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Development of large DNA methods for plants: molecular cloning of large segments of *Arabidopsis* and carrot DNA into yeast

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ABSTRACT

Procedures for the preparation, analysis and cloning of large DNA molecules from two different plant species are described. *Arabidopsis* and carrot protoplasts were used for the preparation of large DNA molecules in agarose "plugs" or in solution. Pulsed-field gel electrophoresis (PFGE) analysis of large plant DNA preparations using a contour-clamped homogeneous field (CHEF) apparatus indicated that the size of the DNA was at least 12 Mb. Large DNA preparations were shown to be useful for restriction enzyme analysis of the *Arabidopsis* genome using both frequent and infrequent cutting enzymes and for the molecular cloning of large segments of DNA into yeast using artificial chromosome (YAC) vectors. PFGE and blot hybridization analysis of *Arabidopsis* and carrot DNA-containing YACs indicated that both unique and highly repeated DNA sequences were represented in these libraries.

INTRODUCTION

There are many cases in pure and applied plant science where genes may be mapped and characterized genetically, yet these genes cannot be isolated by conventional procedures. Examples include genes involved in the biosynthesis or recognition of plant hormones (1), genes conferring resistance to plant pathogens (2) and genes critical for developmental pathways (3,4). Clearly, the ability to isolate these genes would greatly facilitate studies of gene function and eventually may lead to methods for manipulation. By mapping relative to restriction fragment length polymorphism (RFLP) probes (5), it is theoretically possible to isolate such genes using the approach known as "chromosome walking" (6). The difficulty with such an approach for most genes is that the closest RFLP hybridization probe may be several hundred kb (kilobase pairs) or even several Mb (megabase pairs) removed (7,8,9,10). The standard walking strategies are cumbersome and time consuming, at best. New methods which allows one to "jump" along a chromosome have been devised (11,12). However, jumping is technically complex and requires the construction of many specialized libraries for each jumping experiment. Furthermore, the distance of jumps may be severely limited by the non-random nature of infrequent cutting restriction sites in an organism genome (e.g. 13).

In addition to problems with chromosome walking, a number of genetic units in higher organisms have been found to span enormous tracts of DNA. For example, in *Drosophila,* the bithorax complex is over 300 kb (14) and the regulatory sequences for the *engrailed* locus act over a similar

distance (15). In humans, the muscular dystrophy gene may be as large as 2500 kb (16). It is likely that plants will possess similarly large genes. Thus, the conventional chromosome walking/gene complementation approach is not only cumbersome and inefficient, it simply will not work for genes with exceptionally large transcripts or cis-regulatory regions. Efficient walking and molecular characterization of these genes may be possible if techniques for the preparation, cloning and transfer of large DNA segments are developed.

The recent development of several technologies should dramatically increase the ease of gene isolation by chromosome walking. Primary amongst these is the development of pulsed-field gel electrophoresis (PFGE) (17,18). This technique has allowed the separation of DNA molecules of up to 12 Mb (19), an improvement upon conventional techniques of over two orders of magnitude. A second important development is the demonstration that segments of DNA as large as 500 kb can be cloned as yeast artificial chromosomes (YACs) (20). This is an improvement of at least an order of magnitude over previously existing cloning procedures. We have begun to develop and apply these techniques to the preparation, analysis and cloning of large segments of DNA from plants, in particular for *Arabidopsis thaliana* and *Daucus carota* (carrot).

MATERIALS AND METHODS.

Yeast Strains and plamids

The *Saccharomyces cerevisiae* strain AB1380 (*Mat* -a, *ade*2-1, *can*1-100, *lys*2-1, *trp*1, *ura*3, *his*5 [psi +]) (20) was used to generate competent spheroplasts for DNA-mediated transformation. Chromosomal DNA markers were prepared from *Schizosaccharomyces pombe* strain 975 (h+) and *S. cerevisiae* strains, AB1380 and YPH274 (yeast strains were provided by D. Vollrath, M. Olson and P. Hieter, respectively). Artificial chromosomes containing plant DNA sequences were constructed in the vector pYAC-4 (20). The yeast plasmid YCp50, obtained from R. Davis (Stanford Univ.) was used to calculate the efficiency of yeast spheroplast transformation.

