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Preparation of HMW DNA from plant nuclei and chromosomes isolated from root tips

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Abstract

Simple, fast and cost-effective method for preparation of DNA with high molecular weight (HMW DNA) from plant nuclei and mitotic chromosomes has been developed. The technique involves mechanical homogenization of formaldehyde-fixed root tips, purification of nuclei and/or chromosomes on sucrose gradient, embedding in low-melting-point agarose, and DNA isolation in agarose plugs. Alternatively, nuclei and chromosomes may be purified using flow cytometry. Majority of DNA obtained is megabase-sized and well digestible by restriction endonucleases. The method is highly efficient as microgram amounts of DNA can be obtained from only several milligrams of plant tissue. Handling negligible amounts of plant material reduces the consumption of chemicals. Furthermore, the use of root tips makes it possible to obtain high-quality DNA even from plant species with leaves that are rigid or rich in secondary metabolites such as polyphenols. It is expected that preparation of HMW DNA from root tip nuclei will facilitate long-range mapping and construction of large-insert DNA libraries also in these species. Successful isolation of HMW DNA from flow-sorted chromosomes opens a way for construction of chromosome-specific large-insert libraries in plants.

Additional key words: DNA isolation, flow cytometry, large-insert DNA library, pulsed field gel electrophoresis, sucrose gradient.

Introduction

Preparation of HMW DNA is a prerequisite of long-range mapping and construction of large-insert DNA libraries, such as bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) libraries. Such libraries are invaluable for genome analysis, including physical mapping and gene isolation. Further increase in the efficiency of the analysis of large plant genomes could be facilitated by chromosome-specific large-insert libraries. However, the construction of such libraries requires protocols for preparation of sufficient quantities of HMW DNA from mitotic chromosomes.

Current protocols for preparation of HMW DNA are based on the use of protoplasts, nuclei or powdered leaf tissue. The protoplast-based procedures (Ganal and Tanksley 1989), despite of high quality of the DNA obtained, are becoming less popular as they are expensive, time-consuming and require expertise in protoplast handling. Protocols using powdered leaf tissue (Guidet *et al.* 1990) are simple but the samples are, similarly as in protoplast-based procedures, contaminated by plastid and mitochondrial DNA. Nuclei-based methods overcome this problem. They are cheaper, simpler and faster compared to protoplast-based procedures. Several modifications and improvements of the nuclei-based methods were described (Espinás and Carballo 1993, Zhang *et al.* 1995, Liu and Wu 1999). However, all of them have some limitations. First of all, large quantities of plant (usually leaf) tissues are needed

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Abbreviations: BAC - bacterial artificial chromosome; DAPI - 4',6-diamidino-2-phenylindole; HMW DNA - high molecular weight DNA (special term, in this journal regularly mass instead of weight is used according to SI); LMP - low-melting-point; PFGE - pulsed field gel electrophoresis; PMSF - phenylmethylsulfonyl fluoride; YAC - yeast artificial chromosome.

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(typically 10 - 100 g). In some species, the use of this approach is compromised by the presence of secondary metabolites such as polyphenols, which significantly reduce the yield and quality of DNA. In plants with rigid leaf tissues, it is difficult to isolate intact nuclei in sufficient quantities.

Previously, we have developed a rapid and high-yield method to prepare suspensions of intact nuclei and chromosomes from root tip meristems (Doležel *et al.* 1992). The procedure is based on mild formaldehyde fixation, after which intact nuclei and chromosomes are released into isolation buffer. With the aim to overcome the problem of nuclei-based methods that involve leaf tissues, we have modified our procedure for preparation of HMW DNA. Furthermore, we have also optimized a protocol for preparation of HMW DNA from mitotic chromosomes.

Materials and methods

Plants: Broad bean (*Vicia faba* L.), barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and banana (*Musa acuminata* Colla.) were used as model species to develop the protocol. Seeds of broad bean, barley and wheat were germinated in moistened, inert substrate and in petri dish with a moistened filter paper, respectively. Three-day-old seedlings were used for the experiment. In banana, roots were collected from healthy plants grown in a greenhouse.

