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A high-yield procedure for isolation of metaphase chromosomes from root tips of *Vicia faba* L.

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Abstract. A new method is described for the isolation of large quantities of *Vicia faba* metaphase chromosomes. Roots were treated with 2.5 mM hydroxyurea for 18 h to accumulate meristem tip cells at the G_1/S interface. After release from the block, the cells re-entered the cell cycle with a high degree of synchrony. A treatment with 2.5 μM amiprophos-methyl (APM) was used to accumulate mitotic cells in metaphase. The highest metaphase index (53.9%) was achieved when, 6 h after the release from the hydroxyurea block, the roots were exposed to APM for 4 h. The chromosomes were released from formaldehyde-fixed root tips by chopping with a scalpel in LB01 lysis buffer. Both the quality and the quantity of isolated chromosomes, examined microscopically and by flow cytometry, depended on the extent of the fixation. The best results were achieved after fixation with 6% formaldehyde for 30 min. Under these conditions, $1 \cdot 10^6$ chromosomes were routinely obtained from 30 root tips. The chromosomes were morphologically intact and suitable both for high-resolution chromosome studies and for flow-cytometric analysis and sorting. After the addition of hexylene glycol, the chromosome suspensions could be stored at 4° C for six months without any signs of deterioration.

Key words: Cell cycle synchronization – Chromosome isolation (plant) – Flow cytometry – Metaphase arrest – Root tip – *Vicia*

Introduction

Reliable and efficient methods for isolation of intact plant chromosomes are urgently needed in many areas of research. High-resolution chromosome studies ranging from chromatin structure to a detection of low copy sequences by in-situ hybridization are difficult to perform using classical squash preparations because of the presence of cytoplasm and cell wall. Increased resolution has been reported after the enzymatic digestion of the cell wall (Ambros et al. 1986; Dillé et al. 1990). However, cytoplasmic remnants associated with chromosomes may still cause nonspecific binding of probes and prevent undisturbed observation. These problems may be overcome if suspensions of isolated chromosomes are used. Another attractive feature of this approach is that thousands of chromosomes can be dried and then analysed on a small area of a slide.

In addition to high-resolution chromosome studies, the availability of suspensions of intact chromosomes permits the analysis and sorting of single chromosome types by flow cytometry (Gray and Cram 1990). Sorted chromosome fractions can be used for construction of chromosome-specific gene libraries and gene mapping (Van Dilla and Deaven 1990). In plants, the progress in flow-cytometric sorting of single chromosome types has been slow, mainly as a consequence of difficulties in the preparation of high-quality suspensions of chromosomes suitable for flow cytometry (De Laat and Blaas 1984; Conia et al. 1987; Arumuganathan et al. 1991).

Most authors have obtained plant chromosomes after hypotonic lysis and-or mechanical rupture of mitotic protoplasts prepared enzymatically from cells cultured in vitro (Hadlaczky et al. 1983; De Laat and Blaas 1984; Conia et al. 1987; Mii et al. 1987; Arumuganathan et al. 1991). Unfortunately, a more general application of this approach is seriously limited by the fact that reproducible protocols for the establishment of rapidly growing cell cultures are available only for some species or even some genotypes within them. Moreover, cells cultured in vitro are karyologically unstable (Karp and Bright 1985; Lee and Phillips 1988) and so they probably cannot be considered a reliable source of chromosomes for construction of chromosome-specific gene libraries or gene mapping.

In contrast to in-vitro cultures, plant root tips represent an experimental system which is cheap, easy to handle and karyologically stable. Interestingly, only few au-

Abbreviations: APM = amiprophos-methyl; DAPI = 4′,6-diamidino-2-phenylindole

thors have reported successful isolation of chromosomes from root tips (Griesbach et al. 1982). This might be explained by difficulties in obtaining sufficient quantities of protoplasts from root-tip cells.

In this paper, we describe an original and reproducible procedure for isolation of large quantities of morphologically intact mitotic chromosomes from root tips of *Vicia faba*. The procedure employs a novel approach in which the chromosomes are released directly into the isolation buffer by chopping up formaldehyde-fixed root tips.

Material and methods

Cell-cycle synchronization and metaphase accumulation. All incubations were performed at 25° C in darkness, and all solutions were aerated. Seedlings of *Vicia faba* L. cv. Inovec with main roots about 2 cm long were incubated for 18 h in a Hoagland solution (Gamborg and Wetter 1975) containing 2.5 mM hydroxyurea. Then the roots were washed in distilled water and immersed in hydroxyurea-free Hoagland solution. Samples of root tips were taken at 1-h intervals up to 12 h for the analysis of mitotic activity and cell-cycle kinetics.

