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ISOLATION AND PURIFICATION OF NUCLEAR DNA FROM EXCISED COTYLEDONS OF *CUCURBITA PEPO* L. (zucchini)

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Summary. High molecular weight genomic DNA was isolated from excised marrow cotyledons (*Cucurbita pepo* L., zucchini) using high concentration of ethidium bromide (400 μg.ml^{-1}) in the grinding buffer and phenol deproteinization of the crude nuclear pellet. This preparation of DNA was free of proteins but contained polysaccharide contaminations which did not allow complete digestion with a number of restriction enzymes. Further purification of nuclear DNA was obtained by adsorbtion on Celite column and elimination of the polysaccharide contaminants. The UV absorbtion spectra of Celite-purified DNA showed that the ratio A260/A280 remained almost unchanged while the ratio A260/A230 significantly increased. As judged from the electrophoretic pattern of DNA restriction fragments in agarose gel as well as from the autoradioraphs after hybridization with ^{32}P-labelled rDNA probe, Celite-purified DNA was completely digested with commonly used restriction enzymes Eco RI, Hind III, Bam HI. Thus, the purified DNA from excised cotyledons served for analysis of the methylation pattern of rDNA unit with methylation sensitive isoschizomers Msp I and Hpa II.

Key words**:** plant genomic DNA, purification of DNA with Celite, methylation of rDNA, *Cucurbita pepo* L. (zucchini), excised cotyledons

Abbreviations**:** EDTA – ethylenediamine tetraacetic acid; EtBr – ethidium bromide; CTAB – cetyltrimethyl ammonium bromide; GuSCN – guanidinium thiocyanate; SDS – sodium dodecyl sulphate; 1× SSC – 4.41 g sodium

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citrate.l^{-1}, 8.76 g.l^{-1} sodium chloride, pH 7.0; TE – 10 mM Tris-HCl, pH 7.4 – 1 mM EDTA, pH 8.0.

Introduction

There are a lot of methods proposed for isolation of nuclear DNA from a variety of plant species, plant organs and tissues. Now it is considered that three main factors are critical for extraction and purification of maximally undegraded and pure genomic DNA: 1. Physical forces used to homogenize the plant tissue i.e. disruption of plant cell walls. 2. The composition of the grinding buffer. 3. Elimination of enzyme-inhibiting polysaccharides (Rogers and Bendich, 1985). The use of liquid nitrogen for grinding of the plant material and homogenization buffers which contain CTAB (Watson and Thompson, 1986) or polyamines like spermine and spermidine (Hewish and Burgoyne, 1973) as well as the addition of EtBr to inhibit the endogenous nuclease activity (Luthe and Quatrano, 1980) allowed to obtain a high molecular weight plant DNA. Most of these methods for extraction and especially for the purification of plant DNA have employed caesium chloride density gradients to eliminate proteins, RNA and the severe polysaccharides contaminations. This method replaced successfully the classical method of phenol/chloroform purification of DNA (Razin, 1988) but it is not conveniant and time consuming since buoyant density centrifugation is required.

Recently a rapid and simple method has been proposed for purification of DNA from human cells (Boom et al., 1990). Briefly, this method is based on both the lysing and nuclease-inactivating activities of the chaotropic agent guanidinium thiocyanate (GuSCN) together with the high binding properties of silica particles or diatoms to DNA in the presence of this agent. In this paper we describe the use of diatoms preparation Celite (the fossilized cell walls of unicellular algae) for purification of crude nuclear lysate DNA or phenol-deproteinized DNA from excised cotyledons of *Cucurbita pepo* L. (zucchini).

Material and Methods

Plant Material

Seeds of *Cucurbita pepo* L. (zucchini) cv. Cocozele, v. Tripolis were soaked for 4 h in a tap water and germinated on a moistened filter paper for 4 days in darkness at 28ºC. After excision of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water for another 24 h in order to reduce the endogenous cytokinins and abscisic acid content. Then the cotyledons were incubated on distilled water (control) or aqueous solution of 45 µM benzyladenine (BA) for 24 h in the dark.

Nuclear DNA isolation and purification

Preparation of nuclei

Crude nuclei preparation from excised cotyledons was obtained as previously described (Ananiev et al., 1987). Cotyledons were ground in liquid nitrogen or in a mortar with precooled bufer A (30 ml buffer per 5 g of plant material). Buffer A contained 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM KCl, 0.6 M sucrose, 30% glycerol, 0.5 mM EDTA and 10 mM 2-mercaptoethanol. 400 $\mu g.ml^{-1}$ ethidium bromide (EtBr) was presented in the grinding buffer as an inhibitor of endogenous DNase and RNase activities (Luthe and Quatrano, 1980). The homogenate was filtured through Miracloth and centrifuged at 3000 rpm at -5ºC for 10 min.