Plant strains and growth conditions

The *Arabidopsis* strain used in this work was, *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia, obtained from G. Redei (Univ. of Missouri). Axenic cultures of *Arabidopsis* were prepared on Murashige and Skoog salts (MS, Gibco) [pH 5.7] supplemented with 10 g/l sucrose, 1 mg/ l thiamine HCl, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 100 mg/l inositol and containing 0.8% agar (bacto-agar, Difco). Seeds were surface-sterilized for 8 min in a solution containing 5% sodium hypochlorite (Clorox) and 0.15 % Tween 20 and then washed 5 times with sterile distilled water. Seeds were plated with 0.8 % low melting agarose in 100 x 15 mm petri plates, cold treated at 4°C for four days and then transferred for germination to 25° C under continuous illumination.

Isolation of high molecular weight (MW) DNA

High MW DNA from *Arabidopsis* was isolated from protoplasts of *in vitro* cultured seedling segments. About 200 sterile seedlings (1 week old) were cut into small segments (~2 mm long)

and transferred to a 125 ml Ehrlenmeyer flask containing 50 ml of RM 28 medium (21). RM 28 medium consists of MS salts supplemented with 0.2 mg/l 2,4-D, 0.05 mg/ml kinetin, 0.1 M sucrose and buffered with 3 mM 2 [N-morpholino] ethanosulfonic acid (MES) at pH 5.8. Flasks were agitated at 130 rev/min at 25° and the medium was changed every 5-7 days by pouring off the spent medium and any free floating cells. After 3-4 weeks, cultures consisted mainly of clumps of very small cells (< 10 μM). Protoplasts were then isolated by replacing the medium with 50 ml of protoplast isolation medium (PIM) which consists of RM 28 medium with 5 mM $CaCl_2$, 0.6 M sucrose, 1 % Cellulysin (Calbiochem) and 0.2 % Pectolyase Y23 (Seishin Pharmaceutical); the mixture was poured into a 150 x 15 mm petri dishes. The cell wall digestion proceeded for 8-15 hours at room temperature with gently shaking (25 rev/min) and was followed by microscopic observation. Protoplasts were filtered through nylon monofilament (60 μm) and pelleted at 300 xg for 15 min in a table top centrifuge. At this point, the protoplasts were used to prepare high MW DNA either in agarose plugs or in solution.

To make agarose plugs, one volume of packed protoplasts was mixed with one-half volume of molten (37°C) 2 % low-melting temperature agarose (Boehringer-Mannheim) by gently pipetting up and down using blunted pipet tips; the final concentration of protoplasts was approximately 1-2 x 10^8/ml. The molten mixture was quickly loaded into 2 x 5 x 10 mm plastic molds and placed at 4°C for 10 min. until the agarose solidified. The plugs were placed into NDS (17) (0.5 M EDTA pH 8, 1 % sodium N-lauroylsarcosine, 2 mg/ml Proteinase K, Boehringer Mannheim), incubated for 24-48 hr at 50°C and then stored at 4°C. For restriction enzyme digestion of *Arabidopsis* DNA plugs, the methods of Smith *et al.* (22) were followed.

High MW DNA in solution was prepared by sucrose gradient sedimentation of a crude cell lysate (23). One volume of packed plant protoplasts was gently resuspended in one-half volume of PIM and slowly mixed with 2 volumes of lysis buffer (3 % sodium N-lauroylsarcosine, 0.5 M Tris-HCl [pH 9], 0.2 M EDTA) in a 250 ml Ehrlenmeyer flask by very gentle rotation. The cell lysate was heated to 68°C for 15 min. and then gently transferred to the top of a crude sucrose gradient. Sucrose gradients were prepared essentially as described (23), with two exceptions: 1) a 5 ml cushion of 50% sucrose was used and 2) the gradients were centrifuged at 5, 000 rpm for 16 hr at 20°C in a Beckman SW 28 rotor. Following centrifugation, the DNA was found associated with a soft slightly yellowish colored pellet which probably also contained carbohydrates and cell wall material. This very viscous pellet was dialysed against TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and then stored at 4°C. At this stage, the DNA was handled as a viscous clump, samples were obtained by cutting the clump with a razor blade or glass coverslip and the volume was measured by gentle pipetting with a blunted pipet tip. These crude plant DNA preparations were reliably digested with restriction enzymes. High MW DNA from carrot was prepared from protoplasts of a suspension culture line (24) following the protocol used for *Arabidopsis*.

