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TECHNICAL ADVANCE

An improved method of plant megabase DNA isolation in agarose microbeads suitable for physical mapping and YAC cloning

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Summary

The isolation of high quality megabase DNA from plant cells that is susceptible to a variety of molecular reagents is a critical first step in the physical analysis of complex genomes. A method for the isolation of such DNA by encapsulating plant protoplasts in agarose microbeads is presented. In comparison with the conventional agarose plug method, microbeads provide a dramatic increase in the surface area yielding megabase DNA that can be treated essentially as an aqueous DNA solution. Examples of the utility of DNA prepared by this technique for physical mapping, partial restriction enzyme digestion and cloning of large inserts as YACs are presented.

Introduction

The advent of pulse-field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984) and yeast artificial chromosome (YAC) (Burke *et al.*, 1987) cloning of large genomic fragments has fueled an explosion of genome research leading to the physical characterization and isolation of many important genes for which only a phenotype and map position were known (Arondel *et al.*, 1992; Rommens *et al.*, 1989; Ton *et al.*, 1991). An essential element of physical mapping and YAC cloning is the ability to isolate and manipulate relatively intact and unsheared chromosomal DNA. The most commonly used method is to embed intact cells or nuclei in agarose plugs followed by cell lysis, proteinase-K digestion and a thorough washing (Schwartz and Cantor, 1984). The agarose support matrix not only prevents DNA shearing during handling, but also provides enough porosity for diffusion of molecular reagents such as reaction buffer and enzymes.

DNA prepared in agarose plugs has been used successfully for physical mapping and YAC cloning in many systems (e.g. Albertson *et al.*, 1990; Anand *et al.*, 1989; Carle and Olson,1985; Cheung *et al.*, 1991; Deng *et al.*, 1989; Ganal *et al.*, 1989; Grill and Somerville, 1991; Ichikawa *et al.*, 1992; Larin *et al.*, 1991; Martin *et al.*, 1992; Poustka *et al.*, 1987; Smith and Cantor, 1987; Ward and Jen, 1990), however, there are a number of problems associated with the use of such DNA. The primary obstacle is the non-uniform surface area surrounding the DNA. Molecular reagents have greater access to megabase DNA near the surface of the plug than in the middle of the plug. As a result, higher concentration and longer incubation time are required to achieve complete restriction digestions (Cheung and Gale, 1990; Ganal and Tanksley, 1989; Honeycutt *et al.*, 1992; Smith and Cantor, 1987; Sobral *et al.*, 1990). Furthermore, many physical mapping and YAC cloning protocols require that megabase DNA be partially digested in a controlled manner. Although some groups have reported success with partial digests in agarose plugs (Albertson *et al.*, 1990; Anand *et al.*, 1989; Hanish and McClelland, 1990; Hoheisel, *et al.*, 1989; Larin *et al.*, 1991), no plant group published to date, has been successful in achieving reproducible partial digestions using comparable conditions. Consequently, many workers have to digest the agarose with agarase to release the megabase DNA, which is susceptible to partial digestions in a reproducible manner (Edwards *et al.*, 1992; Martin *et al.*, 1992). Unfortunately, the DNA is also subjected to shearing which can lead to smaller inserts for YAC cloning.

Many of the problems associated with DNA prepared in plugs can be overcome by simply increasing the surface area around the DNA in a uniform manner. This can be accomplished by mixing the agarose:whole cell mixture with oil to form an emulsion, which when rapidly cooled while being stirred vigorously results in agarose microbeads (Cook, 1984). DNA prepared in microbeads has all the advantages of megabase DNA prepared in plugs. In addition, because of the uniformity of micro-

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beads and about a 1000-fold increase in surface area, the DNA can be handled essentially as if it were in an aqueous solution. Despite these additional advantages, preparation of megabase DNA in microbeads has not been used extensively for genome research. Furthermore, it is yet to be tested with plant systems.

