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Isolation of chromosomes from *Picea abies* and their analysis by flow cytometry

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Abstract

A high-yield method for preparation of suspensions of intact Norway spruce [*Picea abies* (L.) Karst.] chromosomes was developed for the first time. To accumulate meristem root tip cells at metaphase, actively growing roots were subjected to subsequent treatments with 0.625 mM hydroxyurea for 18 h and after 8 h recovery in distilled water with 0.05 % (m/v) colchicine for 8 h. These treatments resulted in 50 % metaphase indices. Synchronized root tips were fixed in 2 % formaldehyde for 10 min and chromosomes were released into a lysis buffer by mechanical homogenisation, producing 5×10^5 chromosomes from 50 root tips, at average. The isolated chromosomes were morphologically intact and suitable for flow cytometric analysis. Flow karyotypes obtained after the analysis of DAPI-stained chromosomes indicated a possibility to sort at least three different chromosome types.

Additional key words: chromosome sorting, chromosome suspensions, flow karyotype, Norway spruce.

Introduction

Norway spruce [*Picea abies* (L.) Karst.], 2n = 2x = 24) belonging to the family *Pinaceae*, is one of the most important conifers in Europe. Unfortunately, recent forest decline caused by pollution concerns mainly this species and emphasise a necessity to study genetic background of the response to biotic and abiotic stresses. The urgent need for genetic improvement contrasts with a poor knowledge of the genome.

Originally, genetic studies in *P. abies* were performed with morphological markers and isoenzymes (Geburek and Wuehlisch 1989, Lagercrantz and Ryman 1990). The first genetic linkage map of *P. abies* was constructed by Binelli and Bucci (1994) using a panel of 72 haploid megagametophytes. The authors employed randomly amplified polymorphic DNA (RAPD) markers, which were mapped to 26 linkage groups. A partial linkage map based on RAPD markers was also obtained by Skov and Wellendorf (1998). The most recent and most complex map of *P. abies* was constructed by Paglia *et al.* (1998). In total, 413 molecular markers were mapped in 29 linkage groups. In addition to suffering from low density, all genetic maps had far more than the expected 12 chromosomal linkage groups.

Genome mapping of *P. abies* genome is complicated by the biology of the species which has a long generation time and which is highly outbreed. Further problems rise from the complexity of the nuclear genome (2C = 37.2 pg, Siljak-Yakovlev *et al*. 2002), which is about 5-times larger than the human genome. One way to simplify its analysis is fractionation into individual chromosomes. Methods for flow cytometric analysis and sorting of mitotic chromosomes (flow cytogenetics) have been developed for a number of species, (for recent review see Doležel *et al.* 2001). Flow-sorted plant chromosomes have been shown useful for physical mapping (Macas *et al.* 1993, Neumann *et al*. 2002), and isolation of

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Abbreviations: HU - hydroxyurea, COL - colchicine.
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chromosome-specific probes (Arumuganathan *et al*. 1994, Požárková *et al*. 2002). Using DNA of flow-sorted chromosomes, Macas *et al.* (1996) constructed a set of chromosome-specific DNA libraries covering the whole genome of broad bean.

Here we report on the development of flow cytogenetics for Norway spruce. A high-yield procedure has been developed for preparation of suspensions of intact chromosomes from formaldehyde-fixed root tips. Isolated chromosomes were shown to be suitable for flow cytometric analysis and sorting.

Materials and methods

Plants: Seeds of Norway spruce [*Picea abies* (L.) Karst.] (2n = 24) were obtained from Lesy České republiky (Forests of the Czech Republic), seed station Týniště nad Orlicí. Seeds were imbibed in distilled water for 24 h at room temperature and germinated on filter paper soaked with distilled water at 25 ± 0.5 °C. All incubations were performed in the dark at 25 ± 0.5 °C, and all solutions were aerated. After 6 - 8 d when the length of primary roots reached 25 - 30 mm the seedlings were used for cell cycle synchronization.

Cell cycle synchronization and accumulation of metaphases: Seedlings were transferred to an open mesh basket positioned on a plastic tray filled with hydroxyurea (HU) at various concentrations (0.625, 1.25 and 2.5 mM) and incubated for 18 h. Then the roots were washed vigorously in distilled water and transferred to a tray filled with distilled water. Samples of root tips were taken at 2 h intervals up to 16 h for analysis of mitotic activity. 2 - 12 h after HU removal seedlings were transferred to 0.05 % (m/v) colchicine (COL) in distilled water to accumulate cells at metaphase. 0.05 % (m/v) colchicine solution was found to be optimal for metaphase arrest in preliminary experiments. Samples of root tips were taken at 2-h intervals from 0 to 20 h after the COL addition to evaluate the frequency of metaphases. Microscopic squash preparations were made to establish mitotic activity and metaphase frequency. Samples of root tips were fixed overnight in ethanol:acetic acid (3:1) and then prepared according to the Feulgen procedure. One thousand cells per slide and five preparations per variant were analysed. Each experiment was repeated three times.