Construction of plant DNA-containing yeast artificial chromosomes

Yeast artificial chromosomes were generated by cloning partial *Eco*RI-digested plant DNA

fragments into the *Eco*RI site of the vector pYAC4. Briefly, 500 µl of DNA "clump", estimated to contain 50-100 µg of DNA, was very gently mixed with 250 µl of 3X *Eco*RI restriction buffer containing 0.1 unit of *Eco*RI (Boehringer Mannheim) and 100 µg/ml BSA. The DNA was digested for 15 minutes at 37°C and the reaction was stopped by heat inactivation at 65°C for 10 minutes. pYAC4 was digested to completion with *Bam*H I and *Eco*R I (Boehringer Mannheim) and treated with calf-intestinal alkaline phosphatase (Pharmacia). A typical ligation reaction consisted of 10 units of ligase (Boehringer- Mannheim), 50 µl plant DNA (5-10 µg), 10 µg pYAC4 DNA in ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT). After ligating overnight at 12° C, nine microliters of the ligation mixture was used to transform yeast using a high efficiency spheroplast transformation procedure (25). Briefly, yeast AB1380 spheroplasts were prepared using a commercially available crude lyticase (Sigma), dissolved in buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 % glycerol) to a concentration of 10,000 units/ ml. Using 1000 units of lyticase, 90% of the yeast cells (AB 1380) were converted into spheroplasts within 16-22 minutes. The number of transformants routinely obtained using this method was 1 $x10^3$/ng YCp50 DNA. The total number of transformants (*Ura+*, *Trp+*, *Ade-*) obtained in a typical YAC/plant DNA ligation experiment was 1-2 x 10^4. Individual transformants were picked, arrayed on selective plates and incubated at 30 °C for 48 hr. Yeast DNA preparations embedded in agarose "plugs" were then prepared for both *Arabidopsis* and carrot DNA-containing yeast transformant (17). For transformants containing *Arabidopsis* YACs, individual yeast colonies were grown overnight at 30 °C in 5 ml of synthetic medium (lacking uracyl and adenine), washed twice in 50 mM EDTA (pH 7.5) and resuspended in 150 µl of the same solution. To this mixture, 5 µl of 10 mg/ml Zymolyase 60,000 (Seikagaku Kogyo Co.) in 10 mM sodium phosphate and 250 µl of 1 % molten (45°C) low-melting temperature agarose containing 125 mM EDTA were added. The mixture was immediately placed on ice to solidify the agarose and the plugs were then incubated overnight at 37°C in 0.5 ml of LET buffer (0.5 M EDTA, 10 mM Tris-HCl [pH 7.5]). To lyse the spheroplasts, LET buffer was replaced with 0.5 ml of NDS and placed overnight at 50°C. In order to speed up yeast plug preparation, both of the overnight incubation steps were subsequently reduced to 4 hr with comparable results. Carrot DNA containing YACs were prepared essentially as described above except that individual yeast transformants were grown overnight in rich medium (YPD) instead of selective medium.

Gel electrophoresis conditions

CHEF gel electrophoresis was preformed as previously described (26, 27) using apparatus with vertical driving electrodes separated by 26.5 cm. Agarose gels (1 %) in 0.5 X TBE (28) were prepared by pouring 50 ml of agarose into a 10 x 10 cm frame (or 75 ml for a 12 x 12 cm frame) which was positioned in the center of the buffer tank where the bottom of the tank had been abraded with medium grade emery paper. Electrophoresis was carried out using 0.5 X TBE running buffer and a constant temperature of 9°C was maintained by recirculation of the buffer through a heat exchanger. DNA samples in 2 x 5 x 10 mm agarose blocks were inserted directly

into the gel wells. Samples not prepared in molds were melted by heating at 65°C for 5 min. before loading and samples of liquid DNA were loaded with a blunted pipet tip and sealed into the well with 1 % low melting temperature agarose. The electrophoresis conditions are described in the figure legends. CHEF gels were stained with 0.5 μg/ml ethidium bromide for 20 min. and destained in water for 20 min.