Nuclear DNA extraction

The nuclear pellet was lysed in a buffer containing 100 mM Tris-HCl, pH 8.0, 1% SDS and 20 mM EDTA. DNA was extracted by gentle shaking in ice-cold water bath overnight, purified by subsequent deproteinization with phenol (2 times), phenol-chloroform and chloroform:isoamilyc alcohol (24:1), precipitated with 3 vol. ethanol and dissolved in TE buffer. Total cellular RNA was digested with RNase A (10 $\mu g.ml^{-1}$) according to Maniatis et al., 1982.

Celite column purification of DNA

Deproteinized DNA or the crude DNA preparation after lysis of nuclei were further purified mainly from polysaccharide contaminants by binding on Celite column (1 µg DNA/2 µg Celite) according to Boom et al., 1990. The Celite column (68/8 mm) was filled with 250 mg Celite (about 20 mm height) and after that was equilibrated with DNA in the presence of lysis buffer (in ratio 3 volumes DNA to 5 vol. lysis buffer) for 30 min at room temperature. The lysis buffer contained 5.5 M GuSCN, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% Triton-X-100. Celite-DNA complex in the column was formed after wetting the column firstly with lysis buffer and than by adding DNA dissolved in TE. The formed Celite-DNA complex was washed three times each with Wash buffer I (4 M GuSCN, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA) and Wash buffer II (200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 M EDTA, 50% ethanol), after that with 70% ethanol and 80% acetone. Purified DNA was eluted from the column with TE buffer at 60ºC, precipitated with 3 vol. ethanol and dissolved in TE buffer. DNA quantity was estimated spectrophotometrically by the absorbtion at 259 nm and the purity of final DNA preparation was checked by absorbtion ratios 259/230 and 259/280 nm with Spectrophotometer Shimadzu (Japan).

Restriction enzyme analysis and molecular hybridization

Single or double digests of purified DNA were carried out with restriction enzymes Eco RI, Hind III, Bam HI, Msp I and Hpa II (1 µg DNA with 2–5 units of enzyme) accor-

ding to the supplier's instructions. All restriction enzymes used in this work and the DNA markers (1 kb DNA ladder and λ-phage DNA digested with Eco RI and Hind III) were purchased from FERMENTAS, Riga, Lithuania. Restriction enzyme digests of nuclear DNA were electrophoreticaly separated in 0.8% agarose gel and transferred to nylon membranes (Hybond N+) according to standard methods as described by Maniatis et al., 1982. As a hybridization DNA probe was used the plasmid clone pBG35 digested with Bam HI which contains the whole rDNA unit from flax (*Linum usitatissimum* L.) (Goldsbrough and Cullis, 1981). rDNA probe was labelled with α[^{32}P]dCTP by the method of "random priming" using hexanucleotides as primers and DNA polymerase I ("Klenow enzyme") according to Maniatis et al., 1982. Hybridizations were carried out at 42ºC in 5×SSC, 50 mM phosphate buffer pH 6.8, 5% SDS and 50% formamide. Nylon membranes were washed 3 times each in 2×SSC, 50 mM phosphate buffer pH 6.8, 1% SDS ; in 1×SSC, 50 mM phosphate buffer pH 6.8 and in 0.1×SSC, 50 mM phosphate buffer pH 6.8, 1% SDS at room temperature. Membranes were then dried and exposed to AGWA films using intensifying screens (Du Pont) for 6–24 h at -70ºC.

Results and discussion

In our experiments DNA preparation extracted from the crude nuclear lysate showed a typical pattern of high molecular mass DNA during electrophoresis in agarose gel and migrated as a homogenous band in the upper region of the gel (Fig. 1). Obviously, the presence of high concentrations of EtBr in the grinding buffer and the gentle dealing during nuclei isolation and DNA extraction led to isolation of maximally undegraded DNA with molecular mass higher than λ-phage DNA (results not shown here)(Quatrano et al., 1980). The high molecular mass of extracted DNA was preserved after further purification with Celite column (Fig. 1, lanes 2 and 3). Elution of DNA from the column at 60ºC in low ionic strength buffer (TE buffer) did not result in any significant loss of DNA integrity. At the same time the thoroughly washing of Celit-DNA complex with washing buffers (see Materials and Methods) led to much higher degree of DNA purification, especially from poly-

