Chromosome analysis and sorting in *Vicia sativa* using flow cytometry

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Abstract

Procedures were developed for flow cytometric analysis and sorting of mitotic chromosomes (flow cytogenetics) of common vetch (*Vicia sativa* L., 2n=12). Suspensions of intact chromosomes were prepared from root tips after cell cycle synchronization, formaldehyde fixation, and mechanical homogenization. On average, 3×10^5 morphologically intact chromosomes could be isolated from 25 root tips. Flow cytometric analysis of DAPI-stained chromosomes resulted in histograms of relative fluorescence intensity (flow karyotypes) containing four peaks, representing particular chromosomes and/or pairs of chromosomes with similar relative DNA content. Peaks I and II were assigned to chromosomes 6 and 5, respectively. These chromosomes could be sorted with a purity exceeding 90 %. The two remaining peaks on the flow karyotype were composite, each of them representing a pair of chromosomes. Chromosomes 1 and 3 were assigned to composite peak III while chromosomes 2 and 4 were assigned to composite peak IV. The chromosomes could be sorted with a purity of 99 % from both composite peaks. Bivariate flow karyotyping after simultaneous staining of chromosomes with DAPI and mithramycin was not found helpful in discriminating additional chromosomes. This study extends the number of legume species for which flow cytogenetics is available and provides a new tool for targeted and effective analysis and mapping of common vetch genome.

Additional key words: cell cycle synchronization, chromosome suspension, common vetch, metaphase accumulation, physical genome mapping.

Introduction

Common vetch is an important multipurpose crop used as forage, fodder and green manure (Van de Wouw *et al*. 2001). The grain is used for feeding ruminants. However, the presence of cyanoalanine toxins in the seeds limits its usefulness in rations for monogastric animals and for human consumption (Tate and Enneking 1992). Common vetch belongs to *Vicia sativa* aggregate, a complex of six closely related taxa that comprise cultivated, weedy and wild forms (Van de Wouw *et al*. 2001, 2003a).

Pod-shattering restricts the use of common vetch as a leguminous forage crop (Elmoneim *et al*. 1993) and its cultivation is negatively affected by many diseases such as downy mildew (Ahmed *et al*. 2000). Breeding of improved varieties could be accelerated by a better knowledge of nuclear genome. Unfortunately, genome analysis and mapping have lagged behind and common vetch may be considered under researched crop. In addition to scarce studies on genetic diversity and evolution (Shiran and Raina 2001, Van de Wouw *et al*. 2001, 2003b), analysis of seed colour inheritance (Chowdhury *et al*. 2004) and cytological studies (Schiffino-Wittman 2000) were carried out. Recent investigations of sequence composition of *V. sativa* repetitive DNA (Macas *et al.* 2000, 2003, Nouzová *et al.* 2001) resulted in identification of several families of satellite repeats with characteristic chromosomal locations which together with genes encoding ribosomal RNAs provided first landmarks for construction of

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Abbreviations: DAPI - 4′,6-diamino-2-phenylindole; FISH - fluorescence *in situ* hybridization; HU - hydroxyurea; PRINS - primed *in situ* DNA labelling.
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basic physical map of this species (Navrátilová *et al.* 2003).

Flow cytogenetics describes the analysis and sorting of mitotic chromosomes using flow cytometry (Doležel *et al*. 2004). In this method, suspension of intact chromosomes is prepared, stained using a DNA fluorochrome, and classified according to relative fluorescence intensity or DNA content. The resulting distribution is called flow karyotype. Any chromosome that can be discriminated on a flow karyotype can be sorted in large quantities and high purity (Doležel *et al*. 2005). This provides small and defined fractions of a genome, which can be used to simplify its analysis. In our previous work, flow-sorted plant chromosomes were found useful in number of applications, including physical mapping, targeted isolation of markers from defined genome regions, and construction of chromosome-specific DNA libraries (for review see Doležel *et al*. 2004, 2005).

Until now, flow cytogenetics has been developed in 17 plant species including economically important cereals and legumes (Doležel *et al.* 2004). In legumes, chromosome analysis and sorting has been reported in *V. faba* (Doležel *et al*. 1992, Lucretti *et al*. 1993), *Pisum sativum* (Gualberti *et al*. 1996, Neumann *et al*. 1998, 2002) and *Cicer arietinum* (Vláčilová *et al*. 2002). However, no attempt have been made to prepare suspension of *V. sativa* chromosomes and analyse them using flow cytometry.