DNA transfer and hybridization conditions

Efficient transfer of DNA from CHEF gels was achieved by treatment with 500 ml of 0.2 N HCl, twice for 15 min. each, followed by treatment with 0.5 M NaOH/1.5 M NaCl, twice for 20 min. each and finally by treatment with 1 M ammonium acetate/0.1 M NaOH, once for 30 min. (29). The DNA was transferred from the gel to Genatran 45 nylon membranes (Plasco) by capillary transfer for 24 hr using 1 M ammonium acetate/0.1 M NaOH. The membranes were baked for 2 hr at 80°C and then prehybridized for 2-6 hr at 42°C in a solution of 50 % formamide, 5X SSPE, 5X BFP (28), 1 % SDS, 100 μg/ml sonicated denatured salmon sperm DNA. Hybridization was carried out for 24-36 hr in the above solution with the addition of 5 % sodium dextran sulfate. Radiolabeled DNA probes (~ 5 x 10^8 cpm/μg) were prepared using the random hexamer priming method (30) and 1-2 x 10^6 cpm/ml of probe was used for each hybridization. After hybridization, membranes were washed twice in 50 % formamide (practical grade), 5X SSPE, 0.2 % SDS at room temperature for 15 min. each, followed by two 30 min. washes at 60°C in 0.1X SSPE, 0.1 % SDS. To remove the probe, the membranes were heated to 95°C in 0.1X SSPE, 0.1 % SDS for 15-30 min.

RESULTS

The first step for the development of large DNA methods for plants is the isolation of high molecular weight (MW) plant DNA. Several different methods for the preparation of high MW *Arabidopsis* DNA were explored. These included standard procedures of DNA preparation such as grinding of various plant tissues followed by CsCl gradient centrifugation or by isolation of DNA from leaf mesophyll protoplasts or nuclei. These methods were largely unsuccessful for obtaining sufficient quantities of *Arabidopsis* DNA which was greater than 150 kb in size (data not shown). The method devised for preparing sufficient quantities of large *Arabidopsis* DNA was to isolate DNA from callus suspension cultures. Procedures for preparing high MW DNA from yeast spheroplasts (17) were adapted for the isolation of large DNA from plant cell protoplasts. One method involves embedding of plant cell protoplasts in low-melting temperature agarose, followed by the infusion of proteinase in the presence of detergents and high EDTA. The final sample consists of high molecular weight DNA within a cavity in the agarose "plug" where the plant cell once resided. It was also possible to prepare unbroken chromosomal DNA from plant cells by first suspending live cells in low melting temperature agarose and allowing the samples to solidify. Chromosomal DNA was then prepared by "protoplasting" cells in the agarose block using cellulase and pectinase, followed by diffusing in proteinase in the presence of detergent and high concentra-

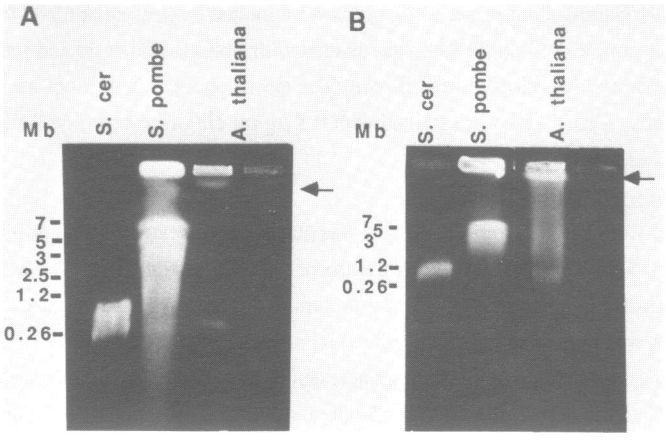

Figure 1. PFGE of large *Arabidopsis* DNA using different electrophoresis conditions. Chromosomal DNA-containing agarose plugs prepared from *S. cerevisiae* strain AB1380, *S. pombe* strain 975 and *Arabidopsis thaliana* ecotype Columbia, were electrophoresed in a 1 % agarose gel using a CHEF apparatus. Following electrophoresis, the gels were stained with ethidium bromide (**A,B**). The conditions for gel electrophoresis were optimized for the resolution of DNA molecules up to 7.5 Mb (**A**, 40 volts, 90 min. pulse length for 168 hrs) or 12.5 Mb (**B**, 30 volts, 180 min pulse length for 192 hrs). Arrows to the right of each gel indicate the position where large *Arabidopsis* DNA migrates. For each gel, two different concentrations (approx. 2 µg or 0.5 µg) of *Arabidopsis* DNA were used.

tions of EDTA. *Arabidopsis* or carrot DNA prepared in this way did not differ significantly in size from the method described above (data not shown).

