Theor Appl Genet (1988) 75:575–584

Mitotic dynamics of micronuclei induced by amiprophos-methyl and prospects for chromosome-mediated gene transfer in plants

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

Research Institute Ital, Mail Box 48, NL-6700 AA Wageningen, The Netherlands

Received July 10, 1987; Accepted September 17, 1987
Communicated by F. Salamini

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 2 or more chromosomes were scattered over the cytoplasm. After 5–6 h of APM treatment, chromosomes decondensed and formed micronuclei. When treatment duration was increased, 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 1 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, in time, chromosomes from one of the parents. 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 distinct conceptionally and operationally 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 (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, i.e., micronuclei surrounded by a thin layer of cytoplasm and plasma membrane, with recipient partners, karyotypically simple hybrid clones were constructed which contained only 1 or a few introduced donor chromosomes (Fournier 1982). Since the transferred genetic material can often be identified using cytogenetic tests, microcells have been proven to be useful not only for

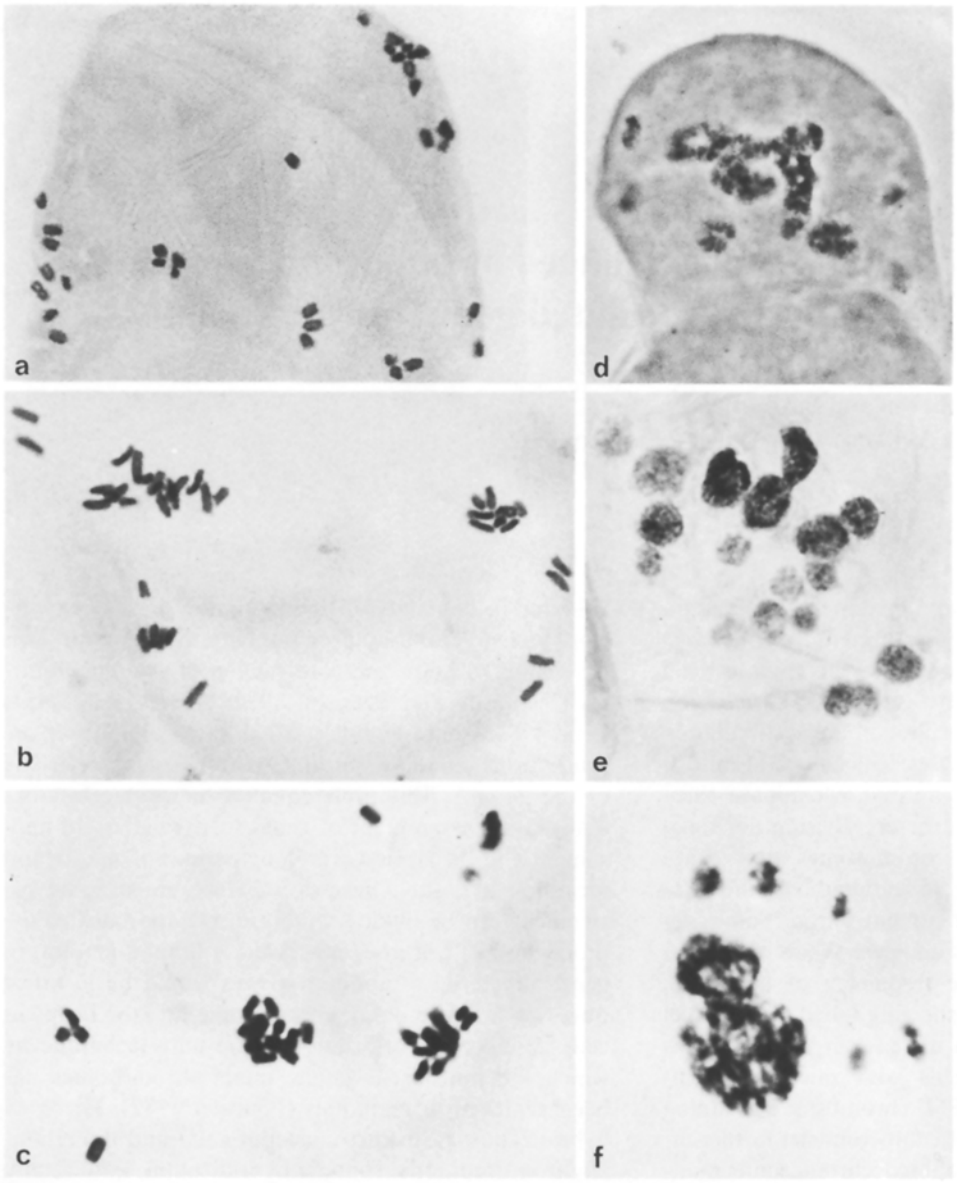