To accumulate mitotic cells in metaphase, the seedlings were transferred 6, 7, or 8 h after incubation in hydroxyurea-free Hoagland solution to containers with 2.5 µM amiprophos-methyl (APM) in Hoagland solution. This concentration was found to be optimal for metaphase arrest (data not shown). Samples of root tips were taken 3 or 4 h after the incubation in APM for analysis of the frequency of metaphases.

Mitotic activity and metaphase frequency were analysed on squash preparations. Samples of root tips were fixed overnight in ethanol:acetic acid (3:1, v/v) and then prepared according to the standard Feulgen procedure (Doležel and Novák 1984). On each slide, 1000 cells were analysed and five preparations were analysed in each variant. The whole experiment was repeated three times.

Chromosome isolation. Immediately after the APM treatment, the roots were cut 1 cm from the root tip, rinsed in distilled water and fixed for periods from 10 to 60 min at 5° C in 6% (v/v) formaldehyde made up in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, pH 7.5) with 0.1% Triton X-100. After three washes (20 min each) in Tris buffer, the meristem tips (1.5–2.0 mm) of 30 roots were chopped with a sharp scalpel in a glass Petri dish containing 1 ml LB01 lysis buffer (Doležel et al. 1989) of the following composition: 15 mM Tris, 2 mM Na_2EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1% Triton X-100, pH 7.5. The suspension of released chromosomes and nuclei was passed through a 50-µm-pore-size nylon filter to remove large tissue and cellular fragments. Then the suspension was carefully syringed twice through a 22G hypodermic needle and finally filtered through a 15-µm-pore-size nylon filter.

To remove interphase nuclei and chromosome clumps, 750 µl of the suspension was layered over 700 µl of 40% (w/v) sucrose in Tris buffer in a 10-ml glass centrifuge tube and centrifuged at 200 rpm for 15 min. The supernatant was transferred into a sterile 1.5-ml Eppendorf tube.

Fluorescence microscopy. Approximately 10 µl of chromosome suspension was dried on a microscope slide. After mounting in LB01 buffer containing 5 µM 4′,6-diamidino-2-phenylindole (DAPI), the slides were analysed using a Leitz Orthoplan microscope. A Ploe-