In this paper we describe the development of the agarose microbead technique for the model crop plant *Lycopersicon esculentum* (tomato) and present evidence of the utility of such DNA preparations for physical mapping and YAC cloning.

Results and discussion

Plant protoplast can be embedded in agarose microbeads

The method of embedding nuclei in agarose microbeads, described first by Cook (1984), to study human chromatin structure has been adapted to whole cells for physical mapping and YAC cloning (Carle and Olson, 1987; Ferrin and Camerini-Otero, 1991; Hall and Hink, 1990; Imai and Olson, 1990; Koob and Szybalski, 1990, 1992; McClelland, 1987; Overhauser and Radic, 1987; Piggot and Curtis, 1987). However, there is no published method using plant cells. Our initial attempts to embed tomato protoplasts prepared in MES protoplasting buffer (0.5 M mannitol; 20 mM 2[*N*-morpholino]-ethanesulfonic acid, pH 5.6; Ganal *et al.*, 1989) in agarose microbeads failed. Some beads did form, however, the majority were very large and about 50 to 100 µl in volume. It was noted that yeast spheroplasts are suspended in SCE (1.0 M sorbitol; 0.1 M sodium citrate; 0.01 M EDTA, pH 5.8) before being embedded into agarose microbeads (Overhauser and Radic, 1987). We found that tomato protoplasts are stable in SCE, however, upon centrifugation at 35 ***g***, the pellets are not quite as tight as with MES. We therefore decided to prepare tomato protoplasts as previously described and perform a final wash and cell suspension in SCE. The protoplasts were embedded in agarose microbeads as described in Experimental procedures and then processed as described in Ganal *et al.* (1989), except that a 5–10-fold lower proteinase-K concentration could be used during lysis. DNA prepared in beads by this method is stable for up to 1 year at 4°C in 50 mM EDTA (pH 8.0).

Restriction enzyme digestion of DNA embedded in agarose microbeads

To test the DNA prepared in microbeads for physical mapping, it was digested with various restriction endonucleases. Typically, for tomato megabase DNA prepared in plugs (Ganal *et al.*, 1989) 40–50 units of a restriction enzyme and an overnight incubation is required for complete digestion. We found that, for the equivalent amount of DNA embedded in microbeads, only 10–20 units of restriction enzyme is required with a 5 h incubation. The latter are standard conditions established for plant DNA used for RFLP mapping. The resulting DNA fragments were resolved by CHEF electrophoresis (Chu *et al.*, 1986) as shown in Figure 1(a). The presence of discrete bands suggests that the DNA was digested to completion.

This gel was blotted (Reed and Mann, 1985), hybridized, stripped and probed sequentially with six radiolabeled (Feinberg and Vogelstein, 1984) tomato RFLP markers. The hybridization pattern for one RFLP marker, TG523 (Giovannoni *et al.*, 1991), is shown in Figure 1(b). TG523 hybridized to discrete bands ranging between 50 and 600 kbp in the *Sma*I, *Sal*I, *Pac*I, *Nco*I and *Mlu*I digests, and to DNA in the unresolved focused region (≥1Mb in size) in the *Sac*II, *Sfi*I, *Rsr*II and *Not*I digests. *Rsr*II and *Not*I are known to produce mean fragment sizes of ≥1Mb (Ganal and Tanksley, 1989) so with the parameters chosen for this CHEF gel, we would only expect to see hybridization with the focused region of the gel. This was the case with all six hybridizations in the *Rsr*II lane and all but one hybridization in the *Not*I lane. RFLP marker TG194 (Tanksley *et al.*, 1992) hybridized to an 800 kb *Not*I restriction fragment (data not shown). Multiple bands were observed in *Pac*I digest, which could be due to incomplete digestion. This contention is supported by presence of multiple bands in subsequent hybridizations with five other RFLP markers (data not shown). No hybridization signal is detected in the *Nhe*I digest, because TG523 hybridized to 4.2 and 4.8 kbp fragments, too small to be retained in a CHEF gel (Rastogi, unpublished results). Subsequent hybridizations with five other RFLP probes showed discrete bands for the *Nhe*I digest in the 50–200 kbp range (data not shown).