Preparation of chromosome suspension: Immediately after metaphase accumulation, the roots were cut 1 cm from the root tip, rinsed in distilled water and fixed for periods from 10 to 30 min at 5 min steps at 5 °C in formaldehyde solutions (1, 2, 3 or 4 % v/v) prepared in Tris buffer (10 mM Tris, 10 mM Na_2EDTA, 100 mM NaCl, pH 7.5) supplemented with 0.1 % Triton X-100. After three 5 min washes in Tris buffer, the meristem tips (1 mm) of 50 roots were cut and transferred to a 5 cm^3 polystyrene tube containing 1 cm^3 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 chromosomes were released by mechanical homogenisation with an *Ultra Turrax* homogenizer (Janke & Kunkel, Staufen, Germany) at 9 500 rpm for 60 s. The suspension was passed through a 50-µm pore size nylon mesh to remove large tissue and cellular fragments. In order to check chromosome yield and morphology, 0.01 cm^3 of chromosome suspension were dried on microscope slides. After mounting in LB01 buffer containing 0.2 µg cm^{-3} 4',6-diamino-2-phenylindole (DAPI), the slides were analysed using an *Olympus AX 70* (Tokyo, Japan) microscope equipped with a filter block for DAPI.

Theoretical flow karyotype: Prior to flow cytometric analysis, theoretical flow karyotype was modelled with the *KARYOSTAR* software (Doležel 1991). The relative chromosome lengths were taken from Köhler *et al*. (1996) and Siljak-Yakovlev *et al*. (2002) and the models were calculated considering 2.5 % coefficient of variation.

Chromosome analysis using flow cytometry: Chromosomes in suspension were stained using DAPI at a final concentration 2 µg cm^{-3} and analysed at rates of 300 - 500 s^{-1} on a *FACSVantage* flow cytometer (*Becton Dickinson*, San José, USA). The instrument was equipped with an argon-ion laser tuned to multiline UV (351.1 - 363.8 nm) and run with a 300 mW output power. Fluorescence of DAPI-stained chromosomes was collected through a 424/44 band-pass filter using linear amplification. Sheath fluid contained 40 mM KCl and 10 mM NaCl, and the instrument was used with a ceramic nozzle tip of 70 µm. Distributions of relative fluorescence intensity were acquired on histograms of FL1 pulse area (FL1-A, linear scale, 1024 channels). For chromosome sorting, gates were set on a dot plot of FL1 pulse area (FL1-A) versus FL1 pulse width (FL1-W). A "counter" sorting mode was used and chromosomes were sorted at a rate of approximately 5 - 10 per second. For microscopic evaluation, 2000 chromosomes were sorted into a 0.015 cm^3 drop of PRINS buffer containing 5 % sucrose on microscope slides (Kubaláková *et al*. 2000). The slides were air dried after sorting, and maintained at room temperature until use.

Results

Cell cycle synchronisation and accumulation of metaphases: Firstly effect of hydroxyurea concentration on mitotic activity was investigated. Treatment of Norway spruce root tips with 0.625, 1.25 and 2.5 mM HU for 18 resulted in mitotic indexes 24.8, 23.6 and 19.8 % reached 10, 14 and 16 h after HU removal, respectively (Fig. 1). After the treatment with 0.625 mM HU, the mitotic activity recovered first and the highest mitotic index was reached. Cells finish reversibile treatment of hydroxyurea after 10-h recovery and cell population becomes asynchronous.

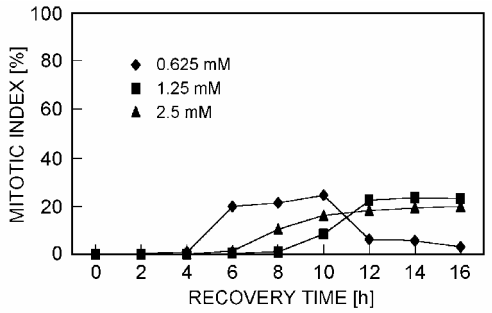

Fig. 1. Mitotic activity in root tips of Norway spruce during recovery after 18-h hydroxyurea (HU) treatment. Mitotic indices were determined on Feulgen stained squash preparations made from root tips fixed overnight in ethanol:acetic acid (3:1). Samples were taken at 2-h intervals after HU removal.