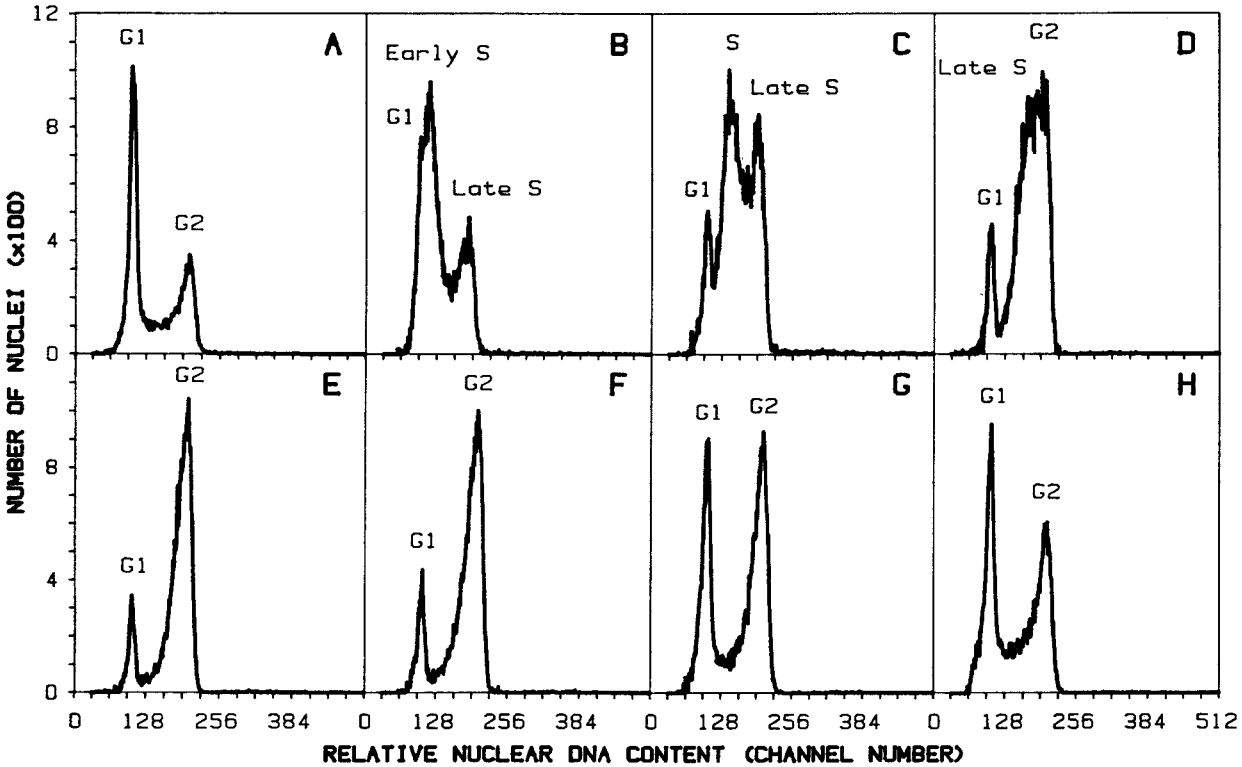

Fig. 1A–H. Histograms of relative nuclear DNA contents obtained by flow-cytometric analysis of DAPI-stained nuclei released from *Vicia faba* root tips. The nuclei were isolated before a treatment with hydroxyurea (**A**) and immediately after the treatment with 2.5 mM hydroxyurea for 18 h (**B**). Further samples of nuclei were isolated from root tips during the recovery from the hydroxyurea block after 2 h (**C**), 3 h (**D**), 4 h (**E**), 6 h (**F**), 10 h (**G**), and 12 h (**H**)

mopak epifluorescent module with filterblock A was used for visualization of DAPI fluorescence. Photographs were taken using a Vario Orthomat 2 automatic microscope camera (Leitz, Wetzlar, FRG).

Flow cytometry. The DNA-specific fluorochrome DAPI was added to suspensions of isolated chromosomes or to suspensions of isolated nuclei to a final concentration of 5 μM. After 30 min, the fluorescence intensity of stained objects was analysed using a Leitz MPV Compact Flow Cytometer at rates of 100–200 counts per s. The histograms were collected over 512 channels and analysed using Flowstar software (Doležel 1989). Prior to analysis, the flow cytometer was aligned using chicken red-blood-cell nuclei (Doležel 1991).

Long-term storage. Chromosome suspensions were stored for several days at 4° C. Alternatively, they were stored for up to six months at 4° C after the addition of hexylene glycol to a final concentration of 0.75 M.

Results

Cell-cycle synchronization. The meristem cells in untreated root tips were mitotically active, the mitotic index being equal to 9.1%. Flow-cytometric analysis of nuclear DNA content showed that a large proportion of the cells were in G_1 phase (45.1%) the rest of the cells being in the S (28.4%) and G_2 (26.5%) phases of the cell cycle (Fig. 1A). The treatment with hydroxyurea resulted in a block of DNA synthesis and in an accumulation of a large fraction of cycling cells at the G_1/S interface (Fig. 1B). As a result, no cells were found in mitosis after 18 h hydroxyurea treatment. After a release from the hydroxyurea block, the meristem cells entered synchronously the S phase (Fig. 1C) and after 6 h reached the G_2 phase. At this time, only 10.2% of cells were found to be in G_1 (Fig. 1F). Simultaneously, the frequency of cells in various stages of mitosis gradually increased and reached its maximum 8 h after the release from the block (Fig. 2) when 44.5% of cells were found in mitosis (Fig. 3A). Subsequently, the frequency of cells