Certain physical mapping and YAC cloning protocols require that megabase DNA be partially digested with restriction enzymes. Initial attempts to establish partial digestions with tomato DNA embedded in agarose plugs failed due to lack of reproducibility (Wing and McCormick, unpublished work). Therefore, we tested DNA in microbeads for its susceptibility to partial digestions by using varying amounts of *Eco*RI. The results shown in Figure 1(c) demonstrate that the partial digestion conditions can be established for DNA embedded in microbeads. The megabase DNA begins to be digested with 5 units of *Eco*RI, the majority of DNA has moved from the well to the compression zone (≥600 kbp). With 50 units of *Eco*RI, all the DNA appears to be completely digested, except for a minor background fraction.

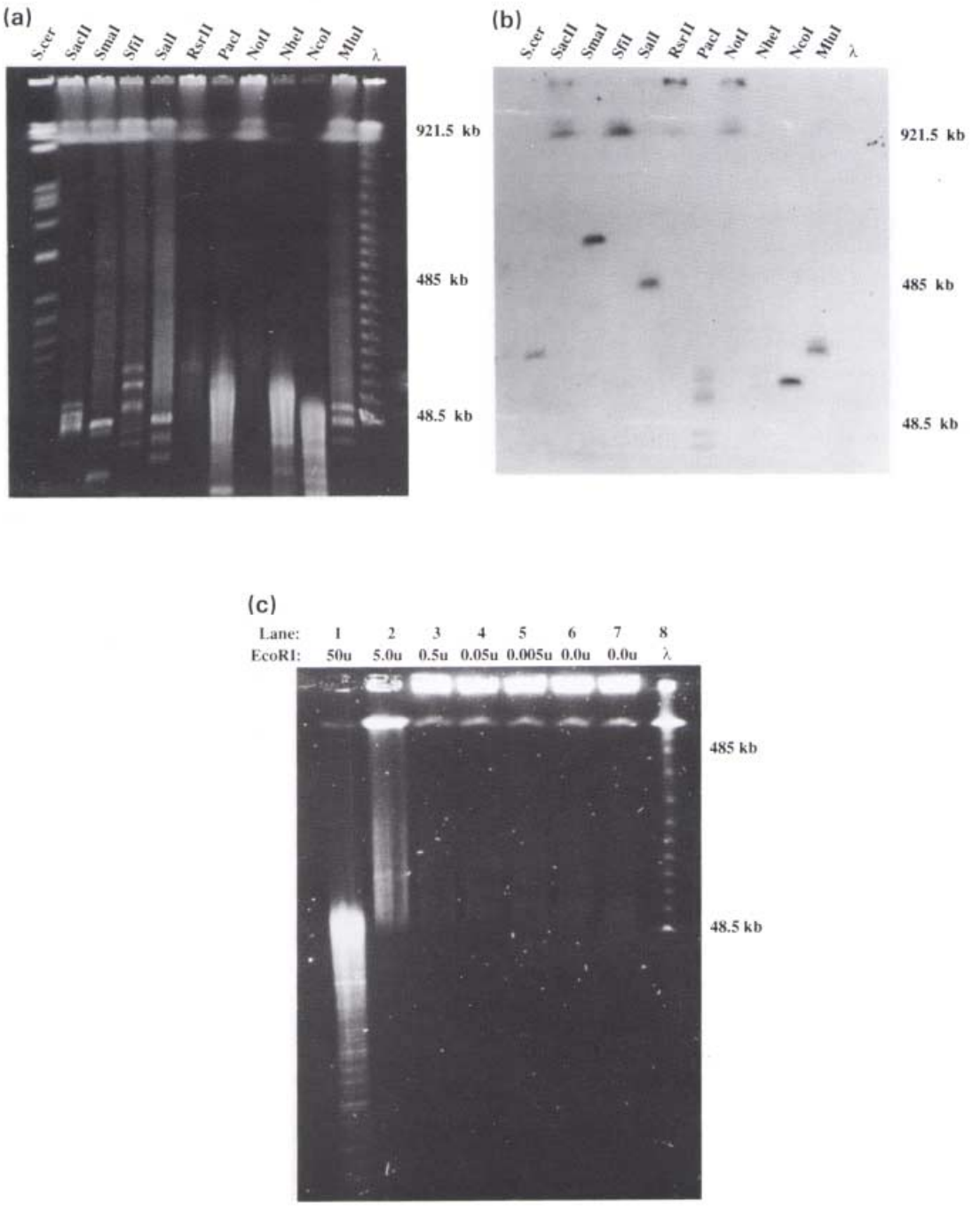

Figure 1. Physical mapping and partial digestion of megabase tomato DNA.
(a) Tomato VFNT cherry megabase DNA in microbeads digested with – lane 2: *Sac*II; lane 3: *Sma*I; lane 4: *Sfi*I; lane 5: *Sal*I; lane 6: *Rsr*II; lane 7: *Pac*I; lane 8: *Not*I; lane 9: *Nhe*I; lane10: *Nco*I; lane 11: *Mlu*I. Lane 1: *S. cerevisiae* containing a 200 kbp tomato YAC. Lane 12: lambda concatamer (FMC, USA). Restriction digestions were carried out in 100 µl volumes containing 88 µl of beads (2 µg); 10 µl of 10 × restriction enzyme buffer (supplied by manufacturer); 2 µl of restriction enzyme (10–20 units) for 5 h at the manufacturer's recommended temperatures. CHEF gel pulse conditions with MJ Research Programable Power Inverter: A = 100 sec; B = –0.05 sec; C = 100 sec; D = –0.05 sec; E = 175 times; F = –0.01 sec; G = –0.01 sec. Gel was run for 40 h with 150 V at 11°C.
