Theor Appl Genet (1996) 92:744–751
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Preparation of pea (*Pisum sativum* L.) chromosome and nucleus suspensions from single root tips

Received: 4 August 1995 / Accepted: 25 August 1995

Abstract A high-yield method for the isolation of intact nuclei and chromosomes in suspension from a variable number of pea root tips (1–10) has been developed. This procedure is based on a two-step cell-cycle synchronization of root-tip meristems to obtain a high mitotic index, followed by formaldehyde fixation and mechanical isolation of chromosomes and nuclei by homogenization. In the explant, up to 50% of metaphases were induced through a synchronization of the cell cycle at the G_1/S interface with hydroxyurea (1.25 mM), followed, after a 3-h release, by a block in metaphase with amiprophos-methyl (10 μM). The quality and quantity of nuclei and chromosomes were related to the extent of the fixation. Best results were obtained after a 30-min fixation with 2% and 4% formaldehyde for nuclei and chromosomes, respectively. The method described here allowed the isolation of nuclei and chromosomes, even from a single root tip, with a yield of 1×10^5/root and 1.4×10^5/root, respectively. Isolated suspensions were suitable for flow cytometric analysis and sorting and PRINS labelling with a rDNA probe.

Key words *Pisum sativum* · Cell-cycle synchronization · Plant chromosome and nuclei isolation · Flow cytometric analysis and sorting · PRINS

Communicated by F. Mechelke

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Introduction

Many attempts have been made to develop reliable methods for isolating chromosomes and nuclei in suspension from animal and plant cells. In plants, such efforts derived mainly from an interest in the possibility of using such suspensions for: (1) high-resolution studies on chromosome structure and morphology (Schubert et al. 1993); (2) detection of DNA sequences by in situ hybridization (Fuchs et al. 1994); (3) sorting of single-type chromosome fractions (Lucretti et al. 1993; Lucretti and Doležel 1995) for the construction of chromosome-specific gene libraries (Wang et al. 1992; Arumuganathan et al. 1994) and gene mapping (Macas et al. 1993). High concentrations of chromosomes in suspension can be obtained only from a large number of plant cells whose cell cycle is synchronized in order to produce a high mitotic index. Because of the subsequent use of isolated chromosomes, those cell populations should be genetically constant.

Fast-growing cell suspensions have been selected as the source of synchronized cells in a number of experiments (Hadlaczky et al. 1983; De Laat and Blaas 1984; Conia et al. 1987; Mii et al. 1987; Arumuganathan et al. 1991), but their use is seriously hampered because they are available only from a limited number of species or genotypes. Furthermore, in vitro cultured cells can be karyologically unstable (Karp and Bright 1985; Lee and Phillips 1988) and rearrangements occurring in their chromosomes could negatively influence the purity of chromosome-specific DNA libraries or gene mapping (Larkin et al. 1989).

With respect to cell cultures, root tips are generally easier to obtain, facile to handle, and karyologically stable. In spite of this, only a few authors have used root tips as a source for chromosome isolation in suspension (Griesbach et al. 1982). More recently, Doležel et al. (1992) demonstrated the possibility of using *V. faba* root tips, synchronized and blocked in metaphasem to isolate high-quality chromosome suspensions avoiding the use

of enzymatic treatments and protoplasts, which could affect both chromosome stability and yield.

Here we describe a highly effective methodology for the isolation of chromosomes and nuclei in suspension by homogenization, which is effective even from a single fixed root tip. The yield and quality of these suspensions are suitable for flow cytometric analysis and sorting. Primed in situ (PRINS) DNA labelling reactions were utilized for the identification of sorted pea chromosome fractions (Macas et al. 1995).

Materials and methods

Plant material

All germinations and incubations were performed at 25 ± 1 °C in the dark, and all solutions were aerated. Seeds of pea (*Pisum sativum* L.) cv Lincoln were imbibed for 24 h in de-ionized H_2O and germinated in perlite for 54 h.

Cell-cycle synchronization and block at metaphase

Pea seedlings with about 3-cm-long main roots were incubated for 18 h Hoagland solution (Gamborg and Wetter 1975) containing 1.25 mM hydroxyurea (HU). Roots were washed in distilled water and incubated for 3 h in HU-free Hoagland solution. Then, to block cells in metaphase, seedlings were transferred into 10 μM amiprophos-methyl (APM) in Hoagland solution. Samples of root tips were taken 2 h after the incubation in APM for squash preparations and an analysis of the frequency of metaphases. This concentration of APM was found to be effective for metaphase arrest in preliminary experiments. Pea-root squashes were prepared after overnight fixation in ethanol:acetic acid (3:1) and stained according to the Feulgen procedure (Doležel and Novák 1984) with some modifications (DNA hydrolysis: HCl 1N for 13 min at 60 °C).