Two different electrophoresis protocols were employed for PFGE of *Arabidopsis* DNA. Varying certain electrophoresis parameters (pulse interval, voltage and run time) has previously allowed the resolution of DNA molecules as large as 7.5 Mb (22,27) or even 12.5 Mb (19). *Arabidopsis* DNA plugs were prepared and examined by PFGE using electrophoresis conditions which were identical to those required to resolve the largest *S. pombe* or *N. crassa* chromosomes. PFGE analysis of these DNA preparations using a contour-clamped homogeneous electric field (CHEF) apparatus (26) revealed the presence of large (>9 Mb) *Arabidopsis* DNA molecules (Fig.1A and 1B). Essentially identical results were obtained using either electrophoresis protocol; large unresolved DNA molecules, which have migrate into the gel, were observed. Further PFG-analysis of *Arabidopsis* DNA using longer pulse intervals (4.0 hr) and longer gel run times (21 days) showed that the majority of this DNA was greater than 12 Mb (data not shown).

The development of large DNA methods for plants requires that the isolated DNA can be used for restriction enzyme mapping and/or cloning experiments. Large *Arabidopsis* DNA embedded in agarose plugs was found to be sensitive to digestion with a number of restriction enzymes. High MW *Arabidopsis* DNA in agarose plugs was digested with the both frequent and infrequent cutting restriction enzymes and the resulting fragments were separated by PFGE. Analysis of an ethidium

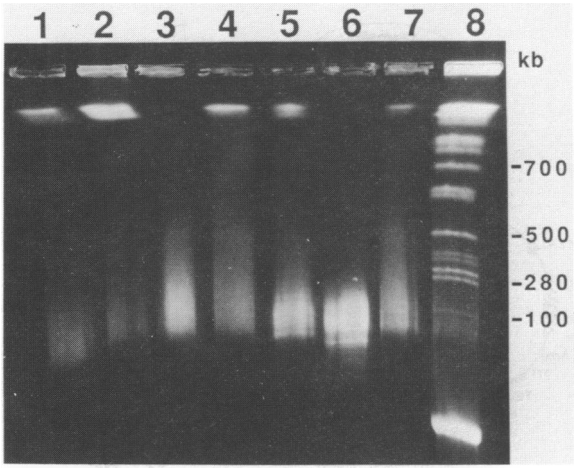

Figure 2. PFGE analysis of restriction enzyme digested *Arabidopsis* DNA plugs. An ethidium bromide stained CHEF gel containing *Arabidopsis* DNA plugs digested with various restriction enzymes. DNA samples were digested overnight with several infrequently cutting restriction enzymes and electrophoresis for 18 hr at 210 V with a switch interval of 80 sec. Lane (**1**) undigested DNA; (**2**) *Not* I; (**3**) *Sfi* I; (**4**) *Pvu* I; (**5**) *Mlu* I; (**6**) *Sma* I; (**7**) *Apa* I and lane (**8**) contains chromosomal DNA from *S. cerevisiae* strain YPH 274 plus an additional 100 kb artificial chromosome.

stained CHEF gel revealed a smear of large MW restriction fragments after digestion with several infrequent cutting enzymes; digestion of *Arabidopsis* plugs with *Not* I or *Pvu* I produced the largest fragments (Fig. 2). In addition, a number of distinct bands between 50-200 kb in size were visible in ethidium bromide-stained gels after digestion with *Sma* I or *Mlu* I and to a lesser extent with *Apa* I and *Sfi* I.

Although large DNA embedded in agarose may be useful for long range mapping of genes using infrequent cutting restriction enzymes, it is not as conveniently used for molecular cloning experiments. These studies are most efficiently carried out using DNA in solution. For example, the preparation of large partially-digested DNA fragments in solution is required for the construction of yeast artificial chromosome (YAC) libraries (20). Large *Arabidopsis* DNA in solution was prepared from cell protoplasts as detailed in Materials and Methods and outlined in figure 3. When compared with DNA embedded in plugs, these "liquid" DNA preparations showed similar profiles on pulsed-field gels; some large DNA molecules (>2 Mb) as well as some DNA sheared down to 150 kb (Fig. 4A, lane u). Further PFG-analysis of *Arabidopsis* DNA in solution using longer pulse intervals (4.0 hr) and longer gel run times (21 days) showed that the average size of this DNA was about 3-5 Mbp (data not shown).

