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Errata

Construction of a Representative Genomic Library from a Hexaploid Wheat[1]

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Abstract: The large genome size and the great amount of DNA repeats make it rather diffiult to obtain a representative hexaploid wheat genomic library. The protocol is given with modifications to phage isolation and to purification of vector and plant DNAs by electrophoresis in low-concentration SeaKem agarose gels. Representative genomic libraries of the soft wheat variety carrying a translocated rye chromosome are 1RS were constructed in a *recD*-minus *E. coli* strain to reduce recombinational loss.

Successful construction of a representative library of plant genomic DNA, depends not only on DNA purity and freedom from polysaccharides and other contaminants but also on the ability to minimize recombinational loss of repeated sequences (Frischauf et al., 1983; Fedoroff, 1985; Wyman et al., 1986). In the case of plants such as hexaploid wheat, the large genome size and the presence of repeated sequences make it difficult to obtain a representative library. Modifications to the standard phage isolation procedure can improve DNA purity, while the use of a *recD*-minus strain of *E. coli* (TAP90) can reduce recombinational loss. The fractionation of DNA digests in the density gradients is a labor-intensive and time-consuming procedure. As a simple and rapid alternative, we separate the vector and insert DNA fragments by electrophoresis in low-concentration agarose gels. Several representative genomic libraries of the soft wheat variety *Aurora* were generated using these approaches.

Abbreviations: EtBr, ethidium bromide; PEG, polyethylene glycol; SM, TAE, TE and TM are defined in *Materials Required.*

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[1] *Erratum*: This is a corrected version of an article that appeared in the *Plant Molecular Biology Reporter* 8(2):85-91.

Materials Required

Bacterial strains and phage vector

EMBL4 lambda replacement vector (Frischauf *et al.*, 1983) and *E. coli* host strains TAP90 (Patterson & Dean, 1987) and K803 (Wood, 1966) were used.

Solutions required

10 M ammonium acetate, filtered through a 0.22 µm membrane
buffer 1: 25 mM Tris HCl pH 7.6, 0.4 M sucrose, 2 mM $CaCl_2$, 0.05% (w/v) 1-octanol, 2 mg polyvinylpyrrolidone/mL (PVP-360, Sigma), 0.4 mg EtBr/mL
buffer 2: 10 mM Tris HCl pH 7.5, 0.2 sucrose, 2 mM $CaCl_2$, 0.1% (v/v) Triton X-100
electroelution buffer: 20 mM Tris HCl pH 3.0, 0.1 mM EDTA, 5 mM NaCl, pH 8.0
ligase buffer: 66 mM Tris HCl pH 7.6, 6.6 mM MgCl, 10 mM dithiothreitol
PEG 8000, 10% (Sigma Chemicals, St. Louis, MO)
PEG 6000, 12% (w/v)
SeaKem agarose, 0.3% (FMC Bioproducts, Denmark)
SM: 10 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris HCl pH 7.5, 0.01% (w/v) gelatin
TAE: 40 mM Tris-acetate pH 8.0, 2 mM EDTA
TE: 25 mM Tris HCl pH 8.0, 20 mM EDTA
TM: 10 mM Tris HCl pH 8.0, 10 mM $MgSO_4$

Procedure

Isolation of EMBL4 λ DNA

- Grow K803 host cells infected by lambda bacteriophage until lysis is complete. After DNAase and RNAase treatment, precipitate phage particles overnight at 0°C with 10% PEG 8000. Collect by centrifugation at 5000 g at 2°C for 20 min.
- Resuspend the pellet in 4 M CsCl in SM buffer and leave overnight at 4°C.[1] Dilute the suspension with SM to 1 M CsCl and centrifuge (10,000 g at 4°C for 10 min).
- Discard the pellet and repeat polyethylene glycol precipitation step.

