1978). Sorting of the micronuclei on the basis of the fluorescence of

ethidium-bromide, followed by analysis of the DNA content by Feulgen staining (chapter three), shows that it is possible to separate micronuclei on the basis of their DNA content by flowcytometry, like it has been shown for isolated plant metaphase chromosomes. Chromosome identification is sometimes possible with isolated metaphase chromosomes (de Laat and Blaas, 1984; Conia et al., 1987a; 1987b). Identification of chromosomes present in a particular micronucleus is not possible. This is due to different degrees of chromosome decondensation in the micronuclei (which influences the fluorescence signal of the fluorochrome-DNA complex by quenching), and due to the various combinations of chromosomes in micronuclei containing more than one metaphase chromosome. This is illustrated by the DNA histograms of isolated micronuclei in chapter two, which lack the specific chromosome peaks, present in metaphase chromosome preparations (chapter four). When micronuclei are present in large numbers, the overall DNA histogram will show no appreciable contribution of a particular type of chromosome combination in micronuclei, since chromosome grouping appears to be a random process, as was shown by the analysis of the number of micronuclei per cell in chapter two, and by cytological data in chapter two and three. Furthermore, the reduction of the number of micronuclei per micronucleated cell, which appears to be the consequence of fusion of micronuclei into a lobed restitution nucleus, gives rise to even more combinations of chromosomes.

The processes, involved in the formation of micronuclei, are studied in chapter three and four. The effects of the anti-microtubular herbicides APM, oryzalin and the alkaloid colchicine, used for metaphase arrest and induction of micronuclei in mammalian cells, on the mitotic index and micronucleus formation are compared. The disruption of the spindle by direct inhibition of microtubule assemble is responsible for the accumulation of cells at metaphase. The concentrations of the inhibitors required for complete metaphase arrest, vary from 3 μM for APM and oryzalin to 500 μM for colchicine, as a consequence of differences in binding specificity (Hertel et al., 1980; Dustin 1984). The differences in the percentage of ball metaphases indicate specific effects of the above mentioned inhibitors on chromosome scattering. Apart from the disruption of the microtubules, APM and oryzalin have been shown to influence the accumulation of calcium in the mitochondria (Hertel et al., 1981). Moreover, oryzalin disturbs the active excretion of calcium by the plasma membrane. These combined effects result in an increased cytoplasmic calcium concentration (Hertel et al., 1980), which will be higher after oryzalin treatment than after APM treatment, due to the reduction of active calcium excretion by oryzalin. Our data suggest that the APM or oryzalin induced increase of the cytoplasmic calcium concentration is involved in both formation and fusion of micronuclei. Colchicine, which does not influence the cytoplasmic calcium concentration, is not effective in the induction of micronuclei. The higher cytoplasmic calcium levels after oryzalin treatment, would increase the fusogenic properties of the nuclear membranes, which would explain why micronuclei exist for a shorter time after oryzalin treatment as compared to APM treatment. This hypothesis will be tested in future experiments by treatments with the calcium ionophore A23187 in combination with the calcium-specific chelator ethyleneglycol-bis-(2-aminoethylether)-N,N'-tetra acetic acid (EGTA), with simultaneous measurements of the cytoplasmic calcium concentrations with the new calcium specific fluorochromes Fluo-3 and Rhod-2 (Haugland, 1989).

In order to obtain both large numbers of micronucleated cells, and large numbers of micronuclei per micronucleated cell, the

effect of DNA synthesis inhibitors was investigated. The results in chapter five show, that a considerable increase in the number of micronucleated cells can be achieved by HU or APH treatments, and that the time at which micronuclei appear can be controlled. The results further indicate that metaphases have to be exposed to APM for at least 12h, before micronucleation occurs, and that their lifetime is in the same order. These data demonstrate that it is possible to manipulate the conditions of the treatments in order to obtain either a high yield of metaphase chromosomes, or a high yield of micronuclei, with little contamination by micronuclei or chromosomes, respectively. In this way, it becomes possible to determine the moment at which the number of micronuclei per cell is at its maximal value.