Preparation of nuclei and chromosome suspensions: Nuclei were isolated from actively growing roots, while chromosomes were isolated from root tips after cell cycle synchronization and metaphase accumulation using a combined treatment with hydroxyurea and amiprophos-methyl (Doležel *et al.* 1992, 1999). Roots were cut 1 cm from a root tip, rinsed in distilled water and fixed for 20 min at 5 °C in 2 % formaldehyde made in Tris buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 7.5). After three 5-min washes in Tris buffer, the meristem tips (0.5 - 1 mm) of 50 roots were cut and transferred into a 5 cm^3 polystyrene tube containing 1 cm^3 of ice-cold isolation buffer IB of the following composition: 15 mM Tris, 10 mM EDTA, 130 mM KCl, 20 mM NaCl, 1 mM spermine, 1 mM spermidine, 15 mM β-mercaptoethanol, 0.1 % Triton X-100, pH 9.4. For banana, which is rich in polyphenols, the concentration of mercaptoethanol was increased to 45 mM. Suspensions of intact nuclei and chromosomes were prepared by mechanical homogenization with *Polytron PT1200* homogenizer (*Kinematica*, Littau, Switzerland) at 9500 rpm for 10 s. Crude suspension was passed through a nylon mesh (50-μm pores) to remove large tissue and cellular fragments.

Purification of nuclei and chromosomes: Nuclei and chromosomes were purified on a sucrose gradient consisting of 50, 30 and 10 % sucrose in IB. The samples were centrifuged at 35 *g* (barley, wheat) or 55 *g* (broad bean, banana) at 4 °C for 15 min. Chromosomes remained in the 10 % fraction whereas the nuclei were collected from the 50 % fraction (barley, broad bean) or 10 and 30 % fraction (banana). The concentration of nuclei or chromosomes was estimated under fluorescence microscope after staining with 2 μg cm^{-3} of 4',6-diamidino-2-phenylindole (DAPI). Samples collected from sucrose gradient were diluted by IB to reach final sucrose concentration of 4 %. Subsequently, nuclei or chromosomes were pelleted at 200 *g* and 4 °C for 25 min.

Alternatively, chromosomes and nuclei were purified using *FACSVantage* flow cytometer (*Becton Dickinson*, San José, USA) equipped with 5 W argon-ion laser (*Innova 305C, Coherent*, Santa Clara, USA). The laser was tuned to multiline UV and adjusted to 300 mW output power. Nuclei or chromosome suspensions were stained with DAPI at final concentration of 2 μg cm^{-3} and analyzed at rates of 200 - 300 particles per second. A "normal-R" sorting mode was used for sorting; sorting gates were set on a dot plot of fluorescence pulse area versus fluorescence pulse width. Nuclei were sorted at rates of 100 - 200 s^{-1} into an equal volume of ice-cold 1.5 × IB to reach final concentration of 0.75 × IB. Approximately 150 000 nuclei were sorted for one agarose plug. Chromosomes were sorted at rates 50 per second into 1.5 × IB; approx. 800 000 chromosomes were sorted for one plug. Nuclei or chromosomes were pelleted at 200 *g* and 4 °C for 25 min.

Preparation of agarose plugs and DNA isolation: Pelleted nuclei or chromosomes were resuspended in 0.04 - 0.24 cm^3 of IB (depending on their concentration in the sample and DNA content in nuclei/chromosomes of particular plant species), warmed to 50 °C and mixed with equal amount of pre-warmed 1.5 % low-melting-point (LMP) agarose made in IB. The mixture was poured into 0.08-cm^3 plug molds to form agarose plugs. The plugs were solidified on ice and incubated in 1 cm^{-3} per plug of lysis buffer (0.5 M EDTA, 1 % lauroyl-sarcosine, 0.1 mg cm^{-3} proteinase K; for banana 45 mM mercaptoethanol was included) at 37 °C for 2 d. For the first 24 h, buffer with pH 9.0 was used. Then it was changed for a fresh buffer with pH 8.0 and the plugs were incubated for another 24 h. After lysis, agarose plugs were rinsed in ET buffer (1 mM Tris, 50 mM EDTA, pH 8.0) and stored in ET buffer at 4 °C. Quality of DNA was checked using pulsed field gel electrophoresis (*CHEF-DR II system, BioRad,* Hercules, USA).