(b) Autoradiogram of alkaline Southern blot (Reed and Mann, 1985) of gel in (a) to Hybond-N^+ (Amersham). Blot was hybridized with ^{32}P-labeled TG523 for 24 h followed by three 30 min washes with 0.5 × SSC and 0.1% SDS at 65°C and autoradiographed.
(c) Tomato VFNT cherry megabase DNA in microbeads digested with increasing amount of *Eco*RI for 15 min at 37°C except no enzyme in lanes 6 and 7, and lane 7 at 4°C. Lane 8: lambda concatamer (FMC, USA). CHEF gel pulse conditions with MJ Research Programable Power Inverter: A = 40 sec; B = 0 sec; C = 40 sec; D = 0 sec; E = 1 time; F = 0 sec; G = 0 sec. Gel was run for 18 h with 150 V at 11°C.

The ability to digest DNA in microbeads to completion with less enzyme, reduced incubation times, and reproducible partial digestions demonstrate that such preparations can essentially be handled as DNA isolated by conventional means. Both of these features can be attributed to the increased surface area provided by the beads which permit rapid diffusion of molecular reagents.

YAC cloning

The establishment of map-based cloning systems for crop plants is a principal objective of our group. An indispensable component of such a system is a complete and representative YAC library. In plants, each of the published YAC libraries have average insert sizes of approximately 150 kbp using DNA partially digested in aqueous solution (Edwards *et al.*, 1992; Grill and Somerville, 1991; Martin *et al.*, 1992; Ward and Jen, 1990). However, in mammalian systems using DNA that was partially digested in agarose, several YAC libraries have been constructed that have average insert sizes ranging from 350 to 700 kbp (Albertson *et al.*, 1991; Imai and Olson, 1990; Larin *et al.*, 1991).