Nuclei and chromosome isolation

Nuclei

Roots were cut at 1 cm from the root tip, rinsed in distilled water and fixed in 2% (v/v) formaldehyde in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, pH 7.5) with 0.1%Triton X-100, for 20 min at 5 °C.

Chromosomes

APM-treated roots were fixed in 4% (v/v) formaldehyde in Tris buffer for 30 min at 5 °C. Then, roots were washed three times (20 min each) in Tris buffer at 5 °C. From one-to-ten meristem tips (1.5–2.0 mm in length) were homogenized (Ultra Turrax T25, with generator probe S25N/8G, Janke and Kunkel, Germany) for 15 s at 9500 rpm in 5-ml polystyrene tubes (Falcon 2054 containing 0.5–1 ml LB01 lysis buffer (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; Doležel et al. 1989).

The suspensions of released chromosomes and nuclei were channelled through a 53-μm nylon mesh to remove large fragments. To reduce the number of clumped nuclei and chromosomes, the suspensions were carefully syringed once through a 22G hypodermic needle and filtered through a 21-μm nylon mesh.

Chromosome purification

Chromosome suspensions were purified from interphase nuclei and clumps by layering, from the bottom, in a 10-ml glass tube 1000 μl of 40% and 500 μl of 10% (w/v) sucrose in Tris buffer, followed by 500 μl of the chromosome suspension, and centrifuging at 40 g for 20 min. After removing 600 μl from the top, purified chromosomes were collected (500 μl) and the volume was brought up to 1 ml with LB01. Chromosomes in suspension were stored at 5 °C in LB01 supplemented with 1 mM NaN_3 for up to several weeks.

Flow cytometry

Nuclei and chromosomes in suspension were stained with 4′,6-diamino-2-phenylindole (DAPI) 5 μM and analysed with a "FACStar-PLUS" flow cytometer and sorter (Becton Dickinson, San Jośe, California, USA) equipped with an argon-ion laser tuned at $\lambda = 351–363$ nm at a 100 mW output power. Their fluorescence was collected through a 400-nm long-pass filter.

Fluorescence signals were monitored according to their height (fluorscence pulse height: FPH) and area (fluorescence pulse area: FPA) on a 1024 channel ADC. Samples were delivered with a motor-driven syringe pump at a flow rate of approximately 200 particles per s^{-1}. A ceramic nozzle tip with an orifice diameter of 75 μm was used for analysis and sorting. The instrument was aligned using chicken red-blood cell nuclei stained with 5 μM of DAPI (full peak CV ≤ 2.5%).

Analysis of pea flow-karyotypes was compared to the theoretical total DNA histogram generated with the software "KARYOSTAR" (Doležel1991), based on pea chromosome lengths, as measured on metaphase spreads. In order to determine the chromosome content of flow-karyotype peaks, sorting gates were set on FPA histograms. Chromosomes were sorted at a rate of 5–20 s^{-1} directly onto coverslips and air dried.

Primed in situ DNA labelling

The PRINS technique (Koch et al. 1989) was performed according to Macas et al. (1995). Small coverslip fragments covered with a dry chromosome suspension or individual sorted chromosome fractions were cut with a diamond cutter and dropped into 45 μl of ice-cold reaction mix in a 0.5-ml tube. The mix consisted of a 1 × DIG labelling mixture (0.1 mM dATP, dCTP and dGTP, 0.065 mM dTTP, 0.035 mM DIG-dUTP) (Boehringer Mannheim, Milan, Italy), 2.8 mM $MgCl_2$, 3U *Taq* polymerase and 1 × PCR buffer (Stratagene, La Jolla, USA). The probe Ver17 (Yakura and Tanifuji 1983), containing parts of the *Vicia faba* 25*s* and 18*s* rDNA genes, was digested with *Hpa*II and *Taq*I and used as primers at a final concentration of 3 μg per reaction. All concentrations were calculated for a 40-μl volume, and an additional 5 μl of H_2O were added to compensate for evaporation during high-temperature incubation. Samples were denatured at 94 °C for 5 min and then incubated at 70 °C for 2 h. The reaction was stopped by submersing the coverslips in 200 μl of pre-warmed (70 °C) stop buffer (0.5 M NaCl, 0.05 M EDTA, pH 8.0) for 2 min. Then, coverslips were transferred to a series of solutions and incubated as follows: (1) 5 min in 500 μl of wash buffer (0.1 M maleic acid 0.15 M NaCl, 0.05% Tween 20, pH 7.5) at room temperature; (2) 20 min in 500 μl of blocking buffer [0.1 M maleic acid, 0.15 M NaCl, 0.5% Blocking Reagent (Boehringer Mannheim, MJilan, Italy)] at 37 °C; (3) 30 min in 50 μl of anti-DIG-fluorescein Fab-fragments (Boehringer Mannheim, Milan, Italy) solution (20 $\mu g \cdot ml^{-1}$) in blocking buffer at 37°C; (4) three rinses (5 min each) in 500 μl of washing buffer at room temperature. Chromosomes were counterstained with 10 $\mu g \cdot ml^{-1}$ propidium iodide (PI) prepared in antifading solution (Vectashield, Vector, Burlingame, CA.).