The isolation and characterization of microprotoplasts from micronucleated suspension cells is described in chapter six. Data obtained from DNA content measurements and flow cytometry demonstrate the presence of up to 40% of subprotoplasts with a DNA content less than the G1-level of the APM treated suspension cells. This indicates that genome fractionation has occurred, and the data on the FDA-staining show that most of the subprotoplasts still possess an intact plasma membrane, since FDA can not be retained by vacuolar membranes only (Lesney, 1986). The viability of the microprotoplasts and other types of subprotoplasts is indicated by the successful culture after gradient fractionation. As it is impossible to measure the DNA-content of microprotoplasts in a non-destructive way, no preselection could be performed to use only microprotoplasts for fusion. In a mass fusion system, the smallest microcells will be the least likely to fuse when electrofusion is used, because their small diameter will prevent alignment and membrane breakdown, which are both related to particle diameter (Zimmermann et al., 1982). Individual selection and fusion could overcome this problem (Koop et al., 1983). This control is essential for the efficient application of microprotoplasts, since the DNA content per microprotoplast will depend upon the DNA content per micronucleus in the cell suspension. Microprotoplast fusion will result in transfer of a part of the total number of chromosomes, directly followed by spontaneous chromosome elimination when two distantly related species are fused, since chromosome elimination seems to be directed by genome dose effects (Graves, 1984; Gilissen et al., 1989). Sofar no successful fusion experiments have been performed, which makes it impossible at the moment to comment on the usefulness of microprotoplasts in chromosome transfer. However, fusion experiments with karyoplasts indicate that it is possible to perform fusions in a controlled way (Spangenberg et al., 1987).

In addition to the microprotoplast fusion, microinjection was developed for transfer of organelles and micronuclei. Glass needles with a large orifice (5μM) were prepared, along with a pressure system, based on the application of mercury. With the injection system, described in chapter seven, it is possible to suck donor material from a donor protoplast, and inject this directly into the recipient. The data on the complementation of the albino tobacco by injection of mature green chloroplasts or etiolated plastids, indicate that protoplasts can survive the injection treatment, and that the injected plastids can be replicated by the recipient. In this way, the organelles to be transferred are not subjected to damaging isolation procedures and they can be preselected visually. Selective transfer of organelles offers a number of advantages when compared to fusion techniques, or transfer of isolated genes. One of the advantages is the protective nature of the membranes associated with chloroplasts, mitochondria and nuclei. Although structural integrity and functionality has been demonstrated for

isolated chloroplasts and mitochondria, it is not known whether isolated organelles are still physiologically intact. The isolation of intact nuclei from plant cells has also been described, with data indicating their structural integrity, as well as their ability to transfer genes into recipient protoplasts (Saxena et al., 1986). Transfer of marker genes does not necessarily implicate the functional integrity of isolated nuclei, since transfer of marker genes can be achieved by uptake of isolated genomic DNA. Preliminary results obtained from experiments with microinjection of micronuclei, indicate that it is possible to remove micronuclei from the donor by suction. Sofar, transfer into a recipient has not been achieved. The kanamycine-resistance, which was introduced into *N.plumbaginifolia* by transformation with *Agrobacterium tumefaciens*, will be used as selectable marker after transfer of micronuclei. The transfer of chromosomes will be tested with species specific repetitive DNA probes, which are able to discriminate between the donor genome (*N.plumbaginifolia* and the recipient (either *Lycopersicon esculentum* or *Solanum tuberosum*). Several probes with the required specificity have already been characterized from a series of highly repetitive sequences, isolated from *N.plumbaginifolia* (data not shown).