To test the cloning properties of tomato DNA in microbeads we constructed partial YAC libraries from VFNT cherry and TA208 plant material. Over 4000 YACs have been obtained using such DNA preparations. Thirty-six randomly chosen YAC DNAs were prepared and analyzed by hybridization for insert size and origin (results not shown). The hybridization results showed that the cloned fragments ranged in size from 57 to 411 kbp, which fell into two broad size classes. Thirty-five per cent of the YACs contained inserts of greater than 350 kbp, while 57% of inserts were smaller than 100 kbp. The remaining 8% YACs contained fragments of 125 kbp. The majority of YACs showed a strong hybridization signal when hybridized to total tomato DNA, indicating their origin from repetitive regions of the genome. Only two of the 35 YACs showed hybridization with chloroplast-derived sequences.

The generation of two size classes of YACs may have resulted from our failure to remove efficiently the small-size fragments (50–150 kbp) from large-sized fragments in the focused region during the size selection steps due to trapping in the CHEF gel. This trapping could be eliminated by using a more stringent size selection method such as the sucrose gradient velocity sedimentation employed by Imai and Olson (1990). If such fragments were eliminated, construction of YAC libraries with average inserts of ≥350 kbp like those recently described for human and mouse systems seems feasible for plants.

Conclusion

This paper presents the first demonstration of the agarose microbead method in plants. A single modification of the protoplast suspension buffer (from MES to SCE) made it possible to generate sufficient quantities of megabase tomato DNA suitable for physical mapping and YAC cloning. In addition, this protocol has been distributed to several laboratories prior to publication and has been shown to be successful in a number of dicot and monocot crop plants (personal communications: lettuce, D. Francis and R. Michelmore, UC Davis; tobacco, S. Whitam and B. Baker, PGEC, Albany, CA; maize, B. Burr, BNL; and sorghum and cotton, S.-S. Woo, A. Paterson and R. Wing, TAMU).

Experimental procedures

Plant materials and growth

Tomato (*Lycopersicon esculentum* cv. TA208 and VFNT cherry) plants were grown in a greenhouse. Young green leaves from 4–5 week-old plants were harvested as required for protoplast isolation.

Preparation of megabase DNA in agarose microbeads

Tomato protoplasts were prepared essentially as described by Ganal and Tanksley (1989) except Cellulysin (Calbiochem Corp., USA) was used instead of Onozuka Cellulase (we generally prepare 40–60 g of leaf material per DNA preparation). The protoplast pellet was washed once with 10 ml of SCE (1.0 M sorbitol; 0.1 M sodium citrate; 10 mM EDTA; pH 7.0) and gently resuspended at a concentration of 2×10^7 protoplasts per ml in SCE. The protoplast suspension was briefly warmed to 45°C and mixed gently with an equal volume of 1% low-melting-point agarose in SCE. Quickly, two volumes of prewarmed light mineral oil (Mallinckrodt, USA) were added and swirled vigorously for less than 5 sec. The emulsion was immediately dumped into 150 ml of rapidly swirling, ice-cold SCE. After 5 min of stirring on ice, the microbeads were pelleted by centrifugation at 500 ***g*** and pooled. A small fraction of the microbeads was found trapped in the aqueous/oil interface. This was also collected, diluted with SCE and recentrifuged. The microbead pellet was pooled and resuspended in 50 ml of a solution containing 0.5 M EDTA (pH 9.0), 1% sarkosyl, and 0.1–0.2 mg ml^{-1} proteinase-K, and incubated for 24–36 h at 50°C with slow agitation. The microbeads were extensively washed (≥1 h) at 4°C with three changes of 10 mM Tris–10 mM EDTA (Tris–EDTA, pH 8.0) solution containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), followed by three changes of the Tris–EDTA solution lacking PMSF. Megabase DNA in microbeads was stored in 50 mM EDTA for long-term storage at 4°C.