High MW *Arabidopsis* DNA in solution was digested with various amounts of the restriction enzyme *Eco*RI and the resulting fragments were separated by PFGE. These large DNA preparations were found to be exquisitely sensitive to digestion with restriction enzymes. Digestion of

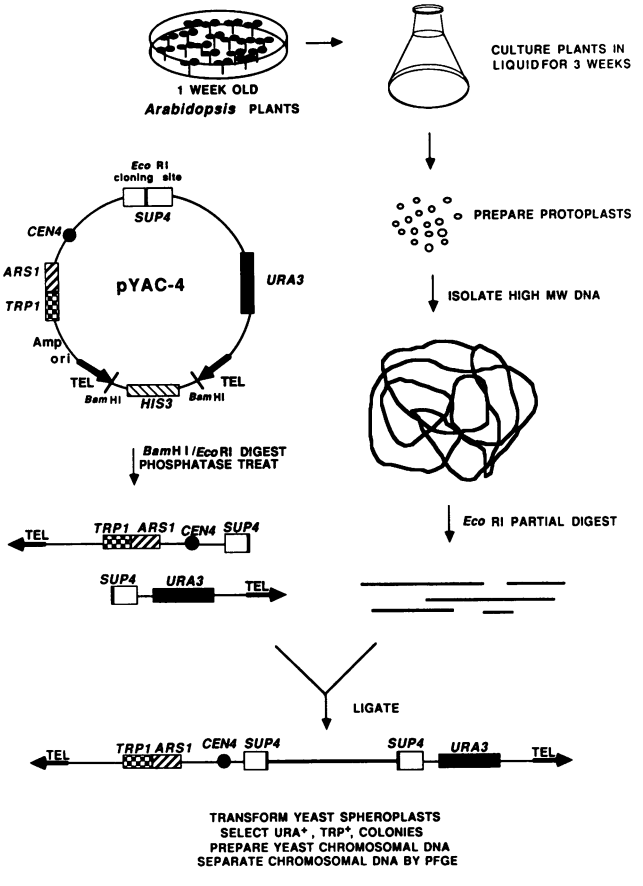

Figure 3. Schematic diagram of the protocol for the preparation and cloning of large segments of *Arabidopsis* DNA as yeast artificial chromosomes. Refer to **Materials and Methods** for details.

Arabidopsis DNA (5.0 μg) with as little as 0.05 units of *Eco*R I resulted in a significant reduction in the average size DNA molecule (Fig. 4A, compare lanes 0 and 0.05 units). Interestingly, *Eco*RI restriction fragments of *Arabidopsis* genomic DNA as large as 580 kb were evident in ethidium bromide-stained CHEF gels after complete digestion with a 20-fold excess of enzyme (Fig. 4A). These large *Eco*R I fragments were not homologous to chloroplast DNA sequences as demonstrated by blot hybridization (Fig. 4C), but hybridized strongly to a total DNA probe (Fig. 4D). Summing the sizes of these fragments indicated that they represented at least 3-4 % of the total nuclear DNA content of *Arabidopsis*.

High MW *Arabidopsis* DNA in solution was also used for restriction enzyme digestion using infrequent cutting enzymes. Complete digestion of *Arabidopsis* DNA with *Not* I or *Sfi* I resulted