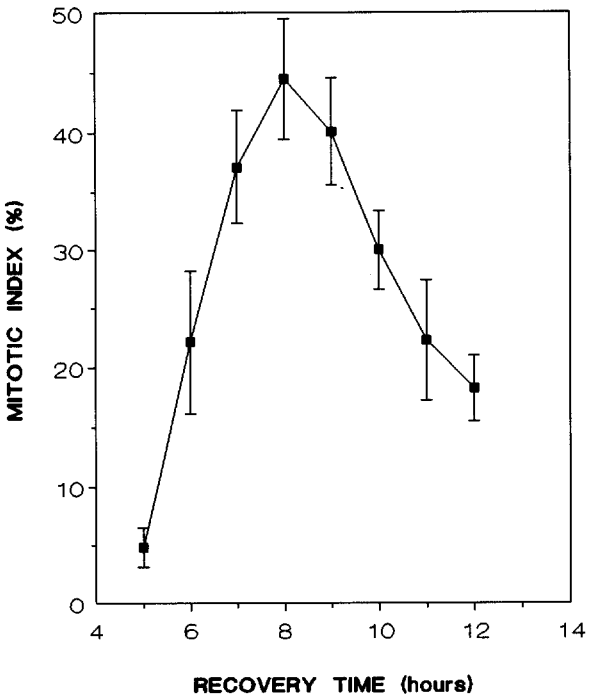

Fig. 2. Mitotic indices in *Vicia faba* root tips (mean ± SE) during the recovery from the hydroxyurea block

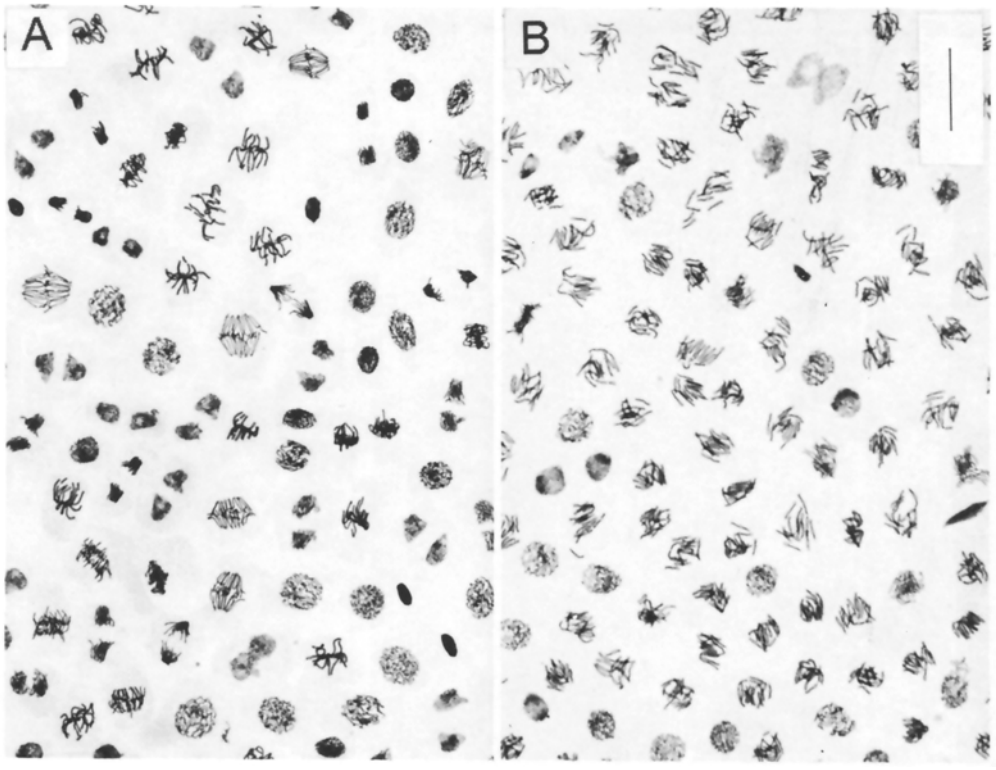

Fig. 3. A Mitotic activity of synchronized *Vicia faba* root tip cells. **B** Metaphase cells in *V. faba* root tips accumulated by the APM treatment. ×260; bar = 50 μm