Eco*RI partial digestion of tomato DNA in microbeads and ligation into pYAC4*

Each assay for partial digestion contained 90 µl of microbead suspension (approx. 2.5 µg DNA), 1 × *Eco*RI buffer (Promega, USA),

2 mM spermidine in a final volume of 116 µl. The reaction was started by adding 4 µl of enzyme solution containing varying amounts of *Eco*RI (ranging from 0.1 to 50 units), incubated at 37°C for 20 min, and stopped by adding 12 µl of 0.5 M EDTA (pH 8.0). Typically, 2.5–10 units of *Eco*RI resulted in appreciable release of fragments in the size range of 90–500 kbp from megabase DNA. For YAC cloning, the partial digestions were scaled up to 12 ml. Using CHEF, fragments of less than 150 kbp were removed from the digested samples. pYAC4 was digested with *Bam*HI and *Eco*RI, and 5' PO_4^- removed by using calf intestine alkaline phosphatase (BRL, USA). Approximately equal µg of pYAC4 and size-selected *Eco*RI fragments were mixed. Equal volumes of 2 × ligation cocktail was added to the DNA mixture. The ligation cocktail (1 ×) contained 50 mM Tris, pH 7.5, 30 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, and 400 units of T_4 DNA ligase (Promega, USA). After 24 h of ligation at 22°C, the agarose was melted at 68°C, and the ligated DNA was size-selected as above. The agarose was then liquefied, and digested with β-agarase (New England Biolabs, USA) at 37°C. DNA thus obtained was dialyzed and concentrated against 10 mM Tris–HCl, 30 mM NaCl, 1 mM EDTA (pH 8.0) solution using colloidin bags and a Schleicher and Schuell concentrator, and stored at 4°C until used further for yeast transformation.

Yeast transformation and YAC analysis

Saccharomyces cerevisiae strain AB1380 (MATa, *ura*3 *trp*1 *ade*2-1 *can*1-100 *lys*2-1 *his*5) was used as host cells in transformation (kindly provided by D. Burke and M. Olson). Plasmid pYAC4 and YCp50 DNAs were used in controls to test transformation efficiency. The media and solution used were prepared as described by Sherman *et al.* (1992) unless otherwise indicated. Transformations were performed according to Burgess and Percival (1987) as modified by Albertson *et al.* (1990). Less than 1/10 volume of ligated DNA (about 1 µg per 150 × 15 mm petri dish) over the spheroplasts and 10 × amount of carrier DNA over that of the ligated DNA were used in transformation. The transformation was plated on to the solidified synthetic complete medium (SD) containing 1.0 M sorbitol and lacking uracil. After 4–7 days of incubation at 30°C red colonies were transferred on to the SD medium lacking uracil and tryptophan. Typically, 1–6 × 10^6 transformants per µg of plasmid DNA were obtained in controls and from 500 to 1500 YACs were obtained per µg of ligated DNA.

Thirty-six random colonies grown on selective plates lacking uracil and tryptophan were grown in 2 ml of YPD broth each with shaking at 250 r.p.m. (20 ***g***) at 30°C overnight. The cells were harvested, converted to spheroplasts, embedded in 100 µl agarose plugs and YAC DNA was isolated as described by Burke *et al.* (1987). The resulting yeast DNA was resolved by CHEF electrophoresis, blotted and hybridized as described in the results.

Pulse-field gel electrophoresis and Southern blotting

PFGE was performed by running DNA through 1% agarose gels in 0.5 × 89 mM Tris, 2.5 mM EDTA, 89 mM Boric acid using the CBS Scientific HEX-CHEF 6000 at 11°C. Switching times and voltages are indicated in figure legends. The gels were blotted as described in Ganal *et al.* (1987).

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References

Albertson, H.M., Abderrahim, H., Cann, H.M., Dausset, J., Paslier, D.L. and Cohen, D. (1990) Construction and characterization of a yeast artificial chromosome library containing seven haploid human genome equivalents. *Proc. Natl Acad. Sci. USA*, **87**, 4256–4260.

