Plant Physiol. (1980) 66, 1140–1143
0032-0889/80/66/1140/04/$00.50/0

Utility of Ethidium Bromide in the Extraction from Whole Plants of High Molecular Weight Maize DNA[1]

Received for publication December 11, 1979 and in revised form June 10, 1980

NAOMI KISLEV AND IRWIN RUBENSTEIN
Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108

ABSTRACT

A procedure is reported for the isolation of high molecular weight maize DNA from whole plant tissue. Nuclei are isolated in the presence of ethidium bromide from leaf, node, and tassel or endosperm tissues and the DNA is extracted and purified. The resulting DNA has a double-strand molecular weight of about 125 kilobase pairs and a single-strand molecular weight of about 125 kilobases. The DNA is cleavable by a number of common restriction endonucleases.

The extraction of high mol wt DNA from whole plant tissue presents many difficulties (1, 3, 7, 8, 10, 12, 15, 19, 26, 33): plant nucleases (16, 20, 21), phenolic compounds (18, 32), cell walls (4, 12, 16, 29), and polysaccharide material (5). In the approach used here, the plant DNA is extracted from nuclei isolated in the presence of EtBr[2]. The DNA has a mol wt of 90 to 170 kbp, is relatively free of single-strand breaks, and is cleavable by a number of restriction enzymes.

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays*, L), inbred line W22rr, were obtained from the Minnesota Crop Improvement Association. Four- to 6-week-old plants (about 60 cm high) were harvested. A 20-cm portion of the plant containing the five to seven uppermost nodes, the tassel, and the whorl of young leaves was cut into small slices, frozen in liquid N_2 in the field, and kept at −80 C. To obtain endosperm material, 22-day-old kernels were collected, frozen with liquid N_2, and stored at −80 C.

Restriction Endonucleases. The restriction endonucleases used in this study were obtained from Bethesda Research Laboratories, Rockville, MD.

Isolation of Nuclei. The frozen plant material (50 g) was suspended in liquid N_2 and powdered with a Waring Blendor. Three cycles of grinding, 1 min each, were applied. The frozen powder was transferred in small portions into 250 ml MNIB at 0 C, while being continuously stirred. The MNIB contains 0.4 M sucrose, 20 mM Tris-HCl (pH 7.6), 2 mM $CaCl_2$, 2% w/v purified gum arabic (15), and 4 mM *n*-octyl alcohol; in addition, 2 mg/ml PVP (Sigma: PVP-360) (18, 32) and 400 μg/ml EtBr (13, 27) were added before use. All subsequent operations were done at 0 to 4 C. Usually the iced slurry was infused for 5 to 10 min (at times, as long as 1 h) under a partial vacuum (50 cm Hg), and then ground in a Brinkman Polytron at maximum speed. The homogenate was filtered through a 60-μm stainless steel screen. The residue was suspended in 150 ml MNIB, reground, and refiltered. The total suspension was filtered twice through a 60-μm and then through a 25-μm Nitex nylon mesh (Tekto Inc., Elmsford, NY). After centrifugation in the HL-4 rotor of the Sorvall GLC-1 centrifuge at 450*g* for 10 min, the pellets were saved; the supernatant was recentrifuged. The pellets were combined and resuspended in 60 ml MNIB and centrifuged at 300*g* for 10 min (HB-4 rotor). The pellets then were resuspended in 60 ml MNIB (without PVP and EtBr) supplemented with 0.15% v/v Triton X-100 (MNIB + Triton). The suspension was centrifuged at 300*g* for 10 min; the pellets were collected, washed in 30 ml MNIB + Triton, and recentrifuged. The pellet was suspended with 25 ml of a buffer containing 0.2 M sucrose, 10 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$ (nuclear suspension buffer) and recentrifuged at 300*g* for 10 min. The nuclei pellet was collected and washed in 20 ml nuclear suspension buffer and recentrifuged. This step was repeated until a clear supernatant was obtained (at least twice). The nuclei were finally resuspended in 20 ml nuclear suspension buffer, a sample for nucleus count was taken, and the suspension was divided into 2-ml portions and stored at −80 C.

Nucleus Count. The nuclei were stained with acetocarmin (0.5% w/v in 45% acetic acid) and counted in a hemacytometer.