Digestion of DNA: To check the purity of HMW DNA, the samples of DNA from barley nuclei were digested with *Not*I, *Pst*I, *Eco*RI and *Bam*HI restriction enzymes (*MBI Fermentas*, Vilnius, Lithuania). Wheat chromosomal DNA was digested with *Hin*dIII. Agarose plugs were washed for six hours in six changes of ice-cold TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). In some experiments, TE buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was used for comparison. 0.04-cm^3 plugs containing about 1.5 μg of DNA were incubated in the appropriate restriction enzyme buffer supplemented with 2 mM spermidine for 1 h on ice. Subsequently, the buffer was changed for a fresh reaction buffer supplemented with 0.2 - 1 mg cm^{-3} BSA and 2 mM spermidine. Total volume of the reaction was 0.2 cm^3. The samples were incubated at 37 °C with 10 or 20 units of the respective enzyme for 3 or 6 h. DNAs of 800 000 flow-sorted wheat chromosomes (approx. 1.2 μg) in 0.08-cm^3 plugs were digested in 0.3 cm^3 reaction volume with 5 or 25 units of *Hin*dIII for 5 min or 2 h. Controls containing no enzyme were included for each reaction. The reactions were stopped by addition of 1/10 volume of 0.5 M EDTA. Quality of digested DNA was checked using pulsed field gel electrophoresis (PFGE).

Results and discussion

The method described here differs from previous methods by using formaldehyde-fixed tissues (root tips) to prepare nuclei and chromosome suspensions. Formaldehyde fixation significantly increases the yield of nuclei and chromosomes (Sgorbati *et al.* 1986, Doležel *et al.* 1992). Furthermore, it makes nuclei and chromosomes mechanically stable and resistant to shearing forces during mechanical isolation. Thus the suspensions prepared from fixed root tips consisted largely from intact nuclei and chromosomes. Surprisingly, the fixation step did not significantly inhibit the activity of nucleases. While DNA of acceptable quality was obtained in broad bean and banana after nuclei isolation in a standard LB01 buffer (Doležel *et al.* 1989), DNA prepared in barley was degraded due to high levels of nucleases (data not shown).

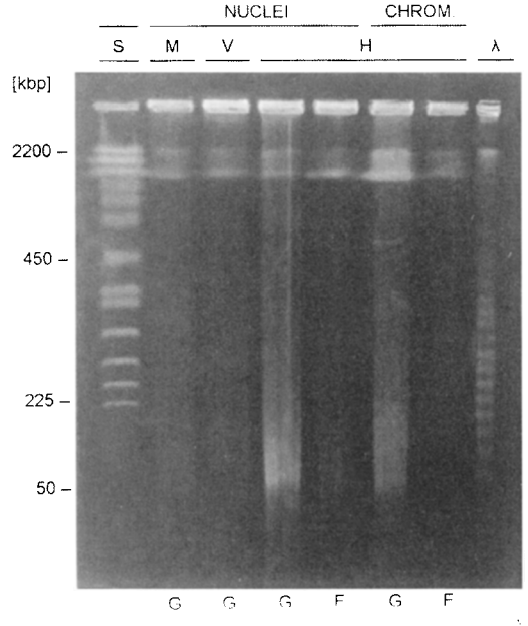

Fig. 1. Pulsed field gel electrophoresis of HMW DNA prepared from nuclei and chromosomes. Isolated nuclei and/or chromosomes of *Musa acuminata* (M), *Vicia faba* (V) and *Hordeum vulgare* (H) were purified on a sucrose gradient (G) or flow-sorted (F), embedded in LMP agarose, treated with proteinase K and electrophoresed. Majority of chromosomal and nuclear DNA remained in the sample wells and in the compression zone. The electrophoresis was run on a 1 % agarose gel in 0.5 × TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3), at 12.5 °C, 6 V cm^{-1} with a 50 s switch time for 18 h. Lane S - chromosomes of *Saccharomyces cerevisiae*, lane λ - lambda ladder used as size markers.

With the aim to reduce the activity of nucleases, we have optimized composition of the isolation buffer named IB, which is based on the original LB01 buffer (Doležel *et al.* 1989). Several approaches used by other authors to protect the DNA against nucleases (Espinás and Carballo 1993, Zhang *et al.* 1995, Liu and Wu 1999) were tested. High pH (9.4) and high EDTA content (10 mM) in isolation buffer (Zhang *et al.* 1995) proved to be essential to inactivate nucleases. Similarly, increased concentration of KCl (130 mM compared to 80 mM in LB01), which helped to protect maize DNA (Espinás and Carballo 1993), was effective also in barley. On the other hand, addition of lysine and EGTA (Liu and Wu 1999) did not result in significant improvement. Using the optimized protocol, HMW DNA could be rapidly and reproducibly isolated in all four species. When analyzing the DNA by PFGE, the majority of isolated DNA remained in the sample well or in the compression zone of the gel suggesting its megabase size (Fig. 1).