In this work, we have developed a high-yield procedure for preparation of suspensions of intact chromosomes from synchronized root meristems of *V. sativa*. Flow-cytometric analyses lead to generation of the first flow karyotype for the crop. Individual peaks on the flow karyotype were assigned to particular chromosomes after FISH analysis of flow-sorted chromosomes with a set of DNA probes that gave chromosome-specific labelling patterns. We have also assessed a potential of bivariate flow karyotyping for discrimination of individual chromosomes in this crop.

Materials and methods

Plant material, cell cycle synchronization and accumulation of metaphases: Seeds of common vetch (*Vicia sativa* subsp. *sativa* cv. Ebena, 2n=2x=12) were obtained from the Plant Breeding Station, Horní Moštěnice, Czech Republic.

The seeds were imbibed in deionized water for 24 h and then germinated on a moistened paper tissue for 24 h. Seedlings with 3-cm-long primary roots were transferred to an open-mesh basket set on a plastic tray filled with 900 cm^3 Hoagland's solution (Gamborg and Wetter 1975) containing hydroxyurea (HU) at three different concentrations (1.25, 2.5 and 5 mmol dm^{-3}) and incubated for 18 h. Then the roots were rinsed in distilled water and incubated in hydroxyurea-free Hoagland's solution. Samples of roots were taken at 1-h intervals for up to 10 h for determination of mitotic activity (mitotic index). To accumulate cells at metaphase, seedlings were treated in Hoagland's solution containing oryzalin (*Eli Lilly and Company*, Indiana, USA) at three different concentrations (2.5, 5 and 10 μmol dm^{-3}) 2, 3, 3.5, 4, 4.5 or 5.5 h after the removal from HU in Hoagland's solution. All incubations were performed at 25 ± 0.5 °C in the dark; all solutions were aerated. Samples of root tips were collected after 2 h of treatment to determine the frequency of metaphases (metaphase index).

Root tips were excised and immediately fixed in freshly prepared ethanol:acetic acid (3:1, v/v) fixative. Squash preparations were made according to the standard Feulgen procedure (Doležel and Novák 1984). On each slide, 1 000 meristematic cells were evaluated. Five preparations per variant were analyzed and each experiment was repeated three times.

Preparation of chromosome suspensions: Immediately after the oryzalin treatment, 25 root tips were cut 1 cm from root tip, rinsed in distilled water and fixed for 15, 20, 25 and 30 min in 1, 2, 3 and 4 % (v/v) formaldehyde in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, 0.1%v/v Triton X-100, pH 7.5) at 5 °C. After three 5-min washes in Tris buffer, root tips were cut and collected in a tube containing 1 cm^3 LB01 lysis buffer (Doležel *et al*. 1989). The chromosomes were released mechanically using a *Polytron PT 1300D* homogenizer (*Kinematica AG*, Litau, Switzerland) with three different speed settings (10 000, 13 000, and 15 000 rpm) and three different times (15, 18 and 20 s). Resulting homogenates were filtered through a 50-μm nylon mesh to remove large cellular debris.

Flow cytometric analysis: Chromosome analysis and sorting were performed on the *FACSVantage SE* flow cytometer (*Becton Dickinson*, San José, USA) equipped with two lasers. To excite DAPI (4′, 6-diamino-2-phenylindole), the first laser was tuned to multiline UV (333.6 - 363.8 nm) and a power output of 300 mW; mithramycin fluorescence was stimulated with the second laser emitting 200 mW at 457 nm. A solution of 50 mM NaCl was used as a sheath fluid and the chromosome suspensions were analyzed at rates of 200 - 400 particles s^{-1}. Approximately 20 000 - 50 000 chromosomes were analyzed in each sample.

For monovariate analysis the suspension of isolated chromosomes was stained with DAPI at final concentration 2 μg cm^{-3}. Fluorescence emission was measured through a 475-nm long pass filter in front of FL1 photomultiplier. Relative fluorescence intensities were acquired on a histogram of FL1 fluorescence pulse area.