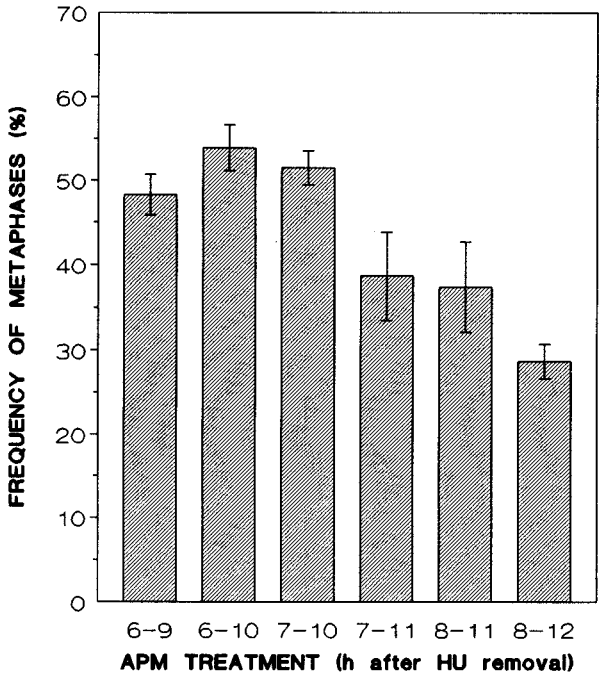

Fig. 4. Frequency of metaphase cells in *Vicia faba* root tips (mean ± SE) treated with 2.5 μM APM for various periods during recovery from the hydroxyurea (*HU*) block

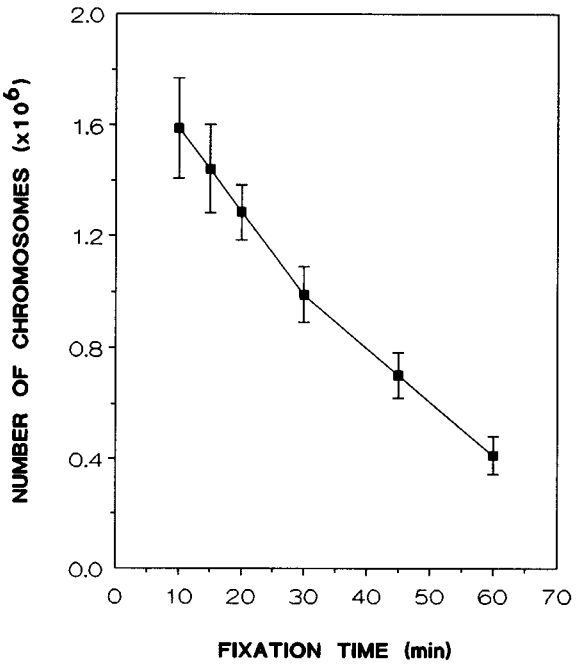

Fig. 5. Number of chromosomes (mean ± SE) released by chopping up 30 *Vicia faba* root tips following varying periods of formaldehyde fixation

in mitosis decreased (Fig. 2) and the cells entered the G_1 phase of the cell cycle (Figs. 1 G, H).

Metaphase accumulation. The frequency of cells blocked in metaphase depended both on the time and length of the APM treatment (Fig. 4). The highest frequency of metaphases (53.9%) was found when the root meristems were treated for 4 h with 2.5 μM APM 6 h after removal of hydroxyurea (Fig. 3 B).

Chromosome isolation. Both the quantity and the quality of isolated chromosomes depended on the extent of the