Fig. 1 a–l. Microphotographs on the sequence of mitotic events leading to the formation of micronuclei after treatment of cell suspensions of *N. plumbaginifolia* with APM, and recovery of cells after removal of APM. **a** Scattering of metaphase chromosomes ("exploded metaphases"). **b, c** Groups of 2, 3 or more chromosomes (*note* single chromosomes). **d** Decondensation of single and grouped chromosomes. **e, f** Micronuclei in different phases. **g–i** Lobed micronuclei. **j–l** Post C-mitotic cells after removal of APM by washing and subsequent culture (*note* multipolar metaphases)

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.

Such a microcell system in plants has not become available because the mitotic arresting- and chromosome-breaking agents induced micronuclei only rarely, at a frequency 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

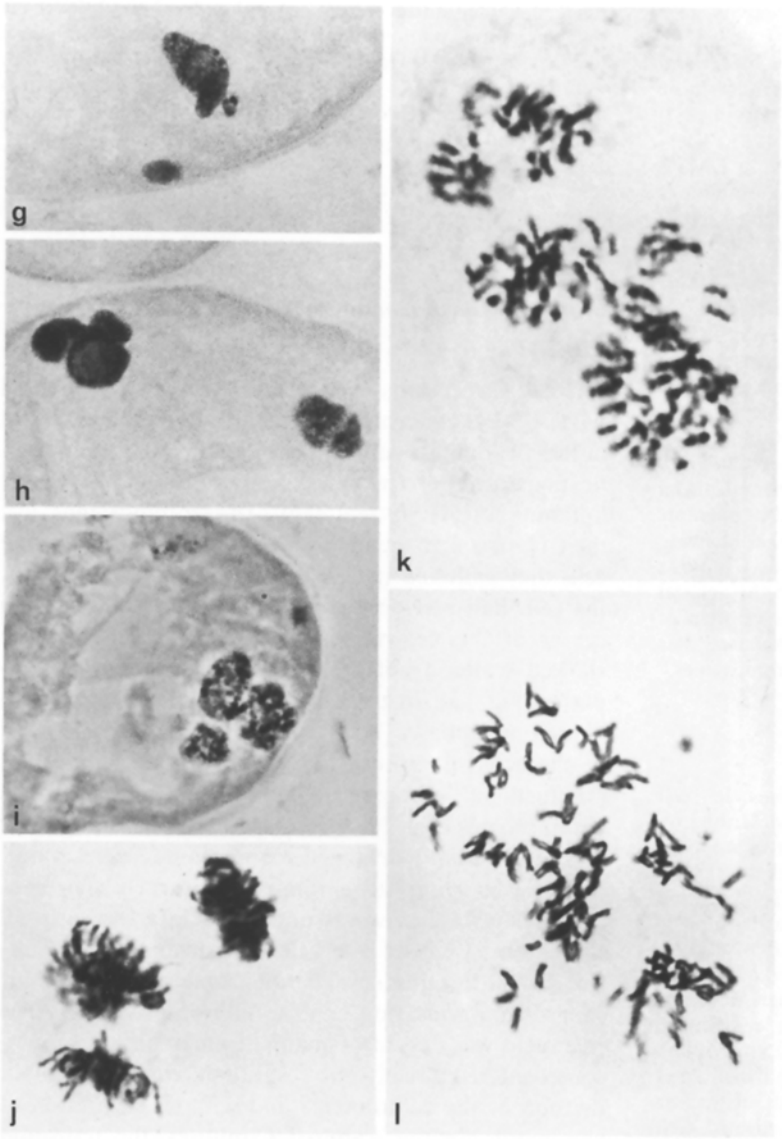

Fig. 1

groups resulting in micronuclei of varying size in cell suspensions of *Medicago sativa* (Lo Schiavo et al. 1980); 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. 1986a, b). Moreover, the mass isolation of individual metaphase chromosomes for sorting by flow cytometry, or transfer into recipient protoplasts, is greatly hindered by the stickiness 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 considered anti-microtubule drugs, because of the disappearance of microtubules after their application and the disruption of the microtubule-dependent process (Morejohn and Fosket 1984). It has been shown that APM directly poisons 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 1 day after subculturing.