For bivariate analysis the suspensions of isolated chromosomes were stained with DAPI at 1, 2, and

3 μg cm^{-3} and mithramycin at 10, 25, 50 and 75 μg cm^{-3}. Because the fluorescence of a complex between DNA and mithramycin (preferentially binding to GC-rich regions) is considerably increased in the presence of Mg^{2+} ions, $MgSO_4$ was added to chromosome suspension at the final concentration of 1 mM prior to staining. Due to the spatial separation of 200 μm of the laser interception points with the liquid jet, a half mirror was used to split DAPI fluorescence to FL1 photomultiplier through a 475-nm long pass filter; mithramycin fluorescence was sent to FL4 photomultiplier through a 585/42-nm band pass filter. Relative fluorescence intensities were acquired on histograms of FL1 and FL4 fluorescence pulse area.

Chromosome sorting: In order to determine chromosome content of each peak on a monovariate flow karyotype, sorting window was set on dot plot of the DAPI fluorescence pulse area versus DAPI fluorescence pulse width. One thousand chromosomes were sorted from each peak into 0.01 cm^3 LB01 buffer containing 5 % (m/v) sucrose on microscope slides, air dried and maintained at -20 °C for FISH (fluorescence *in situ* hybridization) analysis.

FISH (fluorescence *in situ* hybridization): The FISH was carried out using single-stranded oligonucleotide probe specific for VicTR-B (5'-AAA TTT GAA GTG AAT ATA AGT CTT CAG AAA ATC TGA-3') repeat (Macas *et al*. 2000). A probe for VicTR-B labelled by fluorescein at its 5' end was synthesized by *Invitrogen* (Paisley, UK). Before hybridization, the chromosome preparations were aged at 65 °C for 45 min and treated with 4 % (m/v) formaldehyde in 2× SSC for 10 min. The chromosome denaturation was carried out in 1× PCR buffer (*Promega Corp*., Madison, USA) supplemented with 4 mM $MgCl_2$ for 3 min at 94 °C, and followed by dehydration using ice-cold ethanol series. The hybridization mix (2× SSC, 100 ng mm^{-3} sheared calf thymus DNA, 0.125 % SDS and 0.5 ng mm^{-3} *VicTR-B* probe) was denatured at 76 °C for 15 min. Hybridization was done overnight at 37 °C, and post-hybridization washes were performed in 2× SSC at 42 °C for 15 min. Chromosomes were counterstained with DAPI and examined using a microscope *Nikon Eclipse 600* (*Nicon Corp.*, Tokyo, Japan). The images were captured using a b/w CCD camera and analyzed using *Lucia* software (*Laboratory Imaging*, Prague, Czech Republic).

Results and discussion

The protocol for preparation of chromosome suspensions in common vetch developed in this work is based on a procedure originally developed for field bean (Doležel *et al*. 1992). In this protocol, hydroxyurea (HU) is used to arrest cycling cells at the G_1/S interphase. Upon the release from the block, the cells traverse S and G_2 phases and enter mitosis in a synchronous manner. Mitotic cells are then accumulated at metaphase using a mitotic spindle inhibitor (Doležel *et al*. 1999).

A treatment of common vetch root meristems with HU at 1.25 mmol dm^{-3} resulted in a partial cell cycle arrest and consequently in a low degree of mitotic synchrony (32 ± 8.9 %). The highest degree of mitotic synchrony (50 ± 3.8 %) was achieved with 2.5 mmol dm^{-3} HU. A sharp peak of mitotic activity was observed 6 h after the removal from HU (Fig. 1). A treatment with HU at 5 mmol dm^{-3} resulted in a complete cell cycle arrest. However, at this concentration the release from the block was significantly delayed and the peak of mitotic activity was observed only 8 h after the release from HU. Furthermore, the degree of mitotic synchrony was lower (17 ± 4.5 %) as compared to that achieved with HU at 2.5 mmol dm^{-3}.

Oryzalin at 2.5 μmol dm^{-3} did not block all dividing root tips cells in metaphase. On the other hand, at 10 μmol dm^{-3} the compound induced chromosome clumping. The most efficient accumulation of synchronized cells at metaphase was achieved after 2 h treatment with oryzalin at 5 μmol dm^{-3} starting 3.5 h after the HU removal. With this treatment, the average frequency of metaphases was 65 ± 17.6 %. Prolongation of oryzalin treatment to 3 and 4 h resulted in the occurrence of chromatids in chromosome suspensions. Thus, the 2-h treatment was chosen as optimal.