Fluorescence microscopy

All microscope observations were performed with a fluorescence microscope (Leitz Orthoplan, Ernst Leitz Wetzlar, Germany) equip-

ped with an epifluorescent module. Feulgen-stained squashes of treated root tips were analysed with green-light excitation ($\lambda = 530 - 560$ nm), which generated a red fluorescent emission ($\lambda > 580$ nm).

Chromosome suspensions were dried on a microscope slide, mounted in LB01 buffer containing 5 μM of DAPI (excitation $\lambda = 350$–380 nm; dichroic mirror = 400 nm; emission filter $\lambda = 400$–450 nm). PRINS-labelled samples were analysed with a filter set suitable for fluorescein isothyocyanate (FITC) and PI dual analysis (excitation $\lambda = 450$–490; dichronic mirror $\lambda = 515$ nm; emission filter $\lambda = 530$ nm long pass). Photographs were taken using a Leitz "Vario Orthomat 2" automatic microscope camera with ISO 400 Kodak Ecktachrome film and ISO 100 Kodak T-Max black and white film.

Results

Cell-cycle synchronization

Flow cytometric analysis of the DNA content of nuclei isolated from untreated root tips germinated for 54 h showed a high level of DNA synthesis (S phase = 26.6%) with 40% of nuclei in G_1 and 33.4% in G_2 (Fig. 1A). HU affected the cell cycle by blocking DNA synthesis, accumulating a large fraction of cycling cells at the G_1/S interface and inhibiting mitosis. The HU blocking effect was dramatically related to its concentration: 10 mM and 5 mM of HU completely inhibited DNA synthesis while at 2.5 mM and 1.25 mM HU cells were able to escape the block 2 and 4 h before the end of a 18-h HU treatment, respectively (Fig. 1B, C and D). After removing HU from the Hoagland solution, meristem cells entered the S phase synchronously. At 1.25 mM HU, 63% of nuclei reached the G_2 phase after 2 h and the highest MI (35%) was found 4 h after release from HU treament (Fig. 2 and Fig. 3A); this resulted in a shorter synchronizing procedure than with higher HU concen trations. Therefore, in order to allow most of the cells to reach metaphase synchronously, root seedlings were supplied with APM after after a 3-h release from the HU block.

Block at metaphase

The percentage of cells blocked in metaphase depend-end both on the time and the length of the APM treatment (data not shown). The highest frequency of metaphases (50%) was found when the root meristems, synchronized with 1.25 mM of HU for 18 h, were treated with 10 μM of APM for 2 h (Fig. 3B).

Isolation of nuclei and chromosomes

The optimal procedure of isolation in suspension of pea nuclei and chhromosomes was determined in relation to formaldehyde concentration (2–3–4%), the extent of