Based on these results 0.625 mM HU was selected for the following experiments. Colchicine (0.05 %, m/v) was used for metaphase accumulation and was added 2, 4, 6, 8, 10 and 12 h after the recovery from HU block. The samples were taken regularly in 2-h intervals till 20 h during the COL treatment. Addition of COL 2, 4, 6, 8, 10 and 12 h after HU removal resulted in maximal metaphase frequency 8.0, 28.2, 48.3, 50.4, 32.0, and 34.2 % reached after 12, 8, 10, 8, 10, and 6 h of COL treatment, respectively (Fig. 2). Thus the optimal procedure for metaphase accumulation was 18 h of treatment with 0.625 mM HU, 8 h recovery in distilled water followed by 8 h treatment with 0.05 % (m/v) COL. This procedure resulted in metaphase frequency 50.4 %.

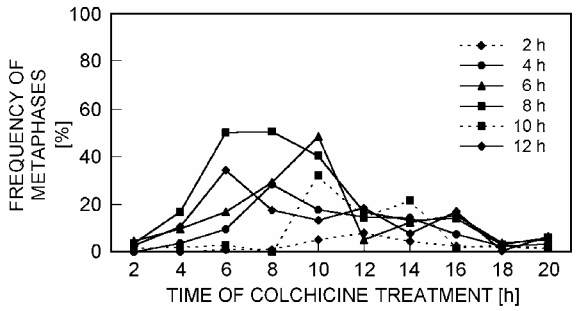

Fig. 2. Metaphase frequency observed in root tip meristems of Norway spruce during treatment with 0.05 % (m/v) colchicine added 2 - 12 h after hydroxurea (0.625 mM for 18 h) removal. Feulgen-stained squash preparations were made from root tips taken in 2-h intervals.

Preparation of chromosome suspensions: The morphology and quantity of chromosomes released from synchronised root tips depended on the extent of fixation with formaldehyde. The weaker the fixation, the higher the number of chromosomes isolated. However, the chromosomes isolated from root tips fixed weakly (1 % formaldehyde for 10 - 15 min) were damaged and chromosome suspensions contained large amounts of chromosomal and nuclear debris. On the other hand, suspensions obtained after homogenisation of strongly fixed root tips (2 - 4 % formaldehyde for 30 min) contained an increased number of chromosome clumps and intact cells. 10 min fixation in 2 % (v/v) formal-

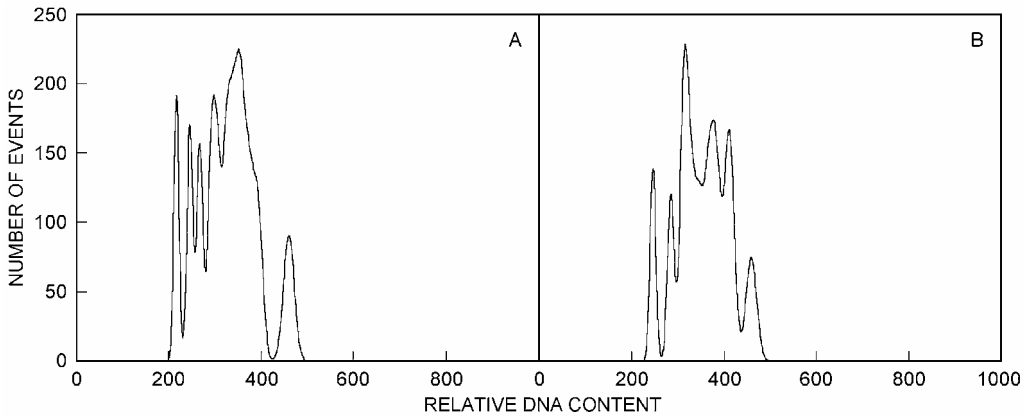

Fig. 3. Theoretical flow karyotypes of Norway spruce. Relative chromosome lengths determined by Köhler *et al*. (1996) (*A*) and Siljak-Yakovlev *et al*. (2002) (*B*) were used for modelling with the *KARYOSTAR* software (Doležel 1991). Models calculated on the base of a coefficient of variation 2.5 % predicted possibility to sort 4 and 3 chromosome types, respectively.

dehyde proved to be optimum producing more than 5×10^5 chromosomes with well-preserved morphology from 50 root tips.

