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Flow-cytometric characterization and sorting of plant chromosomes

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Summary. Flow cytometric measurements of DNA frequency distribution were used to follow the synchronization process in suspension cells from *Haplopappus gracilis* (2n = 4). Metaphase chromosomes were isolated from these synchronized cells and both the acro- and metacentric chromosomes were sorted by flow cytometry based on the different DNA contents. Possible applications of this procedure in fundamental genetics as well as practical plant breeding are discussed.

Key words: Chromosome isolation – Flow cytometry – *Haplopappus gracilis* (Nutt.) Gray – Synchronization

Introduction

Genetic manipulation may offer a valuable addition to conventional plant breeding methods. A main advantage is the potential to transfer parts of genomes containing only one or a few desirable genetic attributes to cells of a productive crop. Moreover, sexual incompatibility barriers are avoided. Transfer of genetic material may be accomplished in various ways. The transgenome may consist of a whole genome (cell hybridization), of intact chromosomes or parts of chromosomes (chromosome transplantation) or of DNA fragments in the kilobase range (monofactorial transformation).

Because of its high specificity, attention is mainly focussed on monofactorial transformation. However, the practical application of the method is largely limited by the lack of suitable procedures for identification of the gene(s) involved in most important plant characteristics. Moreover, the regulation of eukaryotic gene expression, especially in connection with cellular differentiation, is barely understood so far.

Chromosome transplantation may be a powerful technique in mapping of mono- and polygenic plant characteristics and in the identification of plant genes. In addition, chromosome-mediated gene transfer may serve as a transformation technique as such. The fate of a transferred chromosome is unknown but since aneuploidy occurs frequently in plants such chromosomes may be expected to behave autonomously.

Since the first isolation of mammalian chromosomes (Chorazy et al. 1963), considerable progress has been made. Metaphase chromosomes have been sorted by e.g. flow cytometry based on differences in DNA content or base constitution (Carrano et al. 1979). Chromosome-mediated gene transfer has also been achieved (Klobutcher and Ruddle 1981) and chromosome-specific gene libraries have been constructed (Davies et al. 1981).

Recently chromosomes have been isolated from several plant species (Malmberg and Griesbach 1980; Szabados et al. 1981; Griesbach et al. 1982; Hadlaczky et al. 1983).

Our study is focussed on the analysis of the synchronization process and on the purification and sorting of plant metaphase chromosomes by flow cytometry. Cell suspensions of *Haplopappus gracilis* were used as a model system since a) their synchronization has been well established (Eriksson 1966), b) the chromosome number is low (2n = 4) and c) the acro- and metacentric chromosomes are easy to distinguish morphologically and differ considerably in length (Jackson 1957).

Materials and methods

Plant material and growth conditions

Suspension cells of *Haplopappus gracilis* (Nutt.) Gray were initiated and maintained as described in detail by Werry and

Stoffelsen (1978). The synchronization procedure of Eriksson (1966) was adopted with some minor modifications. One day after subculturing, DNA synthesis was inhibited by 5 mM hydroxyurea (HU) for 18 or 24 h. The cells were subsequently washed thoroughly and exposed for 10 h to 0.05% colchicine for accumulation of mitotic cells. At various times during synchronization lactopropionic orcein-stained preparations were made (Dyer 1963) to determine the mitotic indices.

Frequency distributions of the DNA content of these cells were obtained by making protoplasts from log-phase, HU-treated and colchicine-treated cells respectively, according to Puite and Ten Broeke (1983). Cells were then incubated in 5% driselase (Kyowa Makko Kogyo Co, Tokyo, Japan) and 0.5% pectolyase (Seishia Pharmaceutical, Chiba-ken, Japan) in 0.7 M mannitol (pH 5.6) for 3 h at 28 °C in darkness. Protoplasts were washed and treated with 0.1% Triton X-100 and 50 μg ml^{-1} ethidium bromide (EtBr, a fluorescent DNA stain) in 0.5 M mannitol and used for flow cytometry directly.

Chromosome isolation

During the synchronization of the cell suspensions, the temperature was changed from 28 °C to 12 °C at 6 h after the addition of colchicine. Eight hours later, cells were exposed to 5% driselase, 0.5% pectolyase in 3 mM MES, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 370 mM mannitol and 250 mM glucose (pH 5.6) (Hadlaczky et al. 1983) for 2 h. All following steps were carried out on ice. After washing and swelling (300 mM mannitol; 10 min) the nascent protoplasts were lysed in a solution containing 15 mM Hepes, 1 mM EDTA, 15 mM DTT, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 300 mM sucrose, 50 μg ml^{-1} (EtBr) and 1% Triton X-100 (pH 7.0) (Griesbach et al. 1982) and passed twice through a 25G hypodermic needle. Crude samples, thus obtained, were used for flow-cytometric analysis and sorting of the chromosomes.