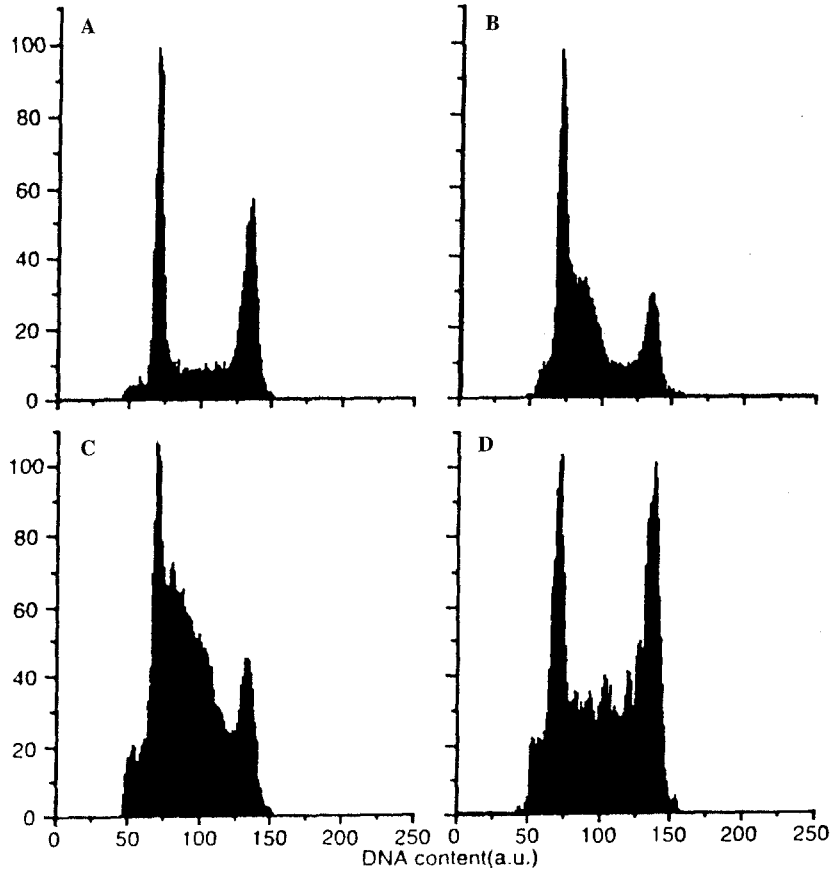


Fig. 1 A–D Histograms of relative nuclear DNA content obtained by flow cytometric analysis of DAPI-stained nuclei released from pea root tips. Nuclei were isolated after 54 h germination before (**A**) and immediately after treatment with HU for 18 h at 5.0 mM (**B**), at 2.5 mM HU (**C**), and 1.25 mM HU (**D**)

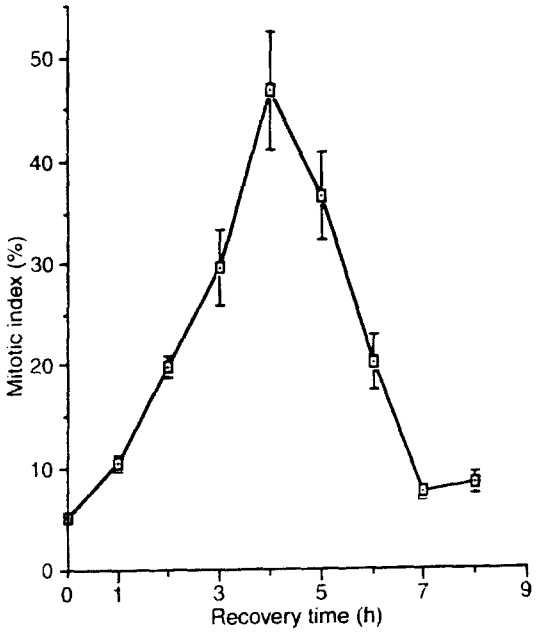
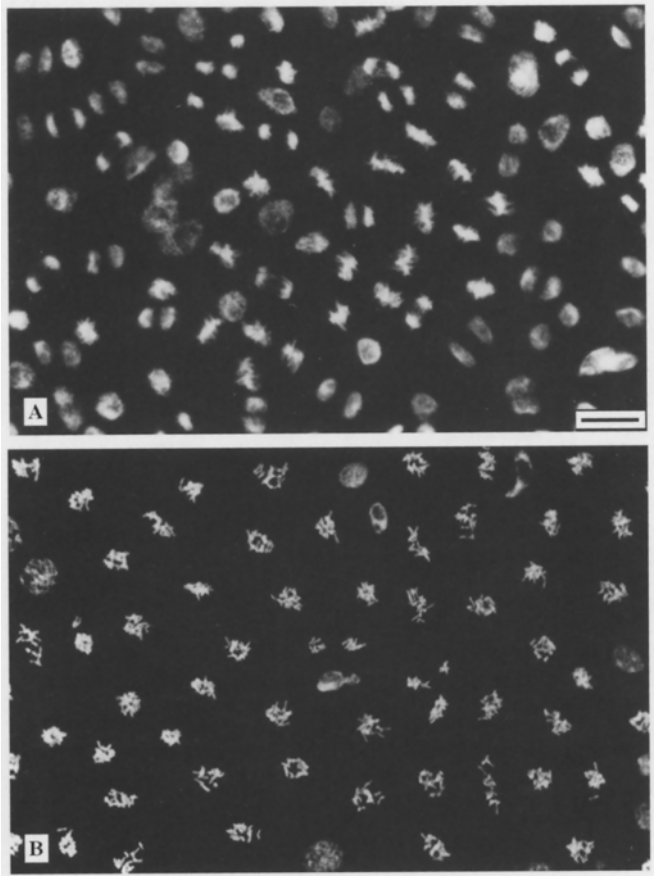