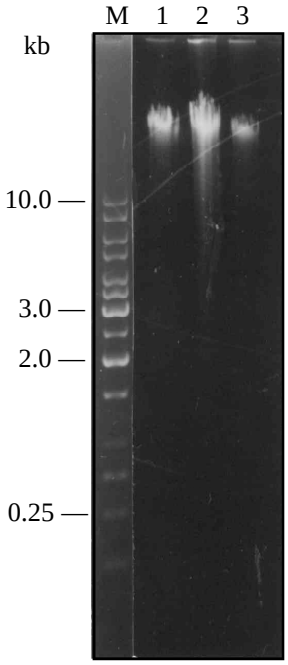

Fig.1. Nuclear DNA isolated from excised cotyledons of *Cucurbita pepo* L. (zucchini). Lane 1 – DNA from crude nuclear lysate before purification with Celite column. Lanes 2 and 3 – purified DNA retained from Celite column (first and second elution respectively). M – DNA marker (1 kb DNA ladder).

saccharide contaminants. If compared, the A260/A280 ratio of phenol/chloroform-deproteinized DNA rested almost unchanged before and after Celite-column purification (1.6 before and 1.8 after Celite column), while the ratio A260/A230 raised from 2.14 to 2.73 after purification with Celite. Therefore, by means of this simple and convenient method we were able further to purify our cotyledonary DNA preparation mainly from polysaccharides. Obviously the presence of high quantities of some acidic polysaccharides in cotyledons as reserve organs interfered with the purity of isolated genomic DNA (Bewley and Black, 1985).

Now it is firmly established that the presence of protein and polysaccharides contaminants in DNA preparations can provoke incomplete digestion of DNA with a lot of restriction enzymes. "Underrestriction" of DNA might contribute to serious problems in different DNA recombination techniques or in the case when a precise physical restriction map of a repeated gene family is needed. So, it was of great importance further to purify DNA from the polysaccharides present in excess. Fig. 2 represents the restriction analysis of DNA before Celite (A) and after Celite column

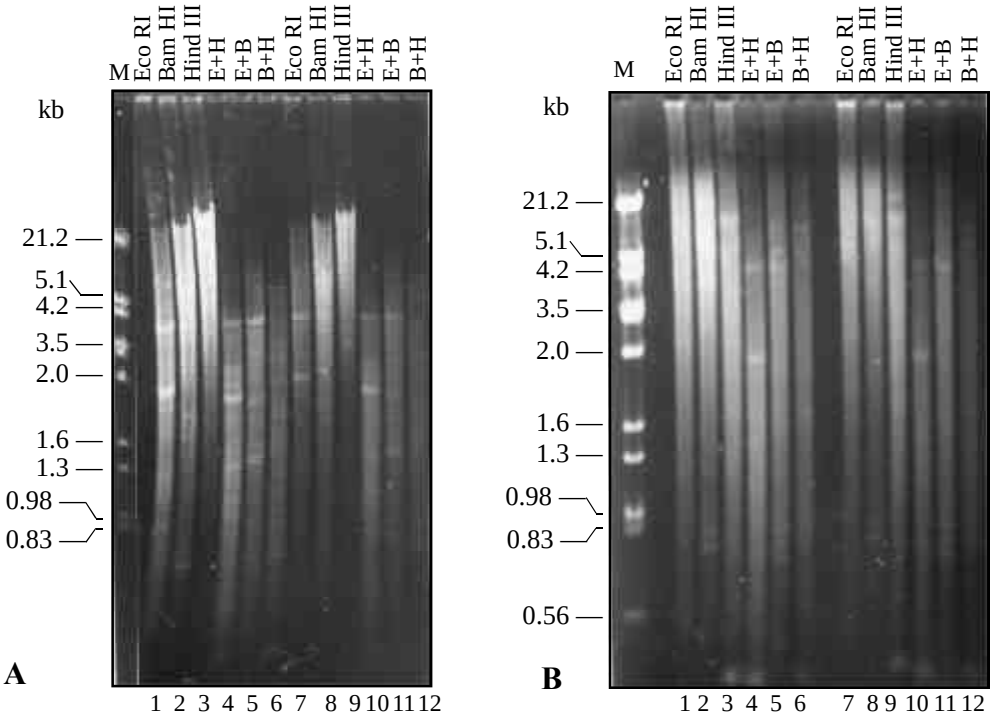

Fig. 2. Restriction enzyme analysis of nuclear DNA isolated from excised cotyledons of *C. pepo.* Phenol/chloroform-deproteinized DNA before (A) and after purification with Celite column (B) was digested with different restriction enzymes as designated on the upper part of the picture representing the EtBr-stained DNA fragments in 0.8% agarose gel. M – DNA marker (λ-phage DNA digested with Hind III and Eco RI).