DNA Extraction. The nuclei were thawed, centrifuged, and resuspended to give a concentration of 1 to 5 × 10^7 nuclei/ml. To 6 ml of the nuclei suspension, 4 ml 5 M NaCl were added. The iced nuclei then were lysed by addition of 10 ml ice-cold buffer containing 10 mM Tris-HCl (pH 8.5), 0.5% (w/v) SDS, 10 mM Na_2-EDTA, 10 mM NaCl and 200 μg/ml Proteinase K (E. Merck, Germany). The suspension was kept on ice for 15 min and mixed gently. The tube then was wrapped in aluminum foil and transferred to a 37 C incubator for 1 h, after which the sample was slowly drawn into a 10-ml pipette about five times and then rocked for 3 to 4 h at 37 C. The nuclei lysate then was transferred to a prewashed, sterile dialysis bag and dialyzed overnight at 37 C against 0.1 M NaCl, 20 mM Tris (pH 8.5), 1 mM EDTA (alkaline buffer). The digested lysate was centrifuged at 20,000*g* for 20 min at 0 C in an RC2-B Sorval centrifuge to remove most of the starch grains and some of the polysaccharides. If there was a large amount of starch, this step was repeated. The supernatant then was placed in a conical glass tube and deproteinized for 15 min with an equal volume of alkaline buffer-saturated phenol (v/v) at room temperature. The mixture was slowly rocked to ensure mixing. A volume of chloroform-isoamyl alcohol (24:1, v/v) equal to that of the phenol was added and the sample was extracted again. The mixture then was centrifuged. Usually little if any interphase was present. The upper phase was re-extracted as above, twice more with chloroform-isoamyl alcohol alone, and then chilled on ice and extracted at least six times with ice-cold ethyl ether. The ether was evaporated and the *A* was measured at

[1] This research was supported by Grant GM24756 from the National Institutes of Health and by Grant PCM76-02600 from the National Science Foundation.

[2] Abbreviations: EtBr; ethidium bromide; Kbp, kilobase pair; Kb, kilobases.

260, 280, and 230 nm. The concentrations of DNA were usually in the range of 20 to 40 μg/ml (this is equal to 30–40 μg DNA/g frozen tissue). The DNA samples for agarose gel electrophoresis were dialyzed against 10 mM Tris (pH 7.4), 1 mM EDTA. The DNA samples contained variable amounts of RNA.

DNA Extraction from 22-Day-old Kernels. The Pitout and Potgieter method (29) was used with a few modifications. The frozen kernels were ground in liquid N_2 in a Waring Blendor for 2.5 min. The N_2 was allowed to evaporate and the powder was transferred to 3 × volume of buffer containing 1% (w/v) SDS, 0.1 M Tris (pH 9), and 5 mM EDTA supplemented with 1 M NaCl. The slurry was filtered through a 120- and a 52-μm stainless steel mesh. The filtrate was centrifuged, phenol extracted, and precipitated by ethanol as described by Pitout and Potgieter (29).

Electrophoresis. Horizontal slab gels were formed and run essentially as described by Fangman (6). Seakem HGT(P) agarose (Lot 10439 M.C., FMC Corp.) was used for gel concentrations of 0.10 to 0.25% (w/v), and Sigma agarose for higher percentage agarose gels. The neutral electrophoresis buffer commonly used contained 36 M Tris base, 3 mM NaH_2PO_4, 25 mM Na_2EDTA (pH 7.8) and 0.5 μg/ml EtBr. The alkaline buffer for denatured DNA contained 30 mM NaOH, 2 mM EDTA (24). The DNA was denatured in 0.3 N NaOH. Samples of 30 μl were loaded slowly into wells and contained 10 mM Tris-HCl (pH 8), 10 mM Na_2EDTA (pH 8), 10% glycerol, and 0.016% (w/v) Bromocresol green. Electrophoresis was carried out at room temperature at a constant voltage (0.5 v to 1.0 v/cm for neutral gels, or 0.5 v to 2.0 v/cm for alkaline gels). The alkaline gels (run without EtBr) were neutralized by soaking for about 2 h in 0.5 M Tris (pH 7), containing 1 μg/ml EtBr. The gels were photographed with Polaroid 665 P/N film by using a shortwave UV illuminator (264 nm) and a Wattern No. 23A filter, and the relative mobilities of the various DNAs were determined (6, 24).