Fig. 2 The mitotic index in pea root tips (mean ± standard error) during recovery from the hydroxyurea block at 1.25 mM HU

Fig. 3 A, B Treated pea meristem tips stained by Feulgen and observed with epifluorescence (λ exc = 530–560 nm, λ ems > 580 nm): **A** Mitotic synchrony after 5 h release from a HU block; **B** metaphase cells accumulated by combining a 1.25 mM HU + 10 μM APM treatment. Bar = 20 μm

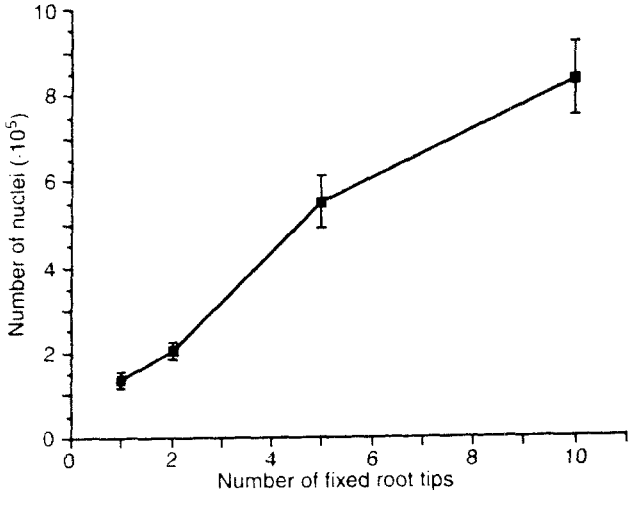


fixation (10-15-20-25-30-40-45 min) and homogenizing speeds (data not shown).

For the isolation of nuclei, the best yield combined with good morphology was obtained after the fixation of root tips with 2% formaldehyde for 30 min and homogenization at 9500 rpm for 15 s in LB01. Following this procedure, $1 \pm 0.2 \times 10^5$ nuclei·ml^{-1} were isolated from a root tip of 1.5 mm in length (about 10 μg fresh weight); the number of released nuclei increased up to $8.3 \pm 0.8 \times 10^5 \cdot ml^{-1}$ when ten root tips were homogenized (Fig. 4).

The extent of fixation with formaldehyde was the principal factor for determining the quantity and the quality of isolated chromosomes. As for the isolation of nuclei, the quality and the number of released chromosomes were dependent on the concentration of formaldehyde and one the length of fixation: the stronger the fixation, the smaller the number of isolated chromosomes, while a reduction of fixation strength produced a large number of damaged chromosomes (data not shown). Flow cytometric analysis of the distribution of chromosome DNA FPA stained by DAPI was used to evaluate the yield and quality of pea chromosome suspensions according to the amount of background debris and the degree of resolution of individual peaks. Based on these criteria, satisfactory results were obtained with a formaldehyde fixation at 4% for 30 min and homogenization at 9500 rpm for 1.5 s in LB01. Different numbers of root tips were homogenized and the quantity of released chromosomes ranged from $1.37 \pm 0.1 \times 10^5 \cdot ml^{-1}$ for one root tip to $4 \pm 0.2 \times 10^5 \cdot ml^{-1}$ for ten root tips (Fig. 5). At the selected conditions, isolated chromosomes showed a well-preserved morphology and the suspension contained a low background of contaminants (e.g. debris and chromosome clumps Fig. 6A) which was significantly reduced by a two-step gradients at 10 and 40% of sucrose and low-speed configuration (Fig. 6B). Such purification was

Fig. 4 Number of nuclei (mean ± standard error) released by homogenization from 1–2–5–10 fixed pea root tips



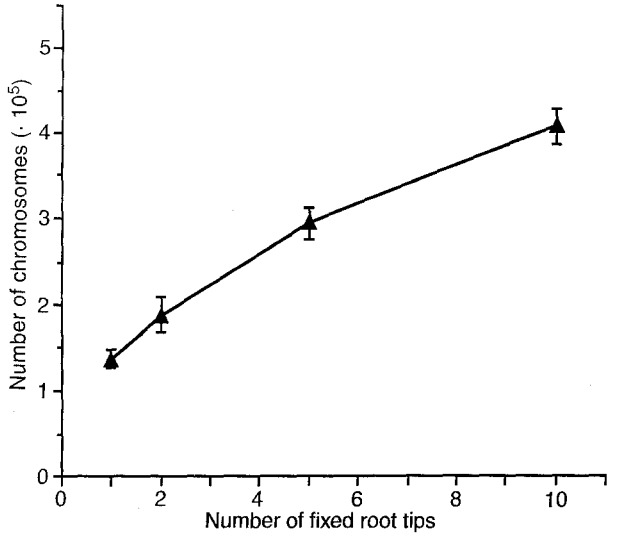


Fig. 5 Number of chromosomes (mean ± standard error) released by homogenization from 1–2–5–10 fixed pea root tips