Cytology

The samples of control and APM-treated cell suspensions were fixed in ethanol/acetic acid (3 : 1 v/v) for 24 h and examined for mitotic division, chromosome behavior 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 600–1,000. Mitotic arrest and metaphase blocking observed after APM treatment were designated as C-mitosis and C-metaphase, respectively in accordance with the general terminology proposed by Levan (1954). For a description of various terms, i.e., "scattered or exploded metaphases, star metaphases and reductional grouping of chromosomes," reference is also made to Levan (1954).

Flow cytometry

For flow cytometric analysis of nuclei and micronuclei, cell samples were fixed for 24 h in buffer solution as described by Blumenthal et al. (1979) with some modification as adopted previously for potato cells (Sree Ramulu and Dijkhuis 1986). The cell suspensions 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 were 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 for 1 min at 1,000 rpm, pellet resuspended in 0.5 ml buffer, stained with 5 μl ethidium bromide (5 mg/ml) and then 25 μl Triton X-100 (20%) was added. The measurements of nuclear DNA content were made with a Fluorescence Activated Cell Sorter (FACS)-IV (Becton Dickinson, Sunnyvale, USA) equipped with a Spectra Physics argon ion laser, model no. 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 fluorescent 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 24 h. 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; the nuclei were stained with Schiffs reagent. After 3 washes in SO_2 water for 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 compact microscope photometer at the wave length of 548 nm, and scanned using a Hewlett Packard-85 computer. These values were transformed to C-values by using the DNA content of nuclei isolated from leaf cells of control plants as a standard (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 1–2 h 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 center of the cell. Ball metaphases with all chromosomes in the center of the cell were also observed. Several cells showed scattering of the chromosomes 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 centromeres inwards towards one point; in a few cases all the chromosomes of one cell were arranged in a star-like shape. In other cells, chromosome groups were arranged in a wide ring or in a line. Mitosis after APM 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 transition "metaphase-telophase" could be followed with all its characteristic changes. In some cases, single metaphase chromosomes decondensed, developing nuclear membrane and forming telophase-like micronuclei. In other cases, 2 or more chromosomes grouped together to form 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.

Figure 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

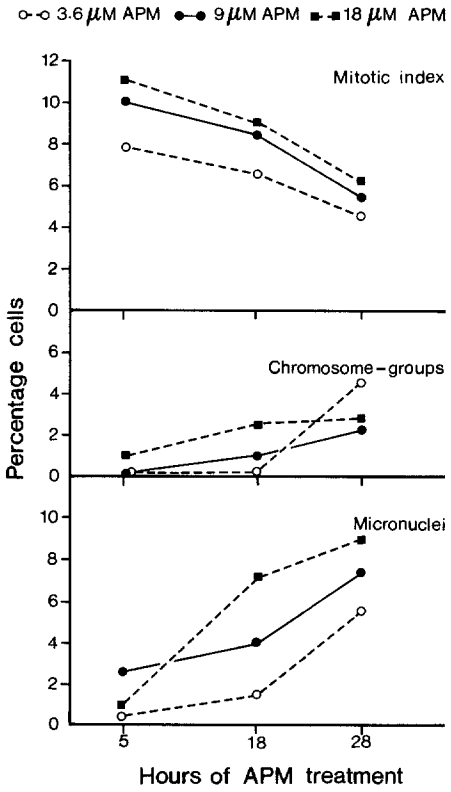

Fig. 2. Mitotic index (*MI*) and the percentage of cells showing chromosome-groups and micronuclei after treatment with various concentrations of *APM* for different periods. The control suspension cells of *N. plumbaginifolia* showed no chromosome-groups or micronuclei (*MI* ranged from 1.5–5.0)