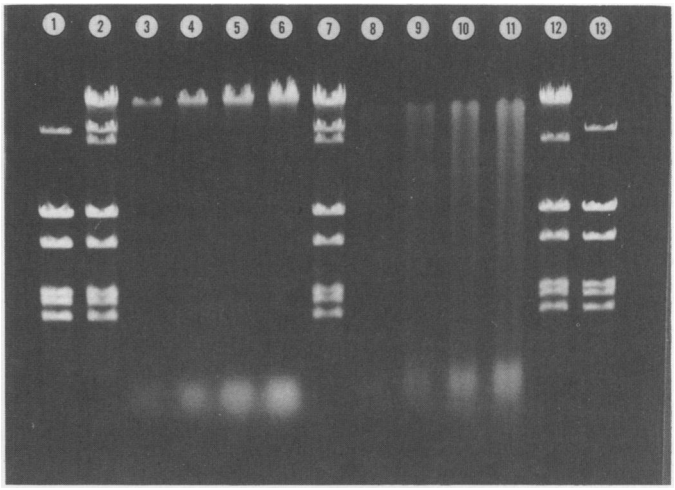

FIG. 1. Neutral agarose gel of maize DNA purified from nuclei that had been isolated in the presence or absence of EtBr. The samples of DNA loaded in lanes 3, 4, 5, and 6 had been isolated with the use of EtBr; the amounts of DNA loaded were about 30, 60, 90, and 120 ng, respectively. The samples of DNA loaded in lanes 8, 9, 10, and 11 had been isolated without the use of EtBr; the amounts of DNA loaded were 30, 60, 90, and 120 ng, respectively. Lanes 1 and 13 contain a mixture of 25 ng λ DNA and about 100 ng λ DNA digested with *Bam* HI. The load for lane 2 contained a mixture of about 100 ng T5 DNA, 30 ng λ DNA, 30 ng T7 DNA, and 100 ng λ DNA cut with *Bam* HI. The load for lane 12 is similar to lane 2 but did not contain λ DNA. The size of the phage DNA standards are 115 kbp for T5 DNA (11), 49 kbp for λ DNA (2, 28), and 40 kbp for T7 DNA (24). The λ DNA cut with *Bam* HI was not heated and, therefore, contains fragments of length 17.4, 12.3, 7.3, 6.5, and 5.5 kbp (9).

RESULTS

Extraction of DNA from Leaf, Node, and/or Tassel Tissues. The utility of EtBr for the extraction of high mol wt DNA from whole corn plant tissue was studied by conducting parallel experiments in which nuclei were isolated from plant node, tassel, and leaf tissues with or without EtBr present in the nuclei isolation solution. To determine the mol wt of the native extracted corn DNA and whether it contained large numbers of single-strand breaks, the DNA preparations were analyzed on neutral and alkaline agarose gels and sucrose gradients. Figure 1 shows a neutral gel which compares the mol wt distribution of native corn DNA isolated from nuclei isolated in the presence of EtBr (lanes 3 to 6) with that of DNA from nuclei isolated in the absence of EtBr (lanes 8 to 11). Figure 2 shows an alkaline gel of the same DNA preparations (lanes 3 to 5, with EtBr; lanes 6 and 7, without EtBr). The results (Fig. 1) indicate that the native corn DNA isolated in the presence of EtBr consists almost entirely of molecules larger than 100 kbp (ranging from 90 to 170 kbp). The upper molecular size was checked by other agarose gel runs with T4 DNA (171 kbp; ref. 14) as a marker (data not shown). In contrast, the DNA isolated in the absence of EtBr appears to be very heterogeneous in molecular weight (ranging from 10 to 140 kbp). The fast-moving band in the corn DNA lanes in Figure 1 represents RNA that was present in these preparations.