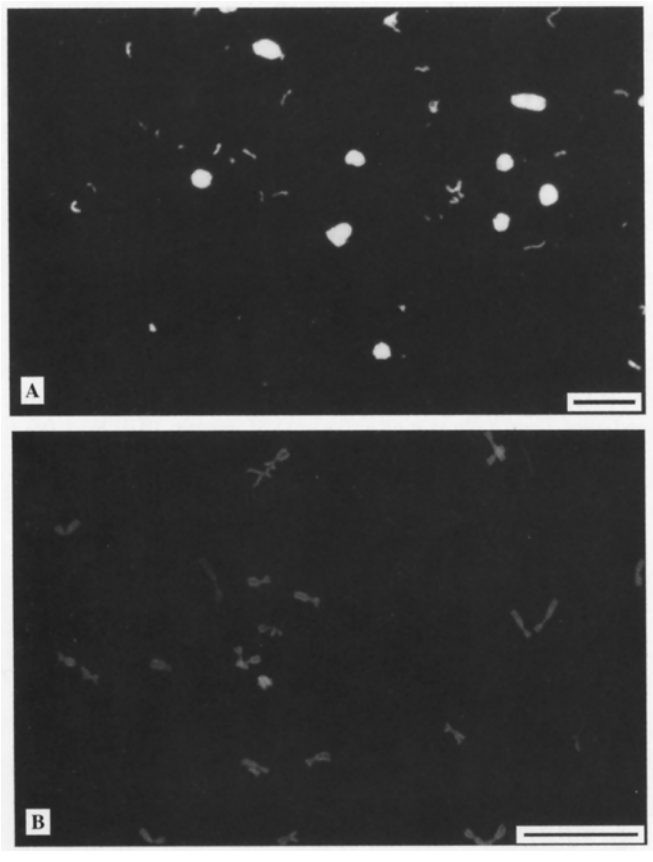


Fig. 6 **A** Crude DAPI-stained chromosome suspension prepared by homogenization of formaldehyde-fixed pea root tips; **B** the chromosome suspension following the purification step. Bar = 20 μm

also useful in lowering the concentration of released cytoplasmic proteins into the isolation buffer, thus decreasing the tendency of chromosome clumping during storage.

Flow-karyotyping, sorting and chromosome labelling

A theoretical pea flow-karyotype was generated according to the different chromosome sizes as measured on metaphase spreads (Fig. 7).

The flow-karyotyping based in a DNA FPA histogram showed four composite peaks representing the seven pairs of pea chromosomes (Fig. 8). Flow sorting was used to confirm the chromosome composition of the peaks and sorting regions were defined on the DNA histogram (R1 = chromosomes I and II; R2 = chromosome VI and III; R3 = chromosome IV and contaminating chromosome III; R4 = chromosomes V and VII).

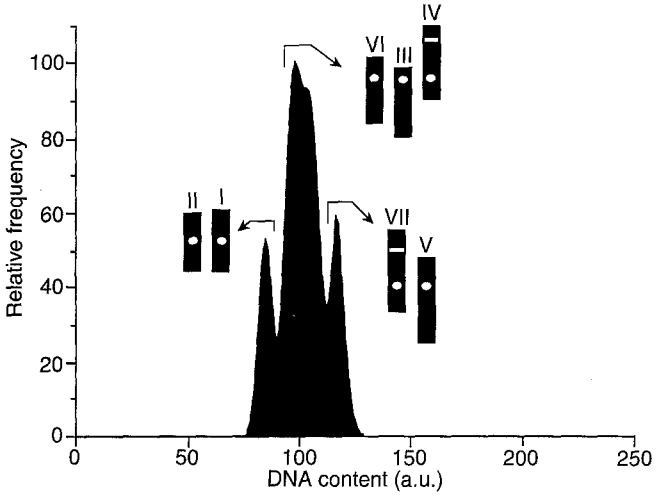


Fig. 7 Theoretical histogram of the DNA content of the *P. sativum* standard karyotype generated according to the total DNA content for a CV = 2.5%: the flow-karyotyping shows three peaks in which the seven pairs of pea chromosomes are arranged according to their relative lengths