- Layer 3 mL of the phage suspension in 1 M CsCl on a step gradient of CsCl composed of 12 mL 1.45 g/mL, 10 mL 1.5 g/mL, and 5 mL 1.7 g/mL, and centrifuge at 80,000 g for 2 h at 20°C.
- Collect the phage band, adjust the density with solid CsCl to 1.75 g/mL, and place on the bottom of 30-mL tube. Overlay with CsCl solutions (10 mL 1.7 g/mL, 10 mL 1.50 g/mL, and 5 mL 1.45 g/mL) and repeat centrifugation. [2]
- Collect the band with phage particles and dialyze against a buffer containing 10 mM Tris HCl pH 8.0, 10 mM NaCl, and 10 mM $MgSO_4$ for 2 h.
- Digest the phage with 40 mg/mL of Proteinase K in 0.5% SDS, 20 mM EDTA at 37°C for 0.5 h; extract sequentially with phenol, phenol/chloroform, and chloroform; precipitate with 2 vols ethanol; and make 2M in ammonium acetate.
- Digest the phage DNA with a three-fold excess of *Bam*HI (Ferment, Vilnius, U.S.S.R); extract with phenol/chloroform; adjust to 2.5 M ammonium acetate, and precipitate with ethanol.

Isolation of wheat nuclear DNA

- Sterilize wheat seeds with 0.1% silver nitrate at room temperature for 30 min and germinate under sterile conditions in the dark.
- Freeze two-week-old seedling shoots in liquid nitrogen, grind with mortar and pestle, and transfer 30 g of the powder into 300 mL of freshly prepared, ice-cold buffer 1 (modification of Kislev & Rubenstein, 1980).
- Homogenize thoroughly and filter through 100- and 40-µm stainless steel screens.
- Centrifuge filtrate at 400 g for 15 min at 2°C. Resuspend the pellets in 50 mL of buffer 2 and centrifuge at 400 g for 10 min. Repeat washing twice, resuspending the pellet in 50 mL of buffer 2 without Triton X-100.
- Resuspend nuclei in 10 mL of ice-cold TE, add sodium N-lauroylsarcosine to 0.5% and proteinase K to 100 µg/mL. Digest for 1 h at 37°C.
- Centrifuge at 5000 g for 15 min and discard the pellet.
- Add 1 g/mL of solid CsCl and 100 µg EtBr/mL to the supernatant. Centrifuge at 200,000 g at 20°C for 24 h.
- Collect the pink band of DNA and remove EtBr by extraction with isopropanol or butanol (Watson & Thompson, 1986).
- Microdialize 300-µl portions in Eppendorf tubes with lids removed (Overall, 1987). Precipitate with two volumes of ethanol; dissolve in TE, without complete drying of the pellets; store at 4°C.

Partial restriction of wheat DNA

- Establish the optimal concentration of *Sau*3A I for producing the maximum number of 20-kb fragments of wheat DNA by digesting with various amounts of the enzyme (Maniatis et al., 1982). Digest 50 to 100 µg of DNA with this optimal concentration of *Sau*3A I.
- Extract with phenol-chloroform (1:1) and chloroform, precipitate with ethanol, dissolve in 50 to 100 µl of water, and store at -20°C.

DNA purification by agarose-gel electrophoresis

- Boil 0.3% SeaKem agarose in TAE buffer for 5 min while stirring. Cool the gel for 30 min before use at 4°C. Load 10 µg of partially digested wheat DNA per well and 1 µg of *Bgl*II-digested λ in wells at each side of the gel.
- After electrophoresis for 3 h at 4 V/cm, stain marker lanes by immersing the edges of the gel in a solution of 0.5 µg EtBr/mL.
- Excise gel slices in the range 13 to 22 kb as determined by the λ markers and soak them for 1 h at 4°C in electroelution buffer.
- Electroelute DNA for 1 h with IBI model UEA (International Biotechnologies, New Haven, Connecticut, USA) or a similar V-channel electroelution device using a cushion of 10 M ammonium acetate cushion without bromphenol blue. Recover DNA by precipitation with 2 volumes of ethanol at -20°C overnight.
- Purify left and right λ arms (19 and 9 kb) as described above, except heat DNA at 65°C for 5 min and cool on ice before loading. Use *Hind*III λ as markers.