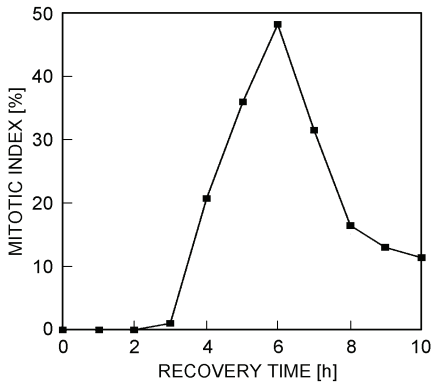

Fig. 1. Frequency of cells in mitosis (mitotic index) observed in common vetch root-tip meristems during recovery from a treatment with 2.5 mmol dm^{-3} hydroxyurea for 18 h.

Procedure for isolation of intact chromosomes from synchronized meristems was optimized with the aim to obtain flow karyotypes with the least amount of debris background and chromosome clumps, and the highest resolution of chromosome peaks (Doležel *et al*. 1999). Four parameters were optimized: concentration of formaldehyde in the fixative, duration of the fixation, speed setting of the mechanical homogenizer and duration of homogenization. The chromosome suspensions containing intact chromosomes and resulting in

high-resolution flow karyotypes were obtained after fixation in 2 % (v/v) formaldehyde for 25 min followed by homogenization at 13 000 rpm for 18 s. Under these conditions, chromosome yield was about 3×10^5.

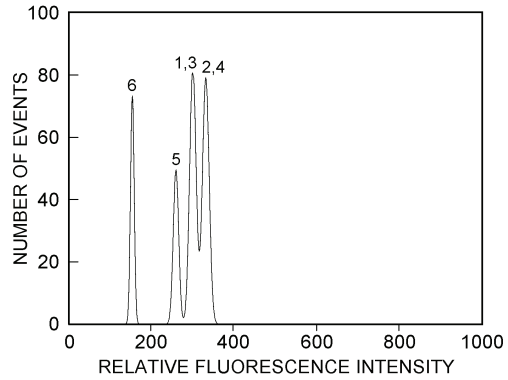

Fig. 2. Theoretical flow karyotype of common vetch calculated using relative chromosome lenghts (Navrátilová *et al*. 2003) and considering 2 % coefficient of variation of DNA peaks. The numbers indicate chromosome content of each peak.

A theoretical flow karyotype was calculated using *Karyostar* software (Doležel 1991), considering the coefficient of variation of chromosome peaks 2 % and relative chromosome lengths as reported by Navrátilová *et al*. (2003). The model predicted that four chromosome peaks would be discriminated on a flow karyotype: peaks representing chromosomes 6 and 5 and two composite peaks represented pairs of chromosomes 1 and 3, and 2 and 4 (Fig. 2). The experimental flow karyotype that was obtained after the analysis of DAPI-stained chromosomes was similar and consisted of four chromosome peaks (Fig. 3). Nevertheless, their spacing differed from the model. This discrepancy could be due to differences in AT/GC ratio among the chromosomes as DAPI binds preferentially to AT-rich regions of DNA molecule

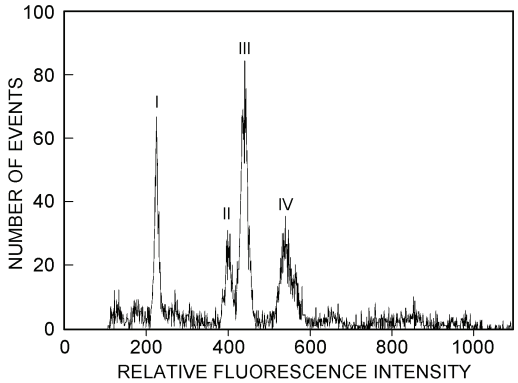

Fig. 3. Experimental flow karyotype of common vetch obtained after analysis of DAPI-stained chromosome suspensions prepared from synchronized root tips. The flow karyotype consists of four peaks. Peaks I and II were assigned to chromosomes 6 and 5, respectively. Composite peak III represent chromosomes 1 and 3, while composite peak IV represent chromosomes 2 and 4.

(Portugal and Waring 1988). However, our results obtained with bivariate flow karyotyping do not indicate significant differences in AT/GC ratio. Other possibility is that the ratio DAPI fluorescence / chromosome length is not the same for all chromosomes within the karyotype. This could be due to differences in chromatin condensation, which may influence the accessibility of DNA to DAPI. Similar discrepancies between theoretical and experimental flow karyotypes were observed in other species (Vláčilová *et al*. 2002, Kubaláková *et al*. 2003).