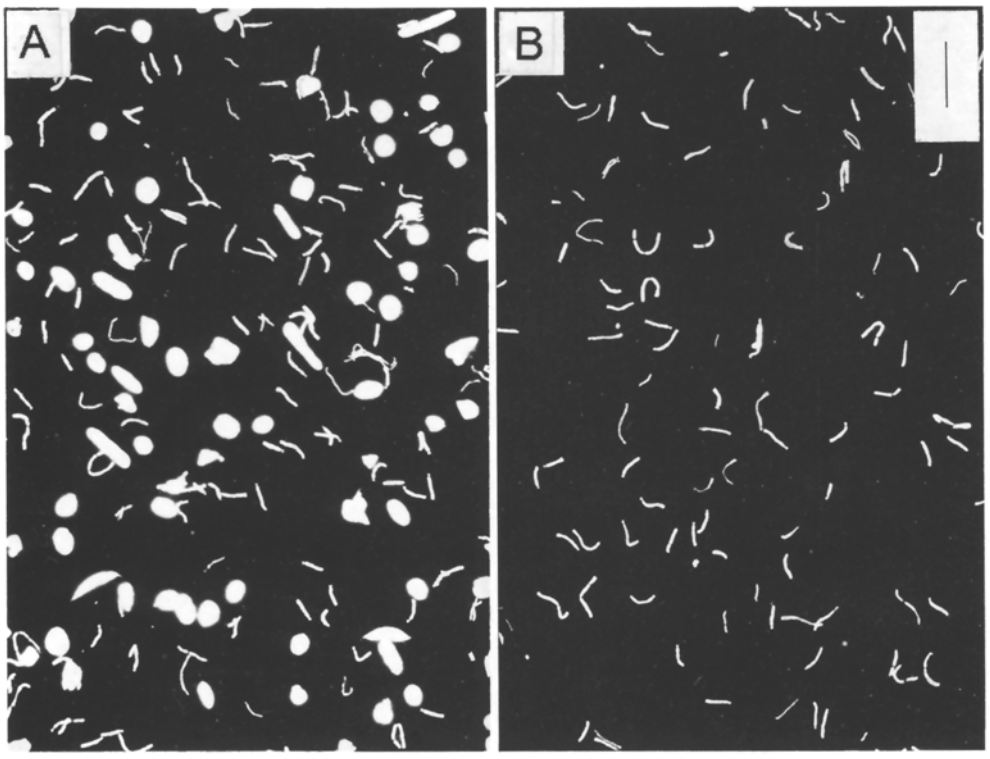

Fig. 6. A Chromosome suspension prepared by chopping up formaldehyde-fixed *V. faba* root tips. Note the presence of interphase nuclei. **B** The chromosome suspension following the purification step. × 390; bar = 25 μm

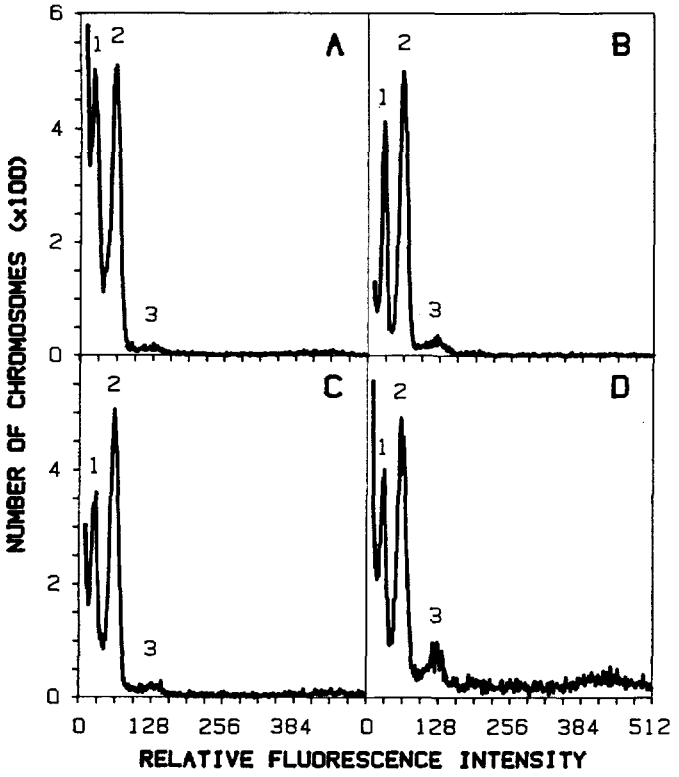

Fig. 7A–D. Histograms of relative fluorescence intensity obtained by flow-cytometric analysis of DAPI-stained chromosome suspensions prepared from *Vicia faba* root tips fixed with 6% formaldehyde for 15 min (**A**), 30 min (**B**), 45 min (**C**), and 60 min (**D**). All histograms show two composite peaks corresponding to five pairs of acrocentric chromosomes (*2*), and to their chromatids (*1*), and a small peak corresponding to a pair of metacentric chromosomes (*3*). Note that the smallest background debris was observed after 30 min of formaldehyde fixation

fixation with formaldehyde. Under our experimental conditions, the number of isolated chromosomes ranged from $4 \cdot 10^5$ to $1.6 \cdot 10^6$ per 30 root tips (Fig. 5). The longer the fixation, the smaller the number of chromosomes isolated. The chromosomes isolated from the root tips fixed for 10 or 15 min were damaged and the chromosome suspensions contained a large amount of chromosomal debris. On the other hand, the suspensions obtained from root tips fixed for 45 or 60 min contained increased numbers of chromosome clumps. Based on these observations, 30 min fixation was chosen as optimal. From 30 root tips, typically $1 \cdot 10^6$ chromosomes were isolated with a well-preserved morphology (Fig. 6A).

Flow-cytometric analysis of the distribution of fluorescence intensity of chromosomes stained by DAPI confirmed visual observations. As can be seen from Fig. 7, the chromosome suspensions isolated from root tips fixed for 30 min contained the least amount of background debris. Two composite peaks corresponding to five pairs of acrocentric chromosomes and to their chromatids, respectively, and a peak corresponding to a pair of large metacentric chromosomes, were clearly resolved. The assignment of individual peaks was confirmed by flow-sorting of objects corresponding to each peak on microscopic slides and examining them with a fluorescence microscope (data not shown).