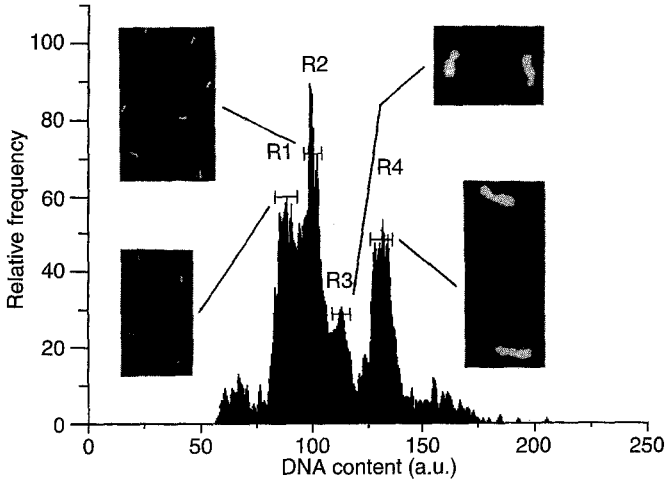


Fig. 8 Flow-karyotyping of DNA integral fluorescence (FPA) of DAPI-stained pea chromosomes. The photographs show sorted chromosomes from regions R1 (I + II) and R2 (VI + III and I), DAPI-stained; and from regions R3 (III + IV) and R4 (V + VII) after PRINS labelling for rDNA

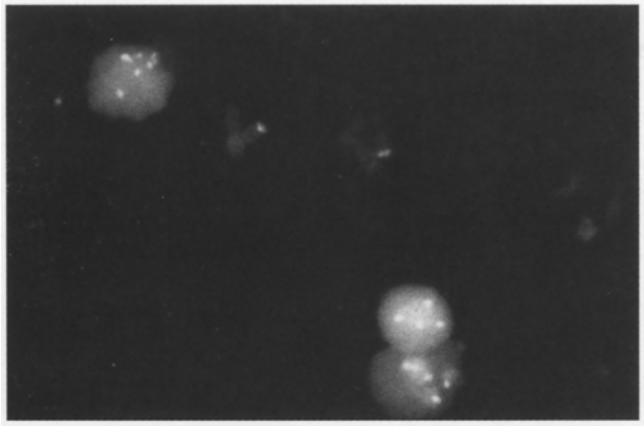


Fig. 9 DNA PRINS labelling of isolated pea nuclei and chromosomes with rDNA: *fluorescent spots* show the location of the secondary constrictions on the PI-counterstained crude suspension. Bar = 20 μm

Sorted fractions were used for PRINS labelling with a rDNA probe. The specificity of the PRINS labelling was controlled on samples containing nuclei and all chromosomes (Fig. 9). Nuclei showed four to eight labelling spots, corresponding to the number of the nucleolar organizing regions (NORs), which depends on their cell-cycle phase; chromosomes IV and VII were labelled at their secondary constrictions. Labelling of rDNA was observed on approximately 30% of the chromosomes, data which correspond to theoretical expectation. Chromosomes sorted from region R4 were identified as V and VII, the latter with a secondary constriction. The percentage of PRINS-labelled chromosomes in R4 was calculated as 45% of the total sorted fraction. Region R3 contained acrocentric chromosome IV (38%) and contaminations of chromosome III, as determined by PRINS. Chromosomes sorted from region R2 and R1 did not exhibit any rDNA labelling and they were identified as acrocentric VI and III, with some contaminations with metacentric I for the former and metacentric I and II for the latter (fig. 8).

Discussion

Here we report on the development of a new method for high-yield isolation of pea chromosomes and nuclei in suspension from single root tips.

Chromosome isolation has already been described in several plants, using protoplasts derived from cell suspensions or mesophyll cells as a source of chromosomes. Only a few authors have reported on the preparation of chromosome suspensions from root-tip cells (Griesbach et al. 1982; Doležel et al. 1994). In fact, roots are easier to obtain from most plants and isolated chromosomes are true-to-type with respect to in vitro cell suspensions where chromosome instability is well documented (D' Amato 1991).

As already pointed out by Hadlaczky et al. (1983) and Doležel et al. (1992), in order to develop an effective chromosome isolation procedure, it is essential to achieve a high metaphase index from already actively dividing plant cells. To fulfil such requirement, several authors applied a metaphase blocking agent, sometimes combined with a partial synchronization by starvation (Conia et al. 1987; Arumuganathan et al. 1991).

A two-step treatment, including synchronization at the G_1/S interface and a block at metaphase, was adopted to achieve a high metaphase index in pea. In this way it was possible to shorten the length of the mitotic-poison treatment, therefore avoiding as much as possible the separation of chromosomes into chromatids and chromosome de-condensation, phenomena which both affect the quality of preparations (Conia et al. 1988; Arumuganathan et al. 1991; Doležel et al. 1992).