With the methods, described in this thesis, the transfer of chromosomes via micronuclei has come within reach. Future work will focus on achieving transfer, and study the fate of the introduced micronuclei. This should provide an answer whether micronuclei can be used as chromosome carriers in plants, as has already been shown in mammalian somatic cell genetics.

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SAMENVATTING

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In dit proefschrift zijn de resultaten gepresenteerd van onderzoek naar de inductie, isolatie en overdracht van microkernen. Het onderzoek is uitgevoerd met het doel een systeem te ontwikkelen, waarmee slechts één of enkele chromosomen van een donor plant via microkernen naar een recipient kunnen worden overgedragen. Met een dergelijke benadering is het mogelijk gebleken, slechts enkele menselijke chromosomen over te brengen naar een muizecellijn. Met de verkregen cellijnen konden de genen op de menselijke chromosomen in kaart worden gebracht via het opheffen van deficienties in de muizecellijn door de genen op de chromosomen van de mens. Behalve voor het opstellen van een chromosomenkaart, zijn plantecellijnen met één extra, vreemd donor chromosoom van belang voor de planteveredeling.

In hoofdstuk een wordt de doelstelling van het onderzoek uiteengezet, en worden literatuurgegevens over chromosoom-overdracht bij dierlijke en plantaardige cellen besproken.

De inductie van microkernen door behandelingen van cel-suspensie cultures van *N.plumbaginifolia* met het herbicide amiprophos-methyl (APM) is het onderwerp van hoofdstuk twee. Het blijkt dat dit herbicide de vorming van de spoelfiguur verstoort, waardoor de chromosomen door de cel verspreid worden. Na ongeveer twaalf uur wordt dit gevolgd door de vorming van microkernen. In dit hoofdstuk wordt het aantal cellen met microkernen, en het aantal microkernen per gemicronucleëerde cel, alsmede het DNA-gehalte per microkern onderzocht. Uit deze resultaten blijkt, dat er vele microkernen voorkomen, die slechts één metafase chromosoom bevatten. Dit wordt bevestigd door het sorteren van de microkernen. De toxische effecten van de behandeling blijken bij de gebruikte APM concentraties niet van belang.

In hoofdstuk drie wordt het gedrag van mitotische cellen tijdens en na de APM-behandeling nader onderzocht. Uit de resultaten blijkt, dat de chromosomen tijdens de metafase geen metafaseplaat vormen, maar in het hele cytoplasma verspreid komen te liggen. Tijdens de metafase vindt er geen deling van de centromeren plaats, zodat zuster chromatiden aan elkaar blijven zitten. Door het ontbreken van de anafase, begint in de telofase de vorming van een kernmembraan om groepjes en vrijliggende chromosomen, wat uiteindelijk resulteert in de vorming van microkernen. Zodra APM verwijderd wordt door uitwassen, vinden er al snel, hoofdzakelijk normale delingen plaats, met een afnemend aantal multipolaire spoelfiguren.

In hoofdstuk vier wordt de werking van de herbicides APM en oryzaline vergeleken met colchicine, dat gebruikt wordt om bij dierlijke cellen microkernen te induceren. Onderzocht zijn de effecten van colchicine, oryzaline en APM op de mitotische index,

chromosoom spreiding en de vorming van microkernen. De resultaten tonen aan, dat colchicine niet geschikt is om cellen in metafase op te hopen voor de isolatie van chromosomen. De resultaten tonen ook aan, dat er door colchicine weinig microkernen gevormd worden in plantecellen. Onder invloed van oryzaline wordt een hoge mitotische index bereikt, die dit middel zeer geschikt maakt voor chromosoomisolatie. Tijdens de oryzaline behandeling worden wel microkernen gevormd, maar deze fuseren bij hogere oryzaline concentraties snel tot een restitutiekern. Hierdoor verkrijgen de cellen een verdubbelde hoeveelheid DNA. Dit is niet het geval bij behandelingen met APM, waarbij microkernen ongeveer twaalf uur kunnen blijven bestaan alvorens te fuseren tot restitutie kern. Het gelijktijdig optreden van verspreide chromosomen en microkernen maakt APM minder geschikt voor het isoleren van metafasechromosomen dan oryzaline.