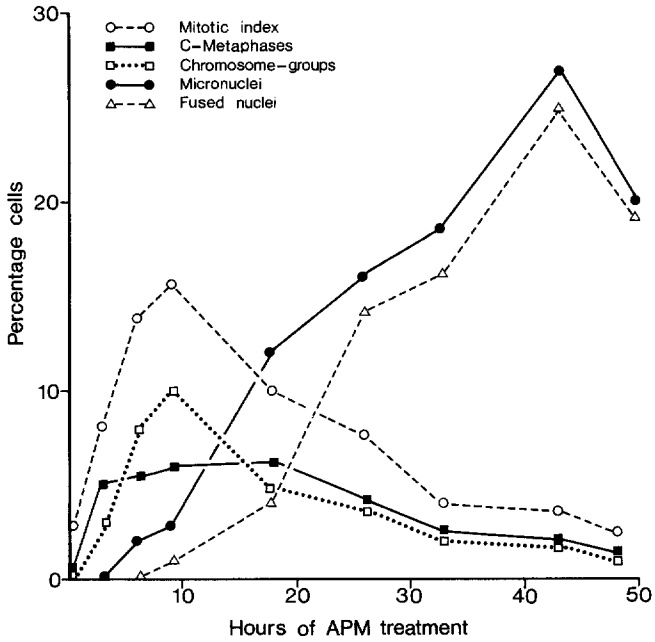

Fig. 3. Time course of induction of micronuclei in relation to other mitotic events after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM). In this, as in Fig. 7, *C-Metaphases:* Blocked metaphase cells with well-spread or partially clumped chromosomes arranged in "a single group";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

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 C-metaphases, the frequency of which dropped only after 18 h. When the duration of APM treatment increased from 3 h 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 a further increase in the incubation period (33–48 h), the cells containing 2 chromosome groups increased and those with > 9 groups disappeared.

Each of these groups contained a various number of chromosomes. The data presented in Table 1 show that the relative percentage of groups containing 1, 4 and > 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 and onward, and the frequency of cells with micronuclei gradually increased 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 2–13. 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 per-

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 (36 μM) treatment	No. of chromosome-groups analysed[a]	Relative percentage of groups showing various no. of chromosomes			
		No. of chromosomes per group			
		1	2	3	4 and above
3–9 h	139 (38)	50.6	2.0	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

[a] The number of cells that showed chromosome-groups are given in parentheses

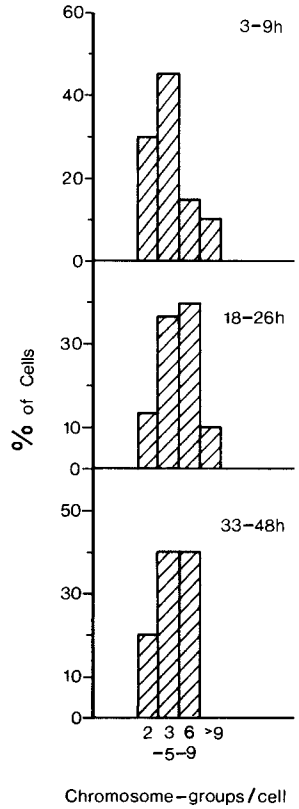

Fig. 4. Relative percentage of cells showing various number of chromosome-groups (*i.e.*, the number of chromosome-groups/cell) after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for various periods. As the data for the periods 3–9 h, 18–26 h and 33–48 h were similar, they were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

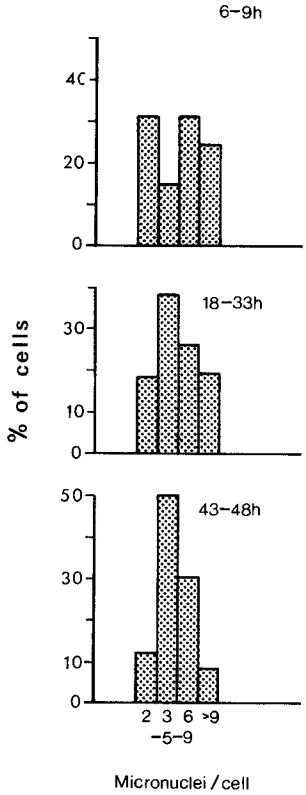

Fig. 5. Relative percentage of multinucleate cells showing various numbers of micronuclei (i.e., number of micronuclei/multinucleate cell) after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for various periods