We have used a HU concentration which allowed meristem cells to escape from the block synchronously and to start DNA synthesis before the actual removal of the synchronizing agent. In this way, it was possible to shorten the period of recovery to 3 h. The phosphoric-amide herbicide APM was used at a concentration four-times higher than in field bean, thus indicating a different sensitivity to APM for tubulin polymerization between different species in legumes. Extending the length of APM treatment for more than 2–3 h did not increase the percentage of metaphase cells, while chromosome initiated a separation into chromatides.

The use of fixed tissues for the isolation of nuclei and chromosomes proved to be a very efficient methodology in pea, field bean and wheat (Sgorbati et al. 1986; Doležel et al. 1992; Schubert et al. 1993). We have optimized the fixation of pea root tips to allow the use of a homogenizing procedure. By this technique it is possible to isolate chromosomes and nuclei in suspension even from a single pea root tip. This efficient procedure could be especially helpful when a very small amount of explant and different material with tiny roots (*Medicago sativa*, *Zea mays*; Doležel and Lucretti, unpublished) need to be analysed (e.g. for cell-cycle parameters).

This approach is faster and more convenient than chopping by a scalpel and permits the isolation of good quality chromosome suspensions in large quantity. Pea chromosomes in suspension would also be very useful for microscope studies and FISH, due to maintenance of their morphology and because isolated chromosomes have very little cytoplasmic residue (Schubert et al. 1993).

During our study, pea chromosomes were analyses for the first time with a flow cytometer to define their flow-karotype and to sort specific fractions.

The pea standard karyotype exhibits little difference in size between chromosomes, which range from the small metacentric I, accounting for 11.72% of the total genome length, to the large chromosome VII, which is 16.16% of that length. A simple discrimination of chromosomes based on total DNA content was not sufficient

to characterize and sort single-type pure fractions from the standard pea complement. The resolution of the flow-karyotype was improved through the use of DAPI as a DNA fluorochrome. Since DAPI shows a higher affinity to A + T-rich DNA regions (Doležel et al. 1994), it has allowed us to discriminate a new peak on the DNA FPA histogram which was not discernible on the theoretical flow-karyotype. In this way, it was possible to define a region, 'R3', which was characterized for its chromosome content by direct sorting and DNA labelling. We have used PRINS DNA labelling with a rDNA probe to identify sorted chromosomes from different regions according to the presence of a secondary constriction, which is not easily distinguishable in fully condensed chromosomes by morphological evaluation. This technique was shown to be faster and simpler than in situ hybridization and it allowed us to perform two consecutive reactions during the same working day.

In previous studies, we have shown that translocation lines may be used to sort single chromosome types in *V. faba* (Lucretti et al. 1993; Doležel and Lucretti 1995). In *P. sativum*, chromosome rearrangements are present in quite a number of lines where defined translocations have occurred (Blixt et al. 1991). Hopefully, such "reconstructed" karyotypes would allow the sorting of pure fractions of single-type chromosomes also in the pea. Specific fluorescent labelling of chromosomes in suspension, and the use of the label to discriminate individual chromosomes, is another approach which can be used to sort single chromosome types (Macas et al. 1995; Pich et al. 1995). Furthermore, combining flow cytometry with this efficient procedure for the isolation of chromosomes and nuclei in suspension, it would be possible to perform a qualitative and quantitative analysis of chromosome and ploidy alterations from a large number of individuals (Gray et al. 1990).

Flow-sorted chromosomes have been used for the localization of seed storage-protein genes (Macas et al. 1993) in *Vicia faba*. The purity of flow-sorted chromosomes was confirmed by the laborious technique of micromanipulation which allows one to distinguish individual chromosomes not only on the basis of their total length but on several other structural rearrangements (e.g. relative arm length).

Flow-sorting of pure fractions of a few hundred pea chromosomes would allow the creation of chromosome-specific libraries by means of PCR-based methods (DOP-PCR, Telenius et al. 1992). Such libraries will be a unique source of probes for in situ hybridization, the detection of chromosome rearrangements and introgression in related genomes, the mapping of DNA sequences and genes, and the definition of linkage groups which are still unclear in the pea (Ellis et al. 1992).

Acknowledgements The authors thank Mrs E. Palmieri for her excellent technical work. A gift sample of APM from the Mobay Corporation (Agricultural Chemicals Division, Kansas City, USA) is gratefully acknowledged. The authors also wish to thank Boehringer Mannheim (Milan, Italy) for providing some of the molecular reagents. Part of this work was supported by grant no. 204/93/1097 from the Grant Agency of the Czech Republic. This is publication no. 119 of ENEA, Biotechnology and Agriculture Sector.

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