Electrophoresis of denatured DNA in alkaline agarose gels can be used to determine the mol wt of single-stranded DNA (24). The single-stranded DNA extracted from nuclei isolated in the presence of EtBr (Fig. 2, lanes 3 to 5) appears to be more uniform and have a higher mol wt than DNA extracted in the absence of EtBr (Fig. 2, lanes 6 and 7). The molecular size of the major portion of the denatured DNA molecules exceeds 100 kb (Fig. 2, lanes 3, 4 and 5) and the modal value of the distribution is 100 to

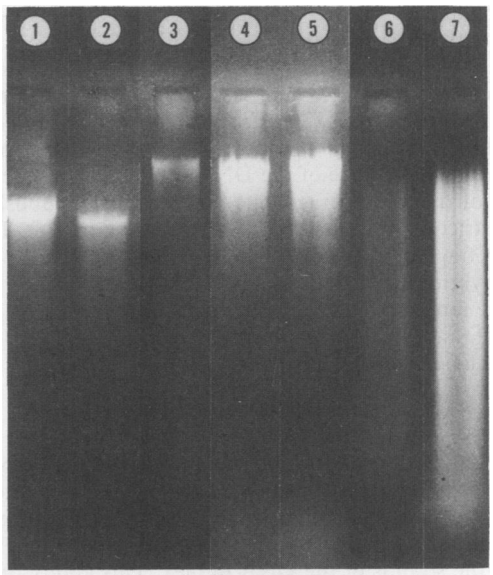

FIG. 2. Alkaline agarose gel of maize DNA. The samples of denatured DNA loaded in lanes 3, 4, and 5 represent portions of the DNA shown in lanes 3 to 6 of Figure 1 and had been isolated with the use of EtBr; the amounts of DNA loaded were about 500, 1,400, and 2,100 ng, respectively. The samples of DNA loaded in lanes 6 and 7 represent portions of the DNA shown in lanes 8 to 11 of Figure 1 and had been isolated without the use of EtBr; the amounts of DNA loaded were 500 and 2,100 ng, respectively. Lane 1 contains denatured λ DNA (about 200 ng) and lane 2 contains denatured T7 DNA (about 100 ng) as size standards.

150 kb. The upper mol wt range was checked by other agarose gel runs by using T5 DNA (115 kb, ref. 11) as a marker (data not shown). In contrast, the mol wt of the single-stranded DNA extracted from nuclei purified in the absence of EtBr were much smaller (having a modal value of 20–40 kb) and were heterogeneous in size (Fig. 2, lanes 6 and 7). DNA isolated from corn nuclei in the presence or absence of EtBr was also analyzed by neutral and alkaline sucrose gradient centrifugation. λ DNA (49 kbp; Refs. 2 and 28) was used as a standard. Most of the duplex corn DNA isolated in the presence of EtBr had mol wt greater than that of λ DNA and ranged in size from 90 to 170 kbp. The single-stranded corn DNA had a similar range of lengths, 90 to 170 kb.

These results were affected by alterations in the extraction procedures. A key factor affecting the degradation of DNA isolated in the absence of EtBr appears to be the time used to infuse the cell fragments and purify the nuclei. The results shown in Figures 1 and 2 were for cell fragments infused for 1 h at 0 C in the absence of EtBr. By shortening this time to about 10 min, much higher mol wt native DNA was obtained, almost the size of the DNA extracted in the presence of EtBr. However, this DNA contained a large number of single-strand breaks. There were a few molecules as large as 110 kb but most were much smaller; the modal value was below 45 kb. In contrast, lengthening the time of infusion and/or nuclei isolation to 1 h did not seem to affect markedly the mol wt of the DNA obtained from nuclei isolated in the presence of EtBr.

Extraction of DNA from Kernels. Most of the native DNA isolated from kernels (29) without the use of EtBr had a size greater than 50 kbp; the range was from 20 to 115 kbp. After denaturation by alkali, the average size of the single stranded DNA molecules was only 30 kb and heterogeneous. This indicates that the DNA preparation contained numerous "nicks" or breaks. Pitout and Potgieter (29) had not characterized the single-strand mol wt of their preparations.

Most of the DNA isolated from kernels in the presence of EtBr was comparable in size to both the native and the single-stranded DNA from the other tissues.

Restriction Endonuclease Cleavage of DNA. An important requirement for the work here is that the DNA be cleaved by restriction endonucleases (30). *Eco* RI, *Bam* HI, *Sst* I, *Xba* I, and *Bst* I all showed complete digestion of the purified DNA as judged by the general size range of the DNA fragments and the number and sizes of the rDNA fragments (D. Benton, unpublished data on the restriction map of the rDNA region of maize). To detect the rDNA fragments, the digests were transferred to nitrocellulose (31) and hybridized with radioactive rRNA. In addition, as judged by the size range of the DNA fragments produced, *Hinc* II, *Hind* III, *Hpa* II, and *Alu* I also appeared to digest the DNA preparations used here to completion.