Chromosome analysis and sorting: Theoretical flow karyotypes were modelled considering relative chromosome lengths reported by Köhler *et al*. (1996) and Siljak-Yakovlev *et al*. (2002) and coefficient of variation of 2.5 % (Fig. 3). Both models predicted that two chromosome 1 and 12 should be well discriminated. In addition, chromosomes 10 and 11 and chromosome 11 should be partially discriminated in Köhler's and Siljak-Yakovlev's model, respectively. All other chromosomes were expected to form a composite peak. Flow karyotype obtained after the analysis of DAPI-stained chromosomes (Fig. 4) was similar to the theoretical model calculated on basis of chromosome lengths given by Köhler *et al*. (1996). However, chromosome peak positions differed slightly, which enabled discrimination of higher number of chromosomes. Thus, in addition to chromosomes 1 and 12, also peak corresponding to chromosome 9 could be resolved. Chromosomes 10 and 11 could be discriminated only partially, while chromosomes 2 - 8 formed a composite peak as predicted. In addition, minor peaks representing chromatids, chromosome fragments and debris were observed. However, the frequency of these particles was low confirming high quality of the chromosome suspension. Microscopic evaluation of chromosome fractions sorted from peaks representing chromosomes 1, 9, and 12 indicated the presence of single chromosome type in each fraction.

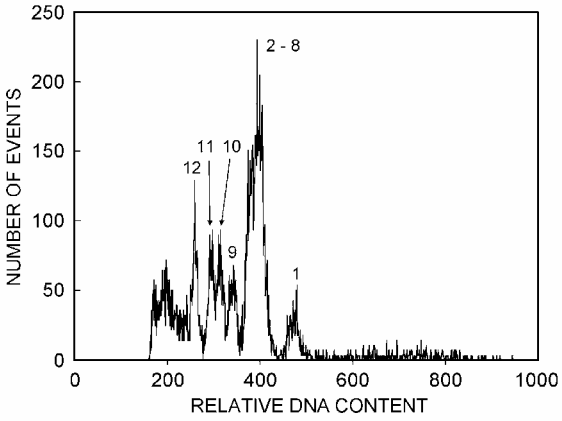

Fig. 4. Flow karyotype of Norway spruce obtained after analysis of DAPI-stained chromosomes. Karyotype contains 5 peaks representing individual chromosome types (1, 9, 10, 11, 12) and one dominant composite peak representing chromosomes 2 - 8.

Discussion

The procedure for preparation chromosome suspensions in Norway spruce is a modification of a procedure, which was originally developed for *Vicia faba* root tip cells (Doležel *et al.* 1992). The use of roots offers important advantages over other systems used to isolate mitotic chromosomes. Seedlings are easy to handle, root meristems are karyologically stable and can be synchronized to obtain a high frequency of metaphase cells (Doležel *et al.* 1999). The degree of the synchrony in spruce root tips achieved after the treatment with a DNA synthesis inhibitor hydroxyurea (approximately 50 % cells in mitosis) is comparable to that of other reports on cell cycle synchronization in root tips.

Norway spruce chromosomes were released from synchronized root tips after a mild formaldehyde fixation. Similarly to other species (Doležel *et al.* 1999), the extent of the fixation determined critically the yield and morphology of isolated chromosomes. The fixation made chromosomes resistant to mechanical shearing forces and thus they could be released from root tissues by a rapid mechanical homogenisation. Plant chromosomes isolated after formaldehyde fixation were shown to be suitable for scanning electron microscopy (Schubert *et al.* 1993), for localisation of DNA sequences using *in situ* hybridization (Fuchs *et al.* 1994), and PRINS (Kubaláková *et al.* 1997), and for immunolocalisation of chromosomal proteins (Binarová *et al.* 1998). Thus the procedure described here might allow similar studies also in spruce.

Flow cytometric analysis of chromosome suspensions confirmed the high quality of isolated chromosomes. To obtain high-resolution flow karyotypes, the chromosomes were stained by DAPI that requires UV excitation. The use of DAPI was critical as the resolution of flow karyotypes obtained after analysis of formaldehyde fixed chromosomes stained by DNA intercalators (*e.g.* propidium iodide) and/or fluorescent antibiotics (*e.g.* mithramycin) show limited resolution (Lucretti and Doležel 1995). Flow karyotype of Norway spruce obtained for the first time in this study agreed fairly well with theoretical model calculated according to the chromosome lengths determined by Köhler *et al*. (1996). Small differences in chromosome peak positions could be due to non-random distribution of AT clusters, which are preferentially stained by DAPI (Portugal and Waring 1988). At least three different chromosome type could be discriminated and sorted. The result of the study will enable using flow cytogenetics to simplify the analysis of the complex genome of Norway spruce.

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