Flow cytometry and sorting

Flow cytometric measurements and sorting experiments were performed with a FACS-IV Cell Sorter (Becton Dickinson, Sunnyvale, USA) equipped with a Spectra Physics argon ion laser, model 164-05, operated at 0.3 W/488 nm with a LP-620 filter in the emission beam (Donner et al. 1972). The fluorescence signal from the EtBr-stained preparations is considered to be proportional to the DNA content of the passing protoplasts, nuclei or chromosomes. The flow rate was approximately 1,000 particles per second. The DNA measurements gave rise to frequency distribution histograms with several peaks representing particles with the same DNA content. About 20,000 particles from each peak were sorted out into an ice-cooled tube containing 50% ethanol. Sorted fractions were kept on ice.

Flurescence microscopy

For identification of the sorted fractions the preparations were restained with EtBr (50 μg ml^{-1}), concentrated on 0.2 μm teflon millipore filters (Sartorius) and embedded in 50% glycerin. Fluorescence microscopy involved the use of a UG-2 exciter filter, a FL-500 dichroic mirror and a LP-580 emission filter.

Results and discussion

In order to isolate chromosomes from plant cells it is essential to dispose of large numbers of mitotic cells. Therefore, log-phase cells from *Haplopappus gracilis* were synchronized by incubation in HU and subsequently in colchicine (Eriksson 1966). In Fig. 1 the time course of the mitotic index (MI) during the synchronization of the suspension cells is demonstrated. During the HU treatment MI decreases from 4% in the log-phase control to less than 1%. From 6 h after washing onwards, MI rapidly increases. Mitotic indices of about 25% were routinely obtained (Fig. 2).

The synchronization process was also studied by flow cytometry by measuring the frequency distributions of the DNA content of burst protoplasts after various incubation periods (Fig. 3). In log-phase suspensions, cells were mainly in G_0/G_1 phase (approx. 80%) whereas only a few cells were found in S (5%) and G_2/M phase (15%) (Fig. 3 A). However, as expected, after incubation in hydroxy urea and colchicine, cells were accumulated in the S phase (Fig. 3 B) and G2/M phase (Fig. 3 C) respectively.

Since the cell wall is a main barrier for the isolation of plant chromosomes, protoplasts were prepared by treatment with cell wall-digesting enzymes in 0.7 M mannitol. However, during protoplast preparation from mitotic cells at 28 °C, we observed a rapid despiralization of the metaphase chromosomes. The chromosomes were largely protected from decondensation by changing the temperature from 28 °C to 12 °C and by using a special buffer solution (Hadlaczky et al. 1983) during a 2 h cell wall digestion. Although cell walls were not completely digested, they were sufficiently weakened to allow mechanical breakage in order to obtain the chromosomes.

The lysis buffer was composed as described by Griesbach et al. (1982) and completed with 50 μg ml^{-1} ethidium bromide (EtBr) and 1% Triton X-100. The intercalator EtBr acted not only as a fluorescent DNA-stain but also as a DNA stabilizing factor (J. Aten, personal communication). Moreover, EtBr can be

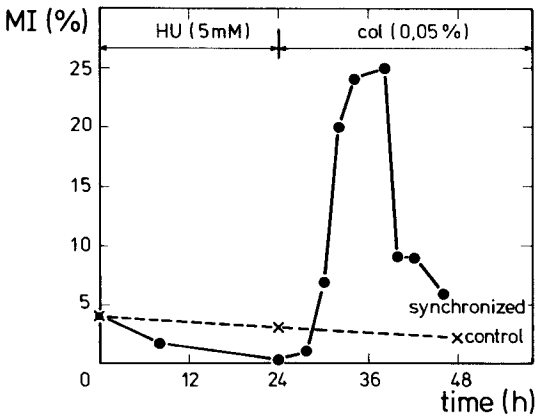

Fig. 1. Time course of the mitotic index (MI) of *Haplopappus gracilis* suspension cells during synchronization