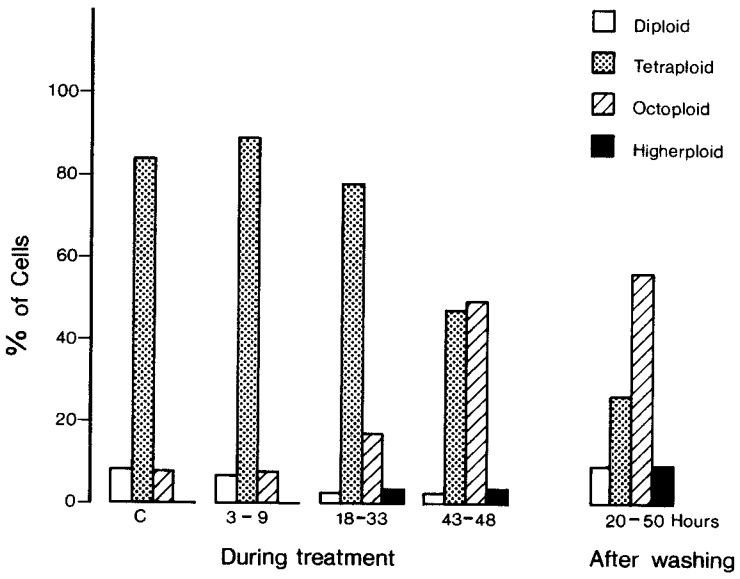

Fig. 6. Relative percentage of diploid (2x = 20), tetraploid (4x = 40), octoploid (8x = 80) and higher ploid (16x = 160) cells after treatment of cell suspensions of *N. plumbaginifolia* with APM (36 μM) for various periods, after washing out APM and subculturing. Chromosome numbers generally varied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

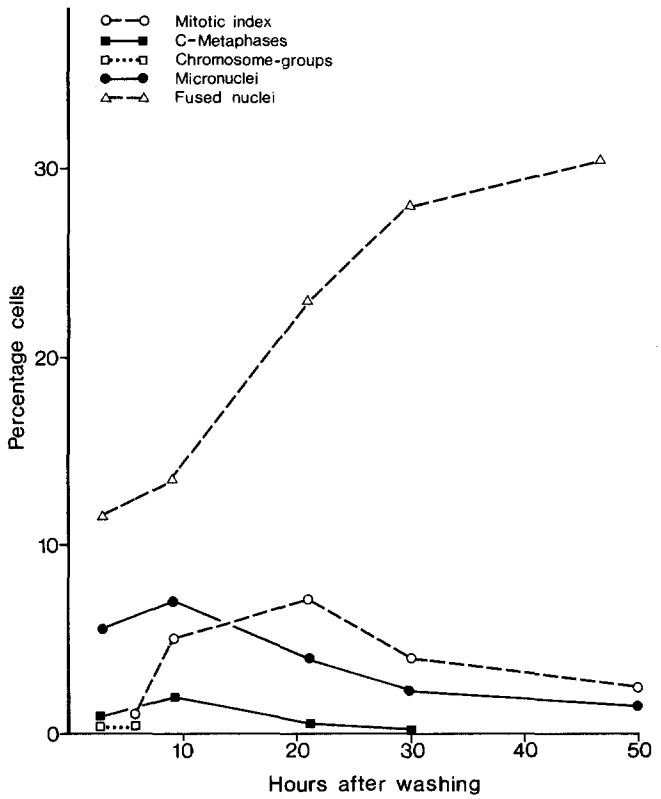

Fig. 7. Mitotic index and frequency of cells showing C-metaphases, chromosome groups, micronuclei and fused (lobed) nuclei after washing out APM (36 μM) and subculturing of cells

centage of cells showing 3–5 micronuclei increased and those with 6–9 remained unchanged.

Lobed nuclei resulting from fusion of two or more micronuclei were observed from 9 h onward (Fig. 1 g–i). When the duration of APM treatment increased, 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 chromosome complement. The proportion of octoploid cells gradually increased with a 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 removing APM and subculturing for 50 h, chromosome grouping almost completely disappeared and C-metaphases and cells with micronuclei gradually decreased