Isolation of nuclei and/or chromosomes from root tips was rapid and very efficient. Nuclei isolation by sucrose gradient purification and embedding in agarose took only about 1.5 h. Furthermore, enough DNA could be obtained from milligram amounts of plant tissues. Typically, six plugs containing about 3 μg of DNA were prepared from one sample (50 root tips) in broad bean, three 3-μg plugs were made-up in barley, whereas only one 2-μg plug was obtained in banana. These differences reflected the differences in genome size (Doležel *et al.* 1994, 1998). In addition to high efficiency, the use of root tips offers two important advantages: *a*) reduced contamination of

samples with plastid DNA, *b*) absence of secondary metabolites. Thus in banana we did not succeed in obtaining high quality DNA from leaf tissues, which are particularly rich in phenolic compounds (data not shown), while the isolation from root tips led to satisfactory results. The use of root tips for DNA isolation is very attractive for species with rigid leaf tissues such as some palms.

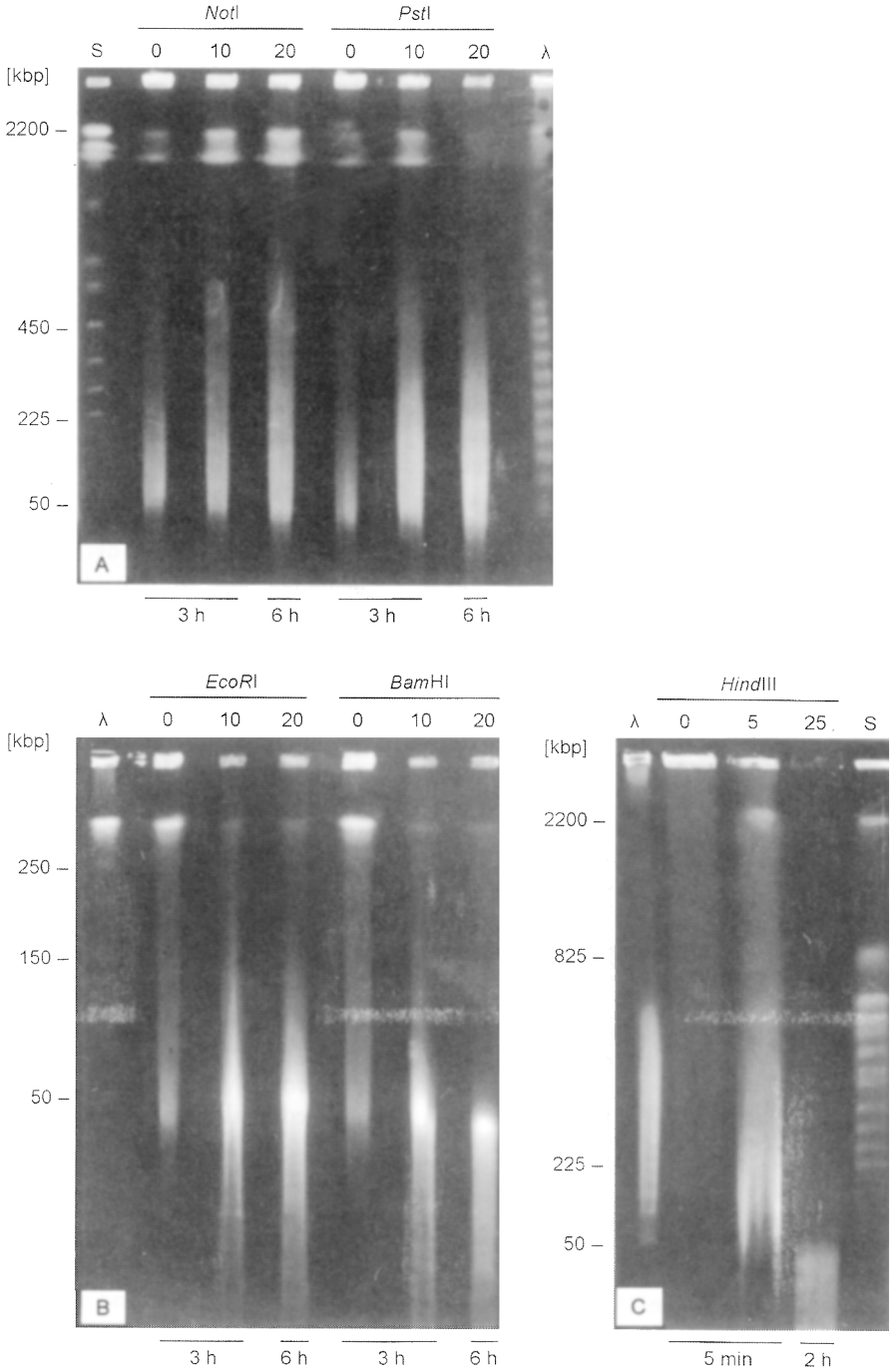

Fig. 2. Pulsed field gel electrophoresis of HMW DNA prepared from gradient-purified barley nuclei (*A*, *B*) and flow-sorted wheat chromosomes (*C*) after digestion with restriction endonucleases. Barley (*H. vulgare*) DNAs in agarose plugs were incubated in appropriate reaction buffers containing 0, 10 or 20 units of *Not*I, *Pst*I, *Eco*RI or *Bam*HI for 3 or 6 h at 37 °C. The electrophoresis was run on a 1 % agarose gel in 0.5 × TBE, at 12.5 °C, 6 V cm^{-1} for 19 h with a 50 s switch time (*A*) or for 15 h with a 5 - 15 s switch time ramp (*B*). Wheat (*T. aestivum*) DNAs in agarose plugs were incubated in *Hind*III reaction buffer containing 0, 5, or 25 units of *Hind*III for 5 min or 2 h at 37 °C. The electrophoresis was run on a 1 % agarose gel in 0.5 × TBE, at 13.5 °C, 6 V cm^{-1} with a 90 s switch time for 20 h. Lanes S - chromosomes of *S. cerevisiae*, lanes λ - lambda ladder used as size markers.

While the basic protocol developed here involved purification on sucrose gradient, we have also tested nuclei and chromosome purification using flow cytometry. Surprisingly, the quality of DNA prepared from flow-sorted particles was higher compared to that obtained after gradient purification (Fig. 1). Thus, a very short exposure of DAPI-stained nuclear and chromosomal DNA to UV light did not cause a detectable damage. The reason why the DNA obtained from flow-sorted particles was better is not clear. However, it may be speculated that it was due to lower contamination of flow-sorted nuclei and chromosome suspensions with remnants of the crude suspension that contained nucleases.