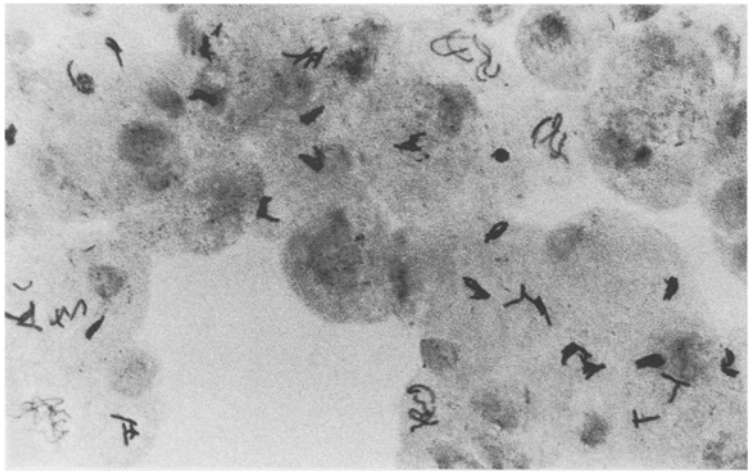

Fig. 2. Lactopropionic orcein-stained squash preparation of synchronized *Haplopappus* cells

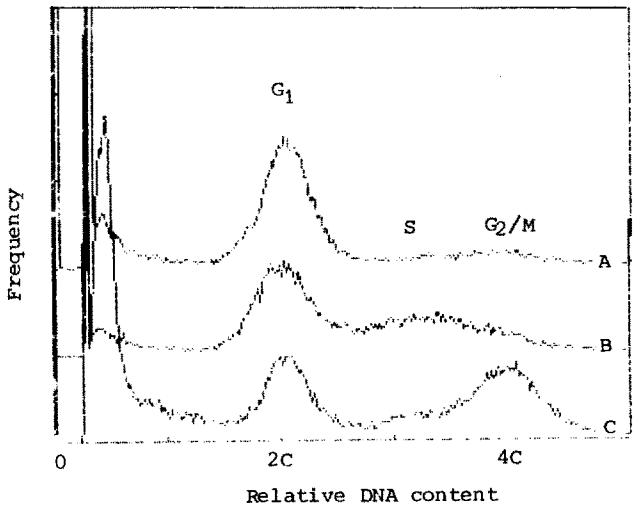

Fig. 3. Frequency distribution of the relative DNA content of protoplasts prepared from log-phase (*A*), HU-treated (*B*) and colchicine-treated (*C*) suspension cells

washed out, probably without affecting the biological activity of DNA. The presence of Triton X-100 (1%) greatly prevented the cell components from aggregation.

The flow cytometric analysis of crude protoplast extracts yielded 8 peaks (Fig. 4 A–H) which were identified by flow sorting and fluorescence microscopy as debris (A) acrocentric chromosomes (B + C), metacentric chromosomes (D + E), G_1 nuclei (F), metaphase nuclei (aggregates of 4 chromosomes) (G) and G_2 nuclei (H). The fluorescence intensity of metaphase nuclei (G), is significantly lower than that of G_2 interphase nuclei (H) containing the same amount of DNA. This is probably a consequence of DNA condensation preventing EtBr to intercalate proportionally or leading to an increased quenching.

The coefficient of variations (CV) for the peaks containing chromosomes and nuclei routinely did not exceed the 4% and 9% level respectively.

Both types of chromosomes were sorted at a rate of 5×10^3 chromosomes min^{-1} (Fig. 5). Contamination of the acrocentric chromosome fraction (B + C) with metacentric chromosomes (D + E) or vice versa did not exceed the 5% level. Both the acrocentric and metacentric chromosome fractions consisted of double peaks, each containing equal amounts of chromosomes. The nature of these double peaks was investigated. For sperm cells, the effects of orientation during passage of the laser beam have already been described as resulting in double peaks (Gledhill et al. 1976). However, since

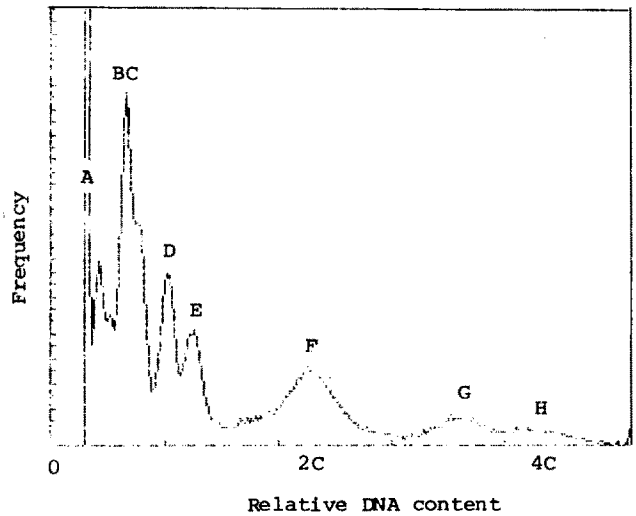

Fig. 4. Frequency distribution of the relative DNA content of EtBr-stained chromosomes and nuclei from mechanically disrupted protoplasts. By flow sorting, peaks were identified as debris (*A*), acrocentric (*B/C*) and metacentric (*D/E*) metaphase chromosomes, G_1 nuclei (*F*), mitotic nuclei (*G*) and G_2 nuclei (*H*)