purification (B). It can be seen from the fluorescence of EtBr-stained DNA fragments in UV light that DNA preparations obtained after phenol deproteinization were not digested to completion (Fig. 2, A). Single digests with the commonly used restriction enzymes Eco RI, Bam HI and Hind III (Fig. 2, lanes 1, 2 and 3; 7, 8 and 9) showed a typical electrophoretic pattern of incomplete digestion when the generated DNA fragments were slowly migrating in the upper part of the gel. Even after precipitation of "single digests" DNA with ethanol and subsequent fragmentation with a second restriction enzyme ("double digestion") it was not possible to achieve complete fragmentation of DNA with the used restrictases (Fig. 2A, lanes 4, 5 and 6; lanes 10, 11 and 12).

Quite different was the restriction pattern of DNA after purification by Celite column adsorbtion (Fig 2, B). The generated DNA fragments were uniformly dispersed in the agarose gel and electrophoretically migrated as previously reported for that species (Leclerc and Siegel, 1987). A good proof for a complete DNA digestion represents the restriction with Hind III, when a prominent band of *ca* 400 bp, probably representing a highly repetitive element was reported earlier (Siegel and Kolacz, 1983; Leclerc and Siegel, 1987; Kelly et al., 1990). In our assays Celite-purified DNA also produced the same low molecular band after digestion with Hind III (Fig. 2, B, lanes 3 and 9) in comparison with its absence in incompletely digested DNA (Fig. 2A, lanes 3 and 9). Therefore, purification with Celite provides a convenient tool for successful digestion of genomic cotyledonary DNA with a number of restriction enzymes.

To prove more precisely the complete digestion of Celite-purified DNA with restriction enzymes further on we made use of the very sensitive method of molecular DNA-DNA hybridization with a specific DNA probe (Southern blotting). As a ^{32}P-labelled DNA probe in this analysis we used the whole rDNA unit from flax harboured in plasmid pBG 35 (Goldsbrough and Cullis, 1981). We studied the methylation pattern of rRNA genes in *C. pepo* using the 4 cutters enzymes Msp I and Hpa II (Fig. 3). DNA was digested with Eco RI and Hind III separately and after that "double digests" (Eco RI + Hind III) were produced in order to obtain fragments with defined length in the repeated rDNA unit (the restriction map of rDNA in *C. pepo* was previously reported by Siegel and Kolacz, 1983 and Ganal and Hemleben, 1986). After that the generated DNA fragments from Eco RI-Hind III double digests were additionally fragmented in the sequence -CCGG- by each of the cytosine-sensitive pair of restriction enzymes-isoschizomers Msp I and Hpa II. Fig. 3 represents the complete digestion of Celite-purified DNA from control and benzyladenine-treated variants as judged from the fluorescence in UV-light of the EtBr-stained DNA fragments (Fig. 3A), as well as from the molecular hybridization analysis using the ^{32}P-labelled rDNA probe (Fig. 3B). It can be seen from lanes 1 and 7 in Fig. 3B, that non-digested DNA from both control and cytokinin-treated variants remained with relatively high molecular mass after electrophoresis in the agarose gel. This result confirmed indeed once again that the purification of DNA with Celite column preserved the integrity of genomic DNA. DNA fragments generated after Hind III digestion (Fig. 3B, lane 2) showed the characteristic