Anand, R., Villasante, A. and Tyler-Smith, C. (1989) Construction of yeast artificial chromosome libraries with large inserts using fractionation by pulsed-field gel electrophoresis. *Nucl. Acids Res.* **17**, 3425–3433.

Arondel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H.M. and Somerville, C.R. (1992) Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. *Science*, **258**, 1353–1355.

Burgess, P.M. and Percival, K.J. (1987) Transformation of yeast spheroplasts without cell fusion. *Anal. Biochem.* **163**, 391–397.

Burke, D.T., Carle, G.F. and Olson, M.V. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science*, **236**, 806–811.

Carle, G.F. and Olson, M.V. (1985) An electrophoretic karyotype for yeast. *Proc. Natl Acad. Sci. USA*, **82**, 3756–3760.

Carle, G.F. and Olson, M.V. (1987) Orthogonal-field gel electrophoresis. *Methods Enzymol.* **155**, 468–482.

Cheung, W.Y. and Gale, M.D. (1990) The isolation of high molecular weight DNA from wheat, barley and rye for analysis by pulse-field gel electrophoresis. *Plant Mol. Biol.* **14**, 881–888.

Cheung, W.Y., Chao, S. and Gale, M.D. (1991) Long-range physical mapping of the α-amylase-1 (α-*Amy*-1) loci on homeologous group 6 chromosomes of wheat. *Mol. Gen. Genet.* **229**, 373–379.

Chu, G., Vollrath, D. and Davis, R.W. (1986) Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science*, **234**, 1582–1585.

Cook, P.R. (1984) A general method for preparing intact nuclear DNA. *EMBO J.* **3**, 1837–1842.

Deng, X.-W., Wing, R.A. and Gruissem, W. (1989) The chloroplast genome exists in multimeric forms. *Proc. Natl Acad. Sci. USA*, **86**, 4156–4160.

Edwards, K.J., Thompson, H., Edwards, D., Saizieu, A.D., Sparks, C., Thompson, J.A., Greenland, A.J., Eyers, M. and Schuch, W. (1992) Construction and characterization of a yeast artificial chromosome library containing three haploid maize genome equivalents. *Plant Mol. Biol.* **19**, 299–308.

Feinberg, A.P. and Vogelstein, S. (1984) A technique for radiolabeling DNA fragments to high specific activity. *Anal. Biochem.* **137**, 266–267.

Ferrin, L.J. and Camerini-Otero, R.D. (1991) Selective cleavage of human DNA: RecA-assisted restriction endonuclase (RARE) cleavage. *Science*, **254**, 1494–1497.

Ganal, M.W. and Tanksley, S.D. (1989) Analysis of tomato DNA by pulsed field gel electrophoresis. *Plant Mol. Biol. Rep.* **7**, 17–27.

Ganal, M.W., Young, N.D. and Tanksley, S.D. (1989) Pulsed field gel electrophoresis and physical mapping of large DNA fragments in the *Tm-2a* region of chromosome 9 in tomato. *Mol. Gen. Genet.* **215**, 395–400.

Giovannoni, J.J., Wing, R.A., Ganal, M.W. and Tanksley, S.D. (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. *Nucl. Acids Res.* **19**, 6553–6558.

Grill, E. and Somerville, C. (1991) Construction and characterization of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. *Mol. Gen. Genet.* **226**, 484–490.

Hall, R.L. and Hink, W.F. (1990) Physical mapping and field inversion gel electrophoresis of *Amsacta moorei* entomopox virus DNA. *Arch. Virol.* **110**, 77–90.

Hanish, J. and McClelland, M. (1990) Methylase-limited partial *Not*I cleavage for physical mapping of genomic DNA. *Nucl. Acids Res.* **18**, 3287–3291.

Hoheisel, J.D., Nizetic, D. and Lehrach, H. (1989) Control of partial digestion combining the enzymes *dam* methylase and *Mbo*I. *Nucl. Acids Res.* **17**, 9571–9582.