DISCUSSION

The presence of EtBr during extraction of nuclei appears to yield higher mol wt DNA. Ethidium bromide is known to inhibit nuclease activity (13, 27), possibly by distortion of the helix (17). This protection is especially important in preventing nicks or breaks in the strands of the DNA duplex. This ability of EtBr to protect the single-strand integrity of DNA appears to be independent of the plant tissue used, at least for the four general types of tissue used.

The DNA extracted from these purified nuclei that had been isolated in the presence of EtBr has a double-strand mol wt of about 125 kbp. In contrast, DNA isolated from nuclei prepared in the absence of EtBr has a very heterogeneous mol wt, ranging from 100 to 10 kbp with the major portion of the molecules being less than 50 kbp in length. The purified DNA appears to be stable during storage at 4 C for at least 6 months.

The yields of nuclei obtained by this procedure range from 1 to 1.5×10^7 nuclei/g of frozen powdered leaf, node, and tassel tissue which is in the range obtained by others (4, 15). The over-all recovery of DNA from the purified nuclei is estimated to be about 25 to 30% of the DNA in the powdered tissue. The amount of RNA in our preparations varies from 5 to 15% by weight.

Our purified DNA is suitable for analysis by cleavage with a number of restriction enzymes. These include *Eco* RI, *Bam* HI, *Sst* I, *Bst* I, *Xba* I, *Hinc* II, *Hind* III, *Hpa* II, and *Alu* I. Other restriction enzymes (*e.g. Pst* II, *Sal* I, *Hae* II, *Hha* I, *Sma* I, and *Xho* I) did not appear to digest the DNA to completion at the concentrations of enzymes used (1–3 units/μg DNA; 1 to 3 h).

Ethidium bromide plays an important role in these experiments. Precautions similar to those used for the handling of phenol were used here, *i.e.* the grinding was done in a hood, gloves were worn, and no pipetting was done by mouth. McCann and coworkers (23) using the Ames test have reported EtBr to be a potent mutagen in the *Salmonella*/microsome mutagenicity test. McCann and Ames (22) strongly suspect that ethidium may be an animal carcinogen, urge caution in its use, and suggest the use of propidium diiodide. There is, however, no indication of *in vivo* animal or human carcinogenicity of EtBr. McKinnell (25) has developed a dominant lethal test for the determination of mutagenicity in which frogs are used as the test animals. This test showed no statistically significant effects of EtBr in the first generation offspring. When compared to triethylene melamine, a known carcinogen, EtBr in an approximately 1,000 times higher dosage (10 mg/kg) failed to produce significant mutations (R. G. McKinnell, personal communication).

Acknowledgments—We thank the following people for their help and advice: John Stout (University of Wisconsin, Madison); and David Benton and Peter Mascia (University of Minnesota, St. Paul). The idea of using EtBr was suggested by Dr. Ruth Kavenoff and Y. Hotta (University of California, San Diego).