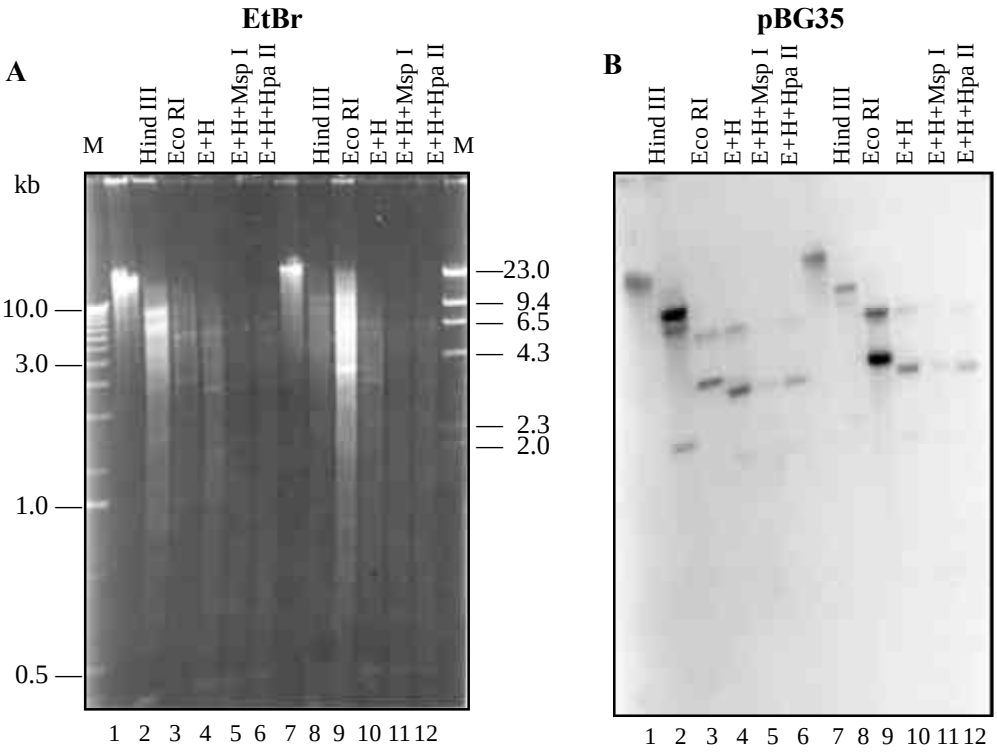

Fig. 3. Restriction enzyme analysis of rDNA unit in *C. pepo* after Celite purification of bulk DNA. **A**. After digestion with designated restriction enzymes and electrophoretical separation of DNA fragments in EtBr-stained 0.8% agarose gel. Lanes 1–6, DNA isolated from control cotyledons; Lanes 7–12, DNA isolated from BA-treated cotyledons. M – DNA marker (1 kb DNA ladder) and λ-phage DNA digested with Hind III. **B**. Hybridization with ^{32}P-rDNA from flax (pBG35).

"two-type" main repeats of rDNA unit in zucchini (major unit of 10 kb length and minor ones with 9.3 kb length) (Ganal and Hemleben, 1986; Torres-Ruis and Hemleben, 1994). The three Hind III-generated fragments with defined length (10 kb, 9.3 kb and 2.4 kb respectively) can appear only after a complete digestion of both types of rDNA units in *C. pepo* with the appropriate enzyme. No "dimmer" forms of rDNA unit of 20 kb or some other DNA bands which could result from partial digestion with Hind III were detected in the autoradiograph. So, by means of the very sensitive method of molecular hybridization with a definitive DNA probe it was proved once again the complete digestion of DNA with the tested restriction enzymes and hence, the purity of Celite-column isolated DNA.

We also demonstrated the complete digestion of repeated rDNA unit with the sensitive restriction enzyme Eco RI which often can meet some difficulties in digestion of genomic DNA preparations. One can easily see the two restriction fragments of 6.3 kb and 3.7 kb generated after complete digestion of the major rDNA unit (10 kb)

with Eco RI as well as the very slight 5.6 kb fragment produced from the minor (9.3 kb) unit with the same enzyme (Fig. 3B, lanes 3 and 9).

It must be noted that in spite of some differences in the quantity of DNA put to analysis (Fig. 3, lanes 2,3 and 8,9), there were no any significant differences in the restriction map of rDNA from control and cytokinin-treated variant.

Conclusions

The main goal of this work was to extract and purify a genomic DNA preparation from excised cotyledons of *Cucurbita pepo* L. (zucchini). By use of Celite column adsorbtion of DNA from crude nuclear lysate or phenol- deproteinized DNA a thoroughly purified DNA was obtained with characteristics as follows:

1. Nuclear DNA retained from Celite column was maximally undegraded with relatively high molecular mass.
2. Celite column purification of nuclear DNA can be applied as for crude nuclear lysate DNA as well as for DNA deproteinized previously by the standard phenol method. UV- absorbtion spectra of DNA and the comparison of the ratios A259/A230 and A259/A280 showed that the effect of Celite purification was mainly due to elimination of polysaccharides contaminants from phenol-deproteinized DNA.
3. Purification of DNA with Celite column resulted in a complete digestion of DNA with a number of commonly used restriction enzymes (Eco RI, Bam HI, Hind III) as judged from electrophoretic pattern of generated DNA fragments as well as from autoradiographs after molecular hybridization with ^{32}P-labelled rDNA probe.
4. Celite-purified DNA from excised cotyledons of *C. pepo* was successfully used for analysis of the methylation pattern of rDNA by means of the cytosine-sensitive 4cutter (-CCGG-) restriction enzymes-izoschizomers Msp I and Hpa II.
5. There are no differences in the restriction map of rDNA unit between control and *in vivo* cytokinin-treated cotyledons.

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