Honeycutt, R.J., Sobral, B.W.S., McClelland, M. and Atherly, A.G. (1992) Analysis of large DNA from soybean (*Glycine max* L. Merr.) by pulsed-field gel electrophoresis. *Plant J.* **2**, 133–135.

Ichikawa, H., Shimizu, K., Saito, A. *et al.* (1992) Long-distance restriction mapping of the proximal long arm of human chromosome 21 with *Not*I linking clones. *Proc. Natl Acad. Sci. USA*, **89**, 23–27.

Imai, T. and Olson, M.V. (1990) Second generation approach to the construction of yeast artificial-chromosome libraries. *Genomics*, **8**, 297–303.

Koob, M. and Szybalski, W. (1990) Cleaving yeast and *Escherichia coli* genomes at a single site. *Science*, **250**, 271–273.

Koob, M. and Szybalski, W. (1992) Preparing and using agarose microbeads. *Methods Enzymol.* **216**, 13–20.

Larin, Z., Monaco, A.P. and Lehrach, H. (1991) Yeast artificial chromosome libraries containing large inserts from mouse and human DNA. *Proc. Natl Acad. Sci. USA*, **88**, 4123–4127.

Martin, G.B., Ganal, M.W. and Tanksley, S.D. (1992) Construction of a yeast artificial chromosome library of tomato and identification of cloned segments linked to two disease resistance loci. *Mol. Gen. Genet.* **223**, 25–32.

McClelland, M. (1987) Site-specific cleavage of DNA at 8-, 9-, and 10-bp sequences. *Methods Enzymol.* **155**, 22–27.

Overhauser, J. and Radic, M.Z. (1987) Encapsulation of cells in agarose beads for use with pulsed-field gel electrophoresis. *Focus*, **9**, 8–9.

Piggot, P.J. and Curtis, A.M. (1987) Analysis of the regulation of gene expression during *Bacillus subtilis* sporulation by manipulation of the copy number of *spo-lacZ* fusions. *J. Bacteriol.* **169**, 1260–1266.

Poustka, A., Pohl, T.M., Barlow, D.P., Frischauf, A.-M. and Hehrach, H. (1987) Construction and use of human chromosome jumping libraries from *Not*I-digested DNA. *Nature*, **325**, 353–355.

Reed, K.C. and Mann, D.A. (1985) Rapid transfer from agarose gels to nylon membranes. *Nucl. Acids Res.* **13**, 7207–7221.

Rommens, J.M., Iannuzzi, M.C., Kerem, B. *et al.* (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*, **245**, 1059–1065.

Schwartz, D.C. and Cantor, C.R. (1984) Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*, **37**, 67–75.

Sherman, F., Fink, G.R. and Hicks, J.B. (1986) *Laboratory Manual for Methods in Yeast Genetics.* New York: Cold Spring Harbor Laboratory Press.

Smith, C.L. and Cantor, C.R. (1987) Purification, specific fragmentation, and separation of large DNA molecules. *Methods Enzymol.* **155**, 449–467.

Sobral, B.W.S., Honeycutt, R.J., Atherly, A.G. and McClelland, M. (1990) Analysis of rice (*Oryza sativa* L.) genome using pulsed-field electrophoresis and rare cutting restriction endonucleases. *Plant Mol. Biol. Rep.* **8**, 253–275.

Tanksley, S.D., Ganal, M., Prince, J. *et al.* (1992) High density molecular linkage maps of the tomato and potato genomes: biological inferences and practical applications. *Genetics*, **132**, 1141–1160.

Ton, C.C., Hirvonen, H., Miwa, H. *et al.* (1991) Positional cloning and characterization of a paired box- and homeobox-containing gene from the *Aniridia* region. *Cell*, **67**, 1059–1074.

Ward, E.R. and Jen, G.C. (1990) Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly-sheared *Arabidopsis thaliana* DNA. *Plant Mol. Biol.* **14**, 561–568.