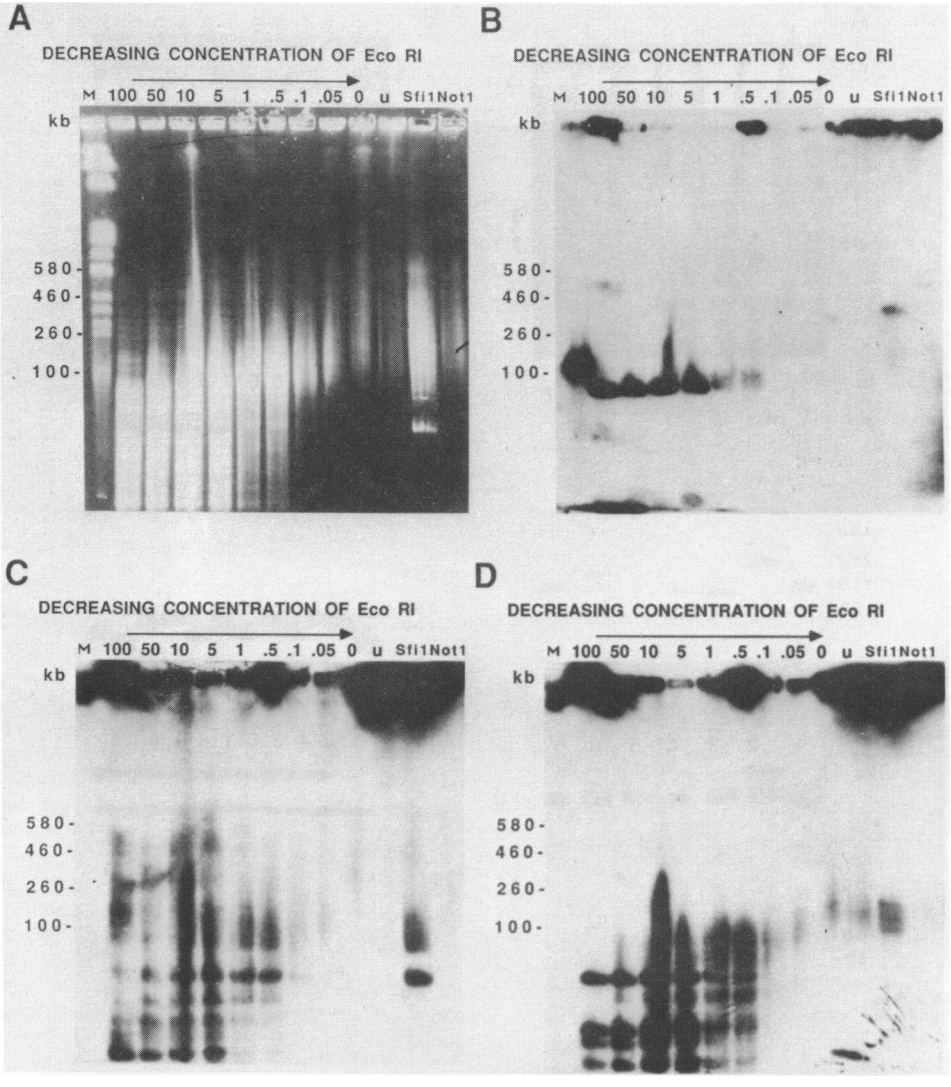

Figure 4. CHEF gel analysis of complete and partial restriction enzyme digested *Arabidopsis* DNA in solution. *Arabidopsis* DNA (~5 μg) was digested with various dilutions of *Eco*R I (100-0.05 units) or with the infrequent cutting restriction enzymes *Not* I and *Sfi* I. The fragments were separated by CHEF gel electrophoresis in a 1 % agarose gel for 18 hr at 210 volts using a switch interval of 80 sec. The gel was then stained with ethidium bromide (**A**). After transfer of the DNA to a nylon membrane, the filter was probed with: (**B**) a radiolabeled cDNA encoding the *Arabidopsis* oxygen-evolving enzyme; (**C**) total *Arabidopsis* DNA or (**D**) with *Brassica napus* chloroplast DNA. Lane M (marker) contains *S. cerevisiae* chromosomal DNA plus an additional 100 kb artificial chromosome. Lane u (untreated) contains *Arabidopsis* DNA not incubated with *Eco*RI restriction buffer. Sizes are in kilobase pairs (kb).