LITERATURE CITED

1. Blamire J, DR Gryer, DB Finkelstein, J Marmua 1972 Sedimentation properties of yeast nuclear and mitochondrial DNA. J Mol Biol 67: 11–24
2. Blattner FR, BG Williams, AE Blechl, KD Thompson, HE Faber, LA Furlong, DJ Grunwald, DO Kiefer, DD Moore, JW Schumm, EL Sheldon, O Smithies 1977 Charon phages: safer derivatives of bacteriophage λ for DNA cloning. Science 196: 161–169
3. Blin N, DW Stafford 1976 A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acid Res 3: 2303–2308
4. D'Alessio G, AR Trim 1968 A method for the isolation of nuclei from leaves. J Exp Bot 19: 831–839
5. Edelman M 1975 Purification of DNA by affinity chromatography removal of polysaccharide contaminants. Anal Biochem 65: 293–297
6. Fangman WL 1978 Separation of very large DNA molecules by gel electrophoresis. Nucleic Acid Res 5: 653–665
7. Graham DE 1978 The isolation of high molecular weight DNA from whole organisms or large tissue masses. Anal Biochem 85: 609–613
8. Gross-Bellard M, P Oudet, P Chambon 1973 Isolation of high molecular weight DNA from mammalian cells. Eur J Biochem 36: 32–38
9. Haggerty DM, RF Schleif 1976 Location in bacteriophage λ DNA of cleavage sites of the site specific endonuclease from *Bacillus amyloliquifacies*. J Virol 18: 659–663
10. Hamilton RH, U Kunsch, A Temperli 1972 Simple rapid procedure for isolation of tobacco leaf nuclei. Anal Biochem 49: 48–57
11. Hamlett NV, B Lange-Guftafson, M Rhoades 1977 Physical map of the bacteriophage T5 genome based on the cleavage products of the restriction endonucleases *Sal* I, *Sma* I, *Bam* I, and *Hpa* I. J. Virol 24: 249–260
12. Hotta Y, A Bassel 1965 Molecular size and circularity of DNA in cells of mammals and higher plants. Proc Natl Acad Sci USA 53: 356–362
13. Jerzmanowski A, K Staron, B Tyniec, K Toczko 1978 Effect of ethidium bromide on the digestion of chromatin DNA with micrococcal nuclease. Biochim Biophys Acta 521: 493–501
14. Kim JS, N Davidson 1974 Electron microscope heteroduplex study of sequence relations of T_2, T_4, and T_6 bacteriophages DNA. Virology 57: 93–111
15. Kuhl L 1964 Isolation of plant nuclei. Z Naturforsch 19b: 525–532
16. Lark G, DE Cress 1978 Cell division and DNA synthesis in plant cells. *In* TA Thorpe, ed, Frontiers of Plant Tissue Culture, International Association of Plant Tissue Culture, Canada, pp 179–189
17. LePecq JB, C Paoletti 1967 A fluorescent complex between ethidium bromide and nucleic acids. J Mol Biol 27: 87–106
18. Loomis WD 1974 Overcoming problems of phenolics and quinones in the

isolation of plant enzymes and organelles. Methods Enzymol 31: 528–544
19. MASCARENHAS JP, M BERMAN-KURTZ, RR KULIKOWSKI 1974 Isolation of plant nuclei. Methods Enzymol 31: 558–565
20. MATILE P 1968 Lysosomes of root tip cells in corn seedlings. Planta 79: 181–196
21. MATILE P, H MOOR 1968 Vacuolation: origin and development of the lysosomal apparatus in root-tip cells. Planta 80: 159–175
22. MCCANN J, BN AMES 1977 The *Salmonella*/microsome mutagenicity test: predictive value for animal carcinogenicity. *In* Origins of Human Cancer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 1431–1450
23. MCCANN J, E CHOI, E YAMASAKI, BN AMES 1975 Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc Natl Acad Sci USA 72: 5135–5139
24. MCDONELL MW, MN SIMON, W STUDIER 1977 Analysis of restriction fragments of T_7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J Mol Biol 110: 119–146
25. MCKINNELL RG, DJ PICCIANO, JW SCHAAD 1979 Dominant lethality in frog embryos after paternal treatment with triethylenemelamine. Cytogenetics, morphology, and swimming capability. Environ Mutagen 1: 221–231
26. OHYAMA K, OL GAMBORG, RA MILLER 1972 Isolation and properties of deoxyribonucleic acid from protoplasts of cell suspension cultures of *Ammi visnaga* and carrot (*Daucus carota*). Plant Physiol 50: 319–329
27. PARKER RC, RM WATSON, J VINOGRAD 1977 Mapping of closed circular DNAs by cleavage with restriction endonucleases and calibration by agarose gel electrophoresis. Proc Natl Acad Sci USA 74: 851–855
28. PHILIPPSEN, P, RA KRAMER, RW DAVIS 1978 Cloning of the yeast ribosomal DNA repeat unit in *Sst* I and *Hin*dIII λ vectors using genetic and physical size selection. J Mol Biol 123: 371–386
29. PITOUT MJ, DJJ POTGIETER 1968 The isolation of highly polymerized deoxyribonucleic acid from maize. Biochim Biophys Acta 161: 188–196
30. SMITH HO 1979 Nucleotide sequence specificity of restriction endonucleases. Science 205: 455–462
31. SOUTHERN E 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503–517
32. STEIN DB, WF THOMPSON 1978 Isolation of DNA from tannin-containing plants. Plant Sci Let 11: 323–328
33. TAUTVYDAS KJ 1971 Mass isolation of pea nuclei. Plant Physiol 47: 499–503