Ligation of intact vector and donor DNA

Mix *Bam*HI-cleaved EMBL4 DNA and 1 µg wheat DNA partially restricted with *Sau*3AI in the proportions 1:2, 1:1, 2:1 in 10 µl of ligase buffer (Maniatis et al., 1982) with 4 to 8 U of T4 DNA ligase. Incubate at 12°C for 16 h, precipitate with ethanol, and dissolve in 3 µl of TM.

Ligation of separated vector arms and donor DNA

- Mix equimolar amounts of λ left and right arms (approximately 0.7 and 0.3 µg) and 0.7 µg of agarose-fractionated wheat DNA. The optimal ratio of vector to insert should be experimentally estimated for every lot of DNA). Ligate in the presence of 12% (w/v) PEG 6000 as described for the intact vector. Add 2 µl of 5 M NaCl and precipitate DNA overnight at 0°C.
- Collect the pellet by centrifugation at 7000 g for 10 min, remove all traces of the ligation mixture and dissolve in 3 µl of TM buffer.
- Package DNA *in vitro* according to Maniatis et al. (1982) or to the

instructions of the packaging extracts manufacturer. Hybridize the sets of 200 to 300 plaques to *Sau*3AI-digested, nick-translated wheat DNA.

- Isolate DNA from 10 to 20 individual plaques (cf. Grossberger, 1987), digest with *Pvu*II, and analyze on 0.9% agarose.

Notes

1. This step significantly increases the yield (Latchman and Brickell, 1986).
2. Consequent purification of bacteriophage from the top to bottom of a step gradient and vice versa allows one to avoid the time-consuming process of equilibrium gradient centrifugation.

Results and Discussion

Wheat DNA was partially digested with the *Sau*3AI, ligated into the λ vector, and packaged as described in brochure. Plating efficiencies obtained for various combinations of vector and insert DNA packaged *in vitro* are shown in Table 1. The vector DNA was cleaved at the *Bam*H1 cloning sites almost completely since on packaging of unligated DNA the plating efficiency was <0.02% that of intact phage DNA. After religation the plating efficiency rose 300 fold.

The highest recombinant plating efficiency was obtained when BamHI-cut vector was ligated to unfractionated, partially digested wheat DNA. A comparison of this value with that of self-ligated BamHI-cut shows

Table 1. Plating efficiencies.

EMBL4 Vector DNA	Insert DNA	p.f.u./μg vector DNA	p.f.u./μg donor DNA
Intact	none	5.0×10^{7}	
*Bam*H1-cut	none	8.0×10^{3}	
*Bam*H1-cut, religated	none	2.5×10^{6}	
*Bam*H1-cut	partial *Sau*3A I	2.5×10^{7}	2.0×10^{7}
*Bam*H1-cut+$Co(NH_3)_6Cl_3$	partial *Sau*3A I	2.5×10^{7}	2.0×10^{7}
*Bam*H1 arms*	none	0	
*Bam*H1 arms*	partial *Sau*3A I*	7.5×10^{4}	1.0×10^{5}