Particular chromosomes were assigned to individual peaks on the flow karyotype after flow sorting on a microscope slide and FISH with the VicTR-B repeat (Navrátilová *et al*. 2003, Fig. 4). Peak I was found to represent the smallest chromosome 6 (subacrocentric). The chromosome could be sorted with 94 % purity. Peak II, which was localized close to the composite peak III, represented the second smallest chromosome 5 (subacrocentric). This chromosome could be sorted with 90 % purity. Chromosomes 1 (metacentric) and 3 (subacrocentric) were assigned to the composite peak III. Fractions obtained after sorting this peak consisted almost entirely of the two chromosomes that could be sorted in a very high purity (99 %). The composite peak IV represented chromosomes 2 (subacrocentric) and 4 (acrocentric). Also these two chromosomes could be sorted with a very high purity (99 %).

Inability to discriminate the two pairs of chromosomes stimulated us to test bivariate flow karyotyping, which is based on simultaneous measurement of fluorescence emitted from a pair of dyes binding preferentially to AT or GC rich regions (Gray *et al*. 1979). The decision was motivated by the observation of Navrátilová *et al*. (2003) who found that the common vetch chromosomes differed in the number of copies of the VicTR-B repeat which is rich in AT bases (Macas *et al.* 2000). A range of combinations of DAPI and mithramycin concentrations was tested. Unfortunately, bivariate flow karyotypes thus obtained suffered from poor resolution, and were not helpful in discriminating particular chromosomes (data not shown). Bivariate analysis is instrumental in human and animal flow cytogenetics, where it facilitates chromosome sorting (Ibrahim and Van den Engh 2004). On the other hand, this approach seems not helpful in plants (Arumuganathan *et al*. 1991, Conia *et al*. 1987, Lucretti and Doležel 1997), most probably, due to small difference in overall AT/GC ratios between the chromosomes. The problem with discrimination of particular plant chromosomes can be overcome using chromosome translocation and deletion stocks (Doležel *et al*. 2004). Until now, these are not available for common vetch.

In conclusion, this work expands the range of legume species for which flow cytogenetics has been developed. The ability to sort particular chromosomes and chromosome pairs at very high purity should facilitate targeted genome analysis and mapping in common vetch. Flow sorting is an expensive and not widely available

method. However, the protocol for preparation of suspensions of intact chromosomes can be established in any laboratory and used to prepare slides with chromosomes free of cytoplasmic remnants and cell walls. Such preparations facilitate high-resolution cytogenetic mapping and analysis of long-range molecular chromosome structure using FISH and PRINS.

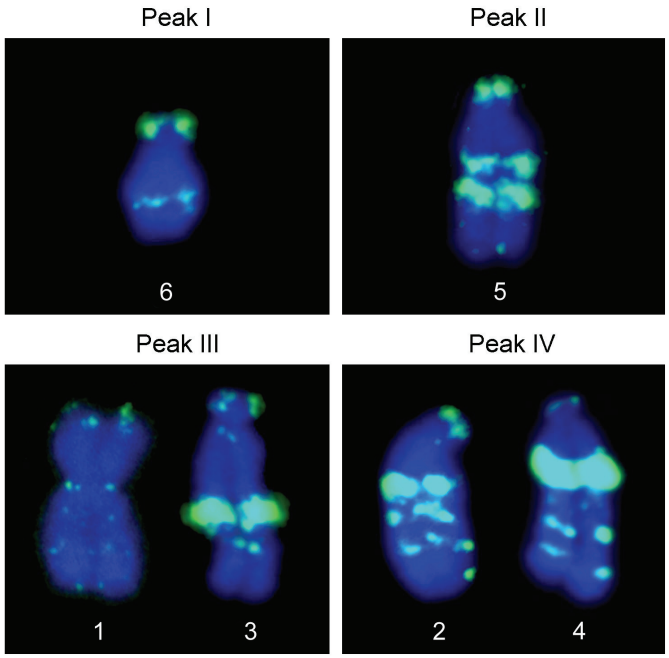

Fig. 4. Assignment of particular chromosomes to the four peaks on flow karyotype of common vetch. The chromosomes were identified after sorting onto microscope slide and FISH with a probe for VicTR-B repeat (*green*). Chromosomes were counterstained using DAPI (*blue*).

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