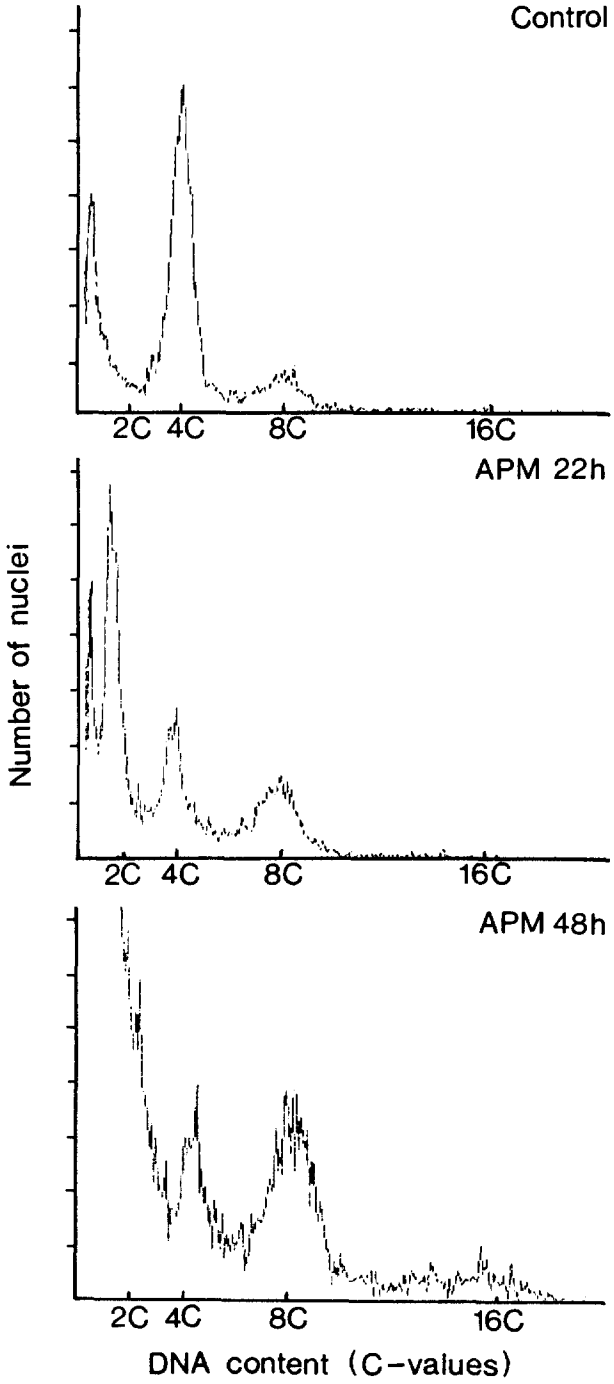

Fig. 8. Flow cytometric analysis of relative DNA contents of nuclei isolated from control and APM (36 μM: 22 h, 48 h) treated cell suspensions of *N. plumbaginifolia*

(Fig. 7). In 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. Figure 1 j–l show some examples of multinucleate cells progressed into post C-mitotic stages.

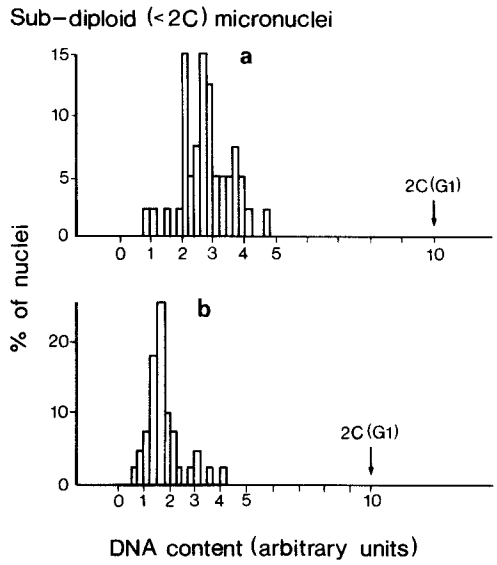

Fig. 9. Feulgen microdensitometric measurement of DNA contents of fraction-**a** and -**b** of sub-diploid (*< 2C*) micronuclei sorted by flow cytometry. The position of *2C (G1)* DNA content of control nuclei isolated from leaves of diploid (2n = 2x = 20) plants of *N. plumbaginifolia* is indicated by *arrow*

DNA content of micronuclei

Flow cytometry was used not only to monitor mass induction of micronuclei after APM treatment, but also to sort and characterize micronuclei containing 1 or a few chromosomes. Figure 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 increased 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 48 h). Figure 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 each from fraction-a and -b were individually measured for DNA content and compared with 80 control nuclei isolated from leaves of diploid (2n = 2x = 20) *N. plumbaginifolia* plant. The results suggest that the smaller micronuclei (fraction-b) contain 1–2 chromosomes, as can be seen from the position of the peak relative to the 2C value, i.e., G1 nuclei of the diploid control plant. 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 mitotic arresting agent which leads to the accumulation of a large number of metaphases and to the formation of high frequency of micronuclei. So far, mass induction of micronuclei by spindle toxins or chromosome-breaking agents has not been reported in plant cells. Amiprophos-methyl has been discovered as early as 1975 (Aya et al. 1975). Since then, this phosphoric amide herbicide has been investigated in several experiments in vitro and in vivo dealing with physiological or biochemical aspects and on microtubule polymerization (Morejohn and Fosket 1984), but not on the induction of micronuclei.