* Electroeluted after fractionation in agarose

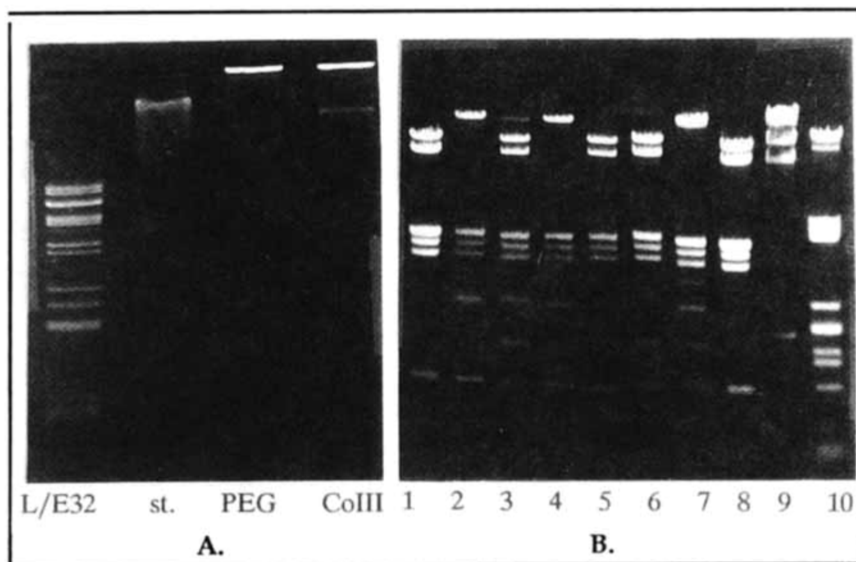

Fig. 1. A. Effect of the presence of PEG 6000 and Co (III) on blunt-end ligation. L/E32, λ DNA digested with Eco32I (Ferment, Vilnus, U.S.S.R.), an EcoRV isoshizomer , before ligation; st., standard ligation conditions; PEG, ligation in the presence of 12% (w/v) PEG 6000; CoIII, ligation in 1 mM $Co(NH_3)_6Cl_3$. **B. Restriction patterns of DNA from randomly selected positive plaques of wheat library.** E, *Pvu*II-cleaved EMBL4 DNA; B, *Bgl*II-cleaved λ DNA; P, *Pst*I-cleaved λ DNA.

that the background of non-recombinant phages does not exceed 10%. This allows the library to be screened directly without amplification under selective conditions. The presence of cobalt hexamine chloride improved the blunt end ligation significantly (Fig. 1A) but had no effect on the plating efficiency when ligating vector and inserts with cohesive ends. Plating efficiency in the latter case was approximately 50% of that of uncut EMBL4 vector.

The use of separated vector arms in place of intact vectors gave a significantly different and potentially more useful result. Ligation of fractionated donor DNA and vector arms gave 300 times fewer plaques than when unfractionated fragments were used. Nevertheless, the complete absence of background in the control experiment (religated arms) indicated that almost all plaques arising from the use of separated arms were recombinant. This was confirmed both by hybridization and restriction analysis. A randomly selected set of 200 plaques all hybrid-

ized with a [^{32}P]-labelled wheat DNA (data not shown). *Pvu*II digestion of DNA isolated from 14 randomly chosen plaques, from the set of 200 (Fig. 1B) showed that most of them had different restriction patterns.

The haploid wheat genome contains 1.7 x 10^{10} bp. In order to prepare a representative library of 20 kb inserts, it is necessary to generate about 3.9 x 10^6 clones ($P<0.01$) (Kaiser & Murray, 1985). This number was exceeded when wheat DNA was ligated to *Bam*HI-cut vector and the library was used directly for screening. Although the library obtained from the use of separated vector arms DNA cannot be considered representative, the absence of background plaques is attractive. The simplicity of the method allows the routine generation of libraries as needed when searching for a desired gene. According to Fedoroff (1985), it is necessary to screen about 10^5 recombinant phages when searching for a clone carrying a unique gene in a maize library. This number may be lowered when searching for repeated sequences or multigene family members.

Plant genomes generally contain large numbers of repeated sequences that may cause recombinational instability when cloned (Kaiser & Murray, 1985). For this reason, amplification of the library was avoided and the libraries were constructed in the host strain *E. coli* TAP90, which carries a mutation in *recD* (Patterson & Dean, 1987). This mutation has been shown (Wyman et al., 1986) to reduce the losses of recombinant phage clones from genomic libraries. TAP90 was also used for DNA minipreps from the individual plaques.

The wheat variety *Aurora* carries a translocated rye chromosome arm and has therefore some positive and negative agronomic traits (Koebner & Shepherd, 1986). Rye DNA sequences from this translocation may be isolated from genomic libraries by probing with rye-specific sequences. These rye translocation-specific sequences may be used for 1RS arm mapping and gene clóning. Several clones were isolated from the EMBL4 library by hybridization with different rye-specific probes and are currently being characterized.

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Erratum

Report of Meeting: VIth NATO Advanced Study Institute–Plant Genomes. Plant Mol. Biol. Report. 8(3):186-198.

p. 190. The sequence defined as ABRE by John Mundy (Copenhagen) should read TACGTGGC not GTGACTGGC as reported.