Om de inductie van microkernen te optimaliseren, is het effect van een behandeling met DNA-syntheseremmers op de mitotische index en de frequentie van microkernen onderzocht (hoofdstuk vijf). Door de periode van DNA-synthese-remming zo te kiezen, dat het hoogste aantal delende cellen door de remming in S-fase opgehoopt wordt, is het mogelijk de maximale mitotische index te bereiken na het uitwassen van de remmer. Deze periode kan eenvoudig bepaald worden via de in dit hoofdstuk beschreven methode. Alle delende cellen kunnen in metafase opgehoopt worden door direkt na uitwassen APM toe te voegen. De opgehoopte mitotische cellen vormen dan twaalf uur na het begin van de toename van de mitotische index microkernen. Dit resulteert in een opbrengst van 45% cellen met microkernen. Microkern inductie kan ten behoeve van de isolatie van metafase chromosomen uitgesteld worden door APM pas zes uur na het uitwassen van de DNA-synthese remmers toe te voegen.

Hoofdstuk zes beschrijft de isolatie van microprotoplasten. Microprotoplasten zijn subprotoplasten met een DNA-gehalte dat overeenkomt met dat van slechts enkele chromosomen. Hiertoe worden van een gemicronucleëerde celsuspensiecultuur protoplasten geïsoleerd, die vervolgens na een korte cytochalasine-B behandeling op een Percoll gradient gefractioneerd worden. Na centrifugatie zijn er verschillende banden in de gradient aanwezig, waarbij een bepaalde fractie meer dan 40% microprotoplasten bevat. Via flowcytometrie en microdensitometrie is aangetoond dat de microprotoplasten een laag DNA-gehalte hebben, en dat ze tevens een intacte plasmamembraan hebben. De levensvatbaarheid van de subprotoplasten is bewezen door ze in kweek te brengen, waarbij van elke fraktie van een gradient microcalli verkregen konden worden. Dit toont aan, dat microprotoplasten gebruikt kunnen worden als fusiepartner in fusie experimenten.

Hoofdstuk zeven behandelt de ontwikkeling en de eerste resultaten van een nieuw microinjectie systeem. Een grote naaldopening (groter dan 5 μm) in combinatie met een op kwik gebaseerd druksysteem, maakt het mogelijk donor organellen op te zuigen uit de donor cellen, en deze direct weer te injecteren in de receptor cel. Met deze techniek is cytoplasma met groene chloroplasten geïnjecteerd in albino protoplasten, waarna verscheidene microcalli verkregen zijn, die de roodfluorescentie van nieuw gesynthetiseerd chlorofyl bezitten.

Hoofdstuk acht geeft de resultaten en conclusies van het onderzoek weer, en plaatst deze tegen de doelstellingen, zoals die in hoofdstuk een geformuleerd zijn.

Curriculum Vitae

Harrie Verhoeven werd geboren op 14 november 1956 te Valkenswaard. Na het behalen van het Gymnasium diploma, met biologie als achtste vak, ging hij in 1976 Electrotechniek studeren aan de Technische Hogeschool Eindhoven. Na een periode van twee jaar veranderde hij van studierichting, en begon in 1977 met zijn studie Biologie aan de Landbouwhogeschool te Wageningen. Hij behaalde het kandidaats diploma in de richting Celbiologie in mei 1981. In 1984 studeerde hij af met als doctoraal vakken dierfysiologie en plantemorfologie en -anatomie. Sinds april 1984 is hij werkzaam als Celbioloog bij de Stichting Ital te Wageningen, eerst op een door de EEG gefinancierd project, en sinds oktober 1987 als projectleider van het onderzoeksproject Organel Transfer.