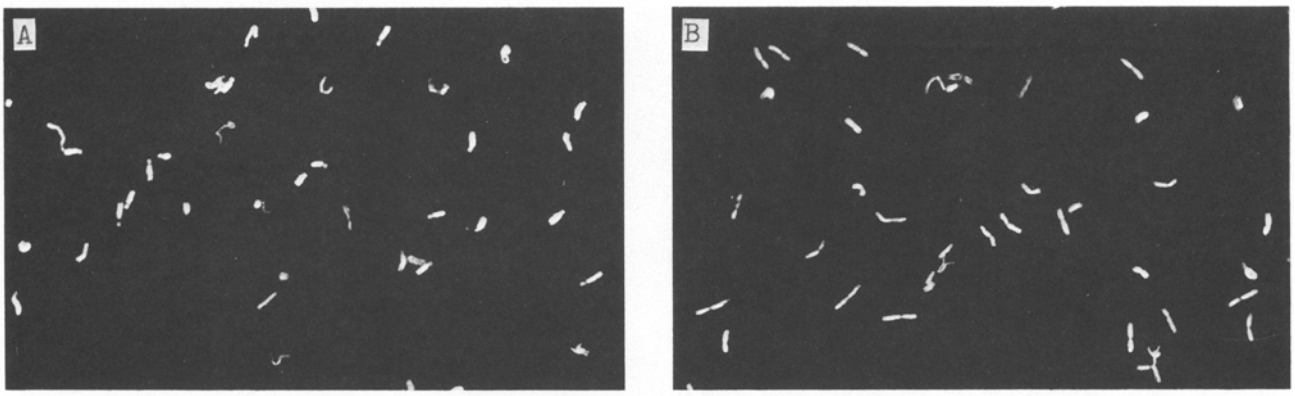

Fig. 5. Acrocentric (**A**) and metacentric (**B**) *Haplopappus* metaphase chromosomes as sorted by flow cytometry

the re-analysis of fraction D and E exhibited only one peak each, the involvement of such reversible chromosome orientation could be excluded. If the double peaks reflect differences in fluorescence between the homologous chromosomes, these differences may result either from differences in DNA base constitution or DNA content. The former, however, is most unlikely since the intercalator, EtBr, is not base specific. To ascertain whether differences in DNA content of both homologues are involved in causing the double peaks, chromosome lengths were measured in synchronized suspension cells. Surprisingly, significant differences were found between the two homologues, the relative mean chromosome-lengths being 100 and 120 for the acrocentric and 155 and 181 for the metacentric chromosomes. This is in good agreement with the relative DNA contents as measured by flow cytometry: 100 and 116 for the acrocentric and 155 and 187 for the metacentric chromosomes. Thus, the differences between homologous chromosomes already existed in our cell suspension and did not result from possible damage during the isolation procedure. Since our cell suspension has been subcultured weekly for at least five years, the observed chromosome dimorphism might have resulted from genetic instability under some selection pressure (D'Amato 1975).

Conclusions

The synchronization process of *Haplopappus* suspension cells, routinely resulting in mitotic indices of about 25%, could be followed easily by flow cytometry. Since DNA histograms can be measured very rapidly, flow cytometry herewith proves its potency in cell cycle analyses, but also in studying genetic instability (determination of ploidy level, aneuploidy and karyotype alterations).

Sorting of plant chromosomes has not been described before. Since the CV for the chromosome peaks is about 4%, the procedure described here enables us to sort chromosomes differing in DNA content by about 10% at a rather useful rate (about 5×10^3 chromosomes per min) apparently without damaging the chromosomes. This provides the opportunity for transplantation of particular chromosomes via fusion techniques (Szabados et al. 1981; Griesbach et al. 1982) or by microinjection (Steinbiss 1983). This would create an important tool for plant breeding as well as gene mapping. Furthermore, chromosome-specific gene libraries may be constructed as has already been done in mammalian cells (Davies et al. 1981).

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