Low-speed centrifugation was used to purify chromosome suspensions. As can be seen from Fig. 6B, purified suspensions were free of contaminating interphase and prophase nuclei and large chromosome clumps.

Long-term storage. The chromosome suspensions were stored in LB01 at 4° C. However, during long-term storage, a tendency to clumping was observed. The addition of hexylene glycol to freshly isolated samples prevented aggregation and allowed them to be stored for up to six months without any sign of deterioration.

Discussion

Isolation of plant chromosomes has already been described for several plant species (Szabados et al. 1981; Griesbach et al. 1982; Hadlaczky et al. 1983; De Laat and Blaas 1984; Mii et al. 1986; Griesbach 1987; Conia et al. 1987, 1989; Arumuganathan et al. 1991). With the exception of Griesbach et al. (1982) all authors prepared chromosome suspensions from in-vitro-cultured cells via protoplast isolation and lysis. In contrast to suspension cultures in vitro, root tips can be obtained from most plant species, are much easier to handle and are karyologically stable. Chromosome instability of cultured cells is well documented (Lee and Phillips 1988). De Laat and Blaas (1984) encountered this phenomenon after isolation of chromosomes from *Haplopappus gracilis* suspension cells. They found differences between homologous chromosomes both in relative length and DNA content.

A prerequisite for preparation of chromosome suspensions is a high metaphase index (Hadlaczky et al. 1983). Unlike other authors (Hadlaczky et al. 1983; Conia et al. 1987; Arumuganathan et al. 1991) and similarly to Mii et al. (1987) we have achieved a very high frequency of metaphase cells by a combined treatment with a DNA-synthesis inhibitor and a mitotic-spindle inhibitor. The advantage of a combined protocol is that accumulation of sufficient numbers of cells in metaphase can be achieved after a relatively short treatment with a mitotic poison. This avoids the chromosome decondensation and micronuclei formation usually observed after longer colchicine treatments (Conia et al. 1987; Arumuganathan et al. 1991).

In this work we have used a phosphoric-amide herbicide, APM, instead of the commonly used colchicine. Amiprophos-methyl is a more effective inhibitor of tubulin polymerization than colchicine (Morejohn and Fosket 1984) and thus could be used at a considerably lower concentration. Although we have developed the protocol for cell-cycle synchronization with the aim to isolate chromosomes, it can be used also in other studies, including cell-cycle regulation and patterns of DNA replication (Sgorbati et al. 1991).

A principal difference between our procedure for preparation of plant chromosome suspensions and other procedures described so far is that mitotic chromosomes

are released directly by chopping up formaldehyde-fixed root tips. This approach avoided the use of enzymes such as pectinases and cellulases which may be contaminated with DNases and proteases and which may cause increased stickness and even disintegration of chromosomes after long treatments (Hadlaczky et al. 1983). We have found that optimal fixation was crucial for release of sufficient numbers of morphologically intact chromosomes. Similarly, Sgorbati et al. (1986) reported an up to sixfold increase in the number of nuclei isolated by crushing of formaldehyde-fixed *Pisum sativum* root tips as compared with non-fixed material.

While non-fixed plant chromosomes are sensitive to mechanical stress (Arumuganathan et al. 1991), optimal formaldehyde fixation results in excellent preservation of chromosome morphology, even during long-term storage, and an increased resistance to mechanical stress. Our chromosome suspensions were syringed several times without a noticeable chromosome breakage. In addition, the chromosomes withstood considerable shearing forces during flow-cytometric analysis and sorting (data not shown). Thus the chromosome suspensions obtained using our method should be suitable for construction of chromosome-specific gene libraries and gene mapping using flow-sorted chromosomes.

Owing to excellent preservation of morphology, and because isolated chromosomes are free of cytoplasmic remnants, the chromosome suspensions prepared according to our protocol are also very suitable for high-resolution chromosome studies including "background-free" in-situ hybridization as shown by Schubert (1992).

It may be concluded that a new protocol has been developed which permits preparation of high-quality suspensions of *Vicia faba* metaphase chromosomes. Isolated chromosomes are suitable for all applications where genetically active chromosomes are not required. Work is in progress to modify the technique for use with other economically important crops.

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