When compared with colchicine-blocked metaphase cells, which mainly 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, either initially as a group or 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).

In 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 features 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 (Levan 1954).

In cell suspensions of *Medicago sativa* griseofulvin affects 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 occurrence of spontaneous 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 frequency of micronuclei (Matsui et al. 1982). Several studies emphasize 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 (for review, see Matsui et al. 1982). Therefore, it is likely that the 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 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., 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 to form lobed nuclei. Light microscopic observations 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 and 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 to grow normally.

Cytological observations and DNA measurements showed that several subdiploid micronuclei containing 1 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.

Acknowledgements. We wish to thank Prof. J. Sybenga, Prof. B. de Groot and Dr. L. van Vloten-Doting for useful suggestions, and W.R.R. ten Broeke for help in flow cytometry. We also thank Prof. M. Terzi for valuable discussions on certain phases of this work, and Prof. V. Nuti Ronchi for making available her papers and unpublished results.

Part of this work was supported by funds from the "Biotechnology Action Programme" of the CEC, contract BAP-0083-NL (GDF). We gratefully acknowledge the receipt of a gift sample of APM from Bayer Nederland B.V., Divisie Agrochemie.

References

Aya M, Takase I, Kishino S, Kurihara K (1975) Amiprophos-methyl, a new herbicide in upland crops. Proc 5th Asian Pac Weed Sci Conf, pp 138–141

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

Blumenthal AB, Dieden JD, Kapp LN, Sedat JW (1979) Rapid isolation of metaphase chromosomes containing high molecular weight DNA. J Cell Biol 81:255–259

Brues AM, Jackson EB (1937) Nuclear abnormalities resulting from inhibition of mitosis by colchicine and other substances. Am J Cancer 30:504–511

Davies KE, Young BD, Elles RG, Hill ME, Williamson R (1981) Cloning of a representative genomic library of the human X chromosome after sorting by flow cytometry. Nature 293:374–378

De Laat AMM, Blaas J (1984) Flow cytometric characterization and sorting of plant chromosomes. Theor Appl Genet 67:463–467

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

De Marco A, Romanelli M, Stazi MA, Vitagliano E (1986) Induction of micronucleated cells in *Vicia faba* and *Allium cepa* root tips treated with nitrilotriacetic acid. Mutat Res 171:145–148

Ege T, Ringertz NR (1974) Preparation of microcells by enucleation of micronucleate cells. Exp Cell Res 87:378–382

Ege T, Ringertz NR, Hamburg H, Sidebottom E (1977) Preparation of microcells. In: Prescott DM (ed) Methods in cell biology, vol 15. Academic Press, New York, pp 339–358

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

Fournier REK, Ruddle FH (1977) Microcell-midiated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc Natl Acad Sci USA 74:319–323

Fournier REK, Frelinger JA (1982) Construction of microcell hybrid clones containing specific mouse chromosomes: application to autosomes 8 and 17. Mol Cell Biol 2:526–534

Fournier REK, Jurecek DK, Ruddle FH (1979) Somatic cell genetic analysis of transgenome integration. Somatic Cell Genet 5:1061–1077

Hesemann CU, Fayed AH (1982) Micronuclei in *Vicia faba* 1. The occurrence and origin. Egypt J Genet Cytol 11:235–244

Kao FT (1983) Somatic cell genetics and gene mapping. Int Rev Cytol 85:109–146

Kiermayer O, Fedtke C (1977) Strong anti-microtubule action of amiprophosmethyl (APM) in micrasterias. Protoplasma 92:163–166

Klein G, Klein E, Klein E (1952) The viability and the average desoxypentosenucleic acid content of micronuclei-containing cells produced by cochicine treatment in the Ehrlich ascites tumor. Cancer Res 12:484–489

Lebo RV (1982) Chromosome sorting and DNA sequence localization. Cytometry 3:145–154

Levan A (1938) The effect of colchicine on root mitoses in *Allium*. Hereditas 24:471–486