To prove that the low migration ability of DNA remaining in the sample well is not due to its insufficient purity, the DNA in agarose plugs was digested with several restriction enzymes (Fig. 2). The results for barley nuclear DNA (Fig. 2*A*,*B*) showed majority of the DNA accessible for restriction endonucleases and thus relieved of proteins. Wheat chromosomal HMW DNA proved to be particularly pure as majority of DNA was partially digested after 5 min treatment with 5 units of *Hin*dIII and no DNA remained in the sample well after 2 h treatment with 25 units of the enzyme (Fig. 2*C*). The results conform to data concerning activity of particular restriction enzymes in agarose and approximate the data for average fragment size obtained for organisms with similar GC content (Birren and Lai 1993). The addition of PMSF to the TE washes before digestion did not change the restriction pattern (data not shown), thus obviating the necessity to use this toxic compound. The utility of the HMW DNA prepared according to this protocol has already been demonstrated in the construction of genomic BAC library in banana, with average insert size of 135 kb (Šafář *et al.*, unpublished).

In summary, this protocol offers a simple, fast and cost-effective approach to the isolation of HMW DNA from plant nuclei and chromosomes. The method is very efficient as microgram amounts of DNA can be obtained from only several milligrams of plant tissue. Handling negligible amounts of plant material reduces the consumption of chemicals. Moreover, isolating DNA from root tips makes it possible to obtain a high-quality DNA even from plant species with rigid leaves and/or leaves rich in polyphenols and other secondary metabolites interfering with DNA isolation and further processing. While HMW DNA is necessary for long-range mapping and large-insert DNA library construction, successful isolation of HMW DNA from flow-sorted chromosomes opens a way for construction of chromosome-specific large-insert libraries.

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