Levan A (1940) The effect of acenaphthene and colchicine on mitosis of *Allium* and *colchicum*. Hereditas 26:262–276

Levan A (1954) Colchicine-induced C-mitosis in two mouse ascites tumours. Hereditas 40:1–64

Lo Schiavo F, Nuti Ronchi V, Terzi M (1980) Genetic effects of griseofulvin on plant cell cultures. Theor Appl Genet 58:43–47

Malmberg RL, Griesbach RJ (1980) The isolation of mitotic and meiotic chromosomes from plant protoplasts. Plant Sci Lett 17:141–147

Marshall I, Bianchi M (1983) Micronucleus induction in *Vicia faba* roots. 2. Biological effects of neutrons below 1 centigray. Int J Radiat Biol 44:163–174

Matsui S, Weinfeld H, Sandberg AA (1982) Factors involved in prophasing and telophasing. In: Rao PN, Johnson RT, Sperling K (eds) Premature chromosome condensation, application in basic, clinical and mutation research. Academic Press, New York, pp 207–233

Mc Bride OW, Peterson JL (1980) Chromosome-mediated gene transfer in mammalian cells. Annu Rev Genet 14:321–345

Morejohn LC, Fosket DE (1984) Inhibition of plant microtubule polymerization in vitro by the phosphoric amide herbicide amiprophos-methyl. Science 224:874–876

Nuti Ronchi V, Bonatti S, Turchi G (1986a) Preferential localization of chemically induced breaks in heterochromatic regions of *Vicia faba* and *Allium cepa* chromosomes. I. Exogenous thymidine enhances the cytologic effects of 4-epoxyethyl-1,2-epoxy-cyclohexane. Environ Exp Bot 26:115–126

Nuti Ronchi V, Bonatti S, Durante M, Turchi G (1986b) Preferential localization of chemically induced breaks in heterochromatic regions of *Vicia faba* and *Allium cepa* chromosomes. II. 4-epoxyethyl-1,2-epoxycyclohexane interacts specifically with highly repetitive sequences of DNA in *Allium cepa*. Environ Exp Bot 26:127–135

Obara Y, Chai LS, Weinfeld H, Sandberg AA (1974) Synchronization of events in fused interphase-metaphase binucleate cells: progression of the telophase-like nucleus. J Natl Cancer Inst 53:247–259

Phillips SG, Phillips DM (1969) Sites of nucleolus production in cultured Chinese hamster cells. J Cell Biol 4:248–268

Sekiguchi T, Shelton K, Ringeretz NR (1978) DNA content of microcells prepared from rat, kangaroo and mouse cells. Exp Cell Res 113:247–258

Smiley JR, Steege DA, Juricek KD, Summers W, Ruddle FH (1978) A Herpes Simplex Virus 1 integration site in the mouse genome defined by somatic cell genetic analysis. Cell 15:455–468

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

Sree Ramulu K, Dijkhuis P, Hänisch ten Cate ChH, De Groot B (1985) Patterns of DNA and chromosome variation during in vitro growth in various genotypes of potato. Plant Sci 41:69–78

Sree Ramulu K, Dijkhuis P (1986) Flow cytometric analysis of polysomaty and in vitro genetic instability in potato. Plant Cell Rep 3:234–237

Stubblefield E (1964) DNA synthesis and chromosomal morphology of Chinese hamster cells cultured in media containing colcemid. In Harris RTC (ed) Cytogenetics of cells in culture. Academic Press, New York, pp 223–298

Szabados L, Hadalaszky G, Dudits D (1981) Uptake of isolated plant chromosomes by plant protoplasts. Planta 151:141–145

Van der Linden PM (1980) Computer analysis of DNA-histograms, PhD thesis, State University, Utrecht, The Netherlands

Verhoeven HA, de Laat AMM, Blaas J (1986) Methodological developments for chromosome-mediated gene transfer in higher plants. In: Magnien E (ed) Biomolecular engineering in the European community, achievements of the research programme (1982–1986). Nijhoff, Dordrecht, pp 977–981

Verhoeven HA, de Laat AMM, Sree Ramulu K, Dijkhuis P, van Vloten-Doting L (1987) Induction, isolation and flow cytometric sorting of metaphase chromosomes and micronuclei from cell suspension cultures of *Nicotiana plumbaginifolia*. In: Genetic and cellular engineering of plants and micro-organisms important for agriculture, Commission of the European Communities meeting, Louvain-la-Neuve, pp 119–120