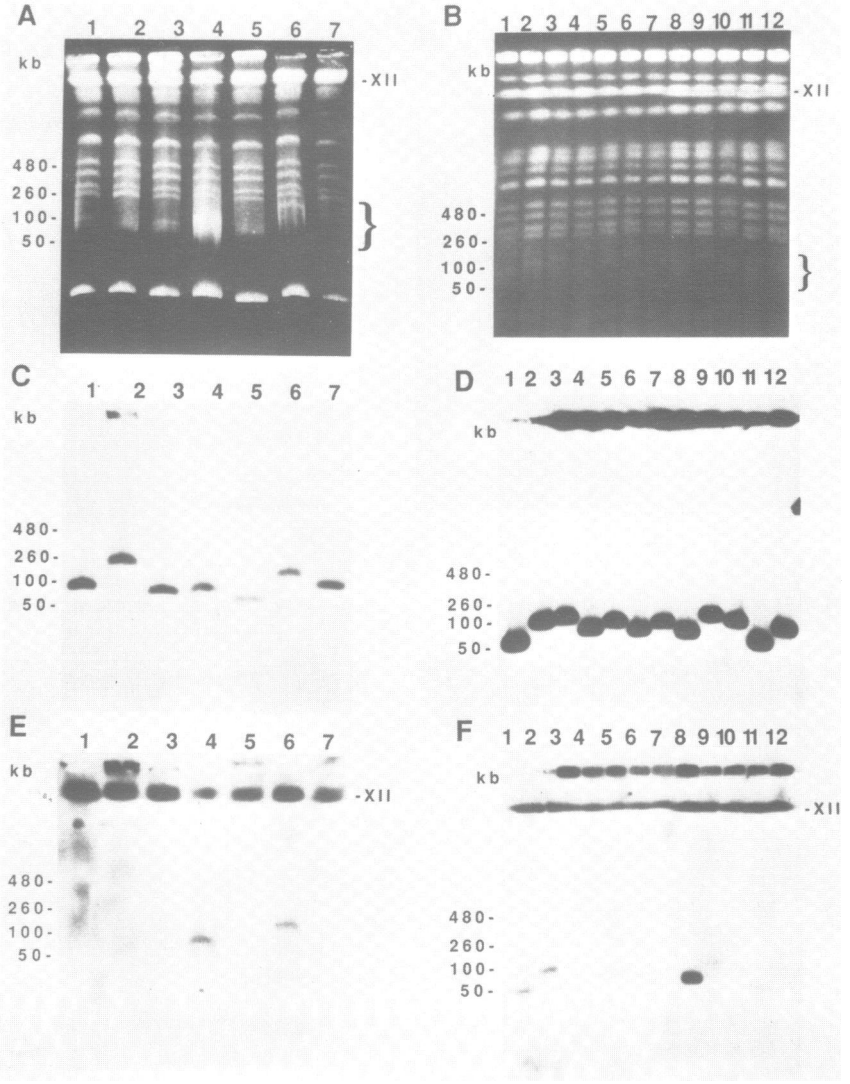

Figure 5. Characterization of carrot and *Arabidopsis* DNA-containing YACs by PFGE and Southern blotting. Yeast chromosome plugs were prepared from individual yeast transformants as described in Materials and Methods. Chromosome size DNAs were then separated by PFGE on a 1 % agarose gel for 18 hr at 210 V using a switch interval of either 30 seconds (**A**) or 80 seconds (**B**). The gels were then stained with ethidium bromide. Carrot (**A**) and *Arabidopsis* (**B**) yeast artificial chromosomes are weakly visible as bands ranging in size from 75-260 kb (indicated by brackets). The gels were then blotted to nylon membranes and probed with radiolabeled pBR322 DNA (**C,D**). The blots were then stripped and reprobed with radiolabeled total carrot (**E**) or *Arabidopsis* (**F**) DNA. The position of migration of yeast chromosome XII is indicated. Chromosome XII contains rDNA sequences that are homologous to the total carrot and *Arabidopsis* DNA probes (**E,F**).

in a smear of very large restriction fragments that ranged in size from 50 kb up to greater than 2.5 Mb (Fig. 4A). After blotting and hybridization with a radiolabeled cDNA probe encoding the *Arabidopsis* oxygen-evolving enzyme (provided by K. Ko and A. Cashmore, Univ. of Pennsylvania), single *Not* I and *Sfi* I fragments of sizes 500 kb and 400 kb were revealed (Fig. 4B). Interestingly, a prominent *Sfi* I fragment (*Sfi* I repeat) of about 15 kb in size, which was visible in ethidium bromide stained gels (Fig. 4A), hybridized strongly with total DNA (Fig. 4C) but not with chloroplast DNA (Fig. 4D).

High MW plant DNA preparations were then used for the construction of linear yeast artificial chromosomes. High MW carrot DNA prepared from a suspension culture line (23) was partially-digested with *Eco*R I and ligated to *Eco*R I/*Bam*H I-digested phosphatase-treated pYAC4 (20) DNA (Fig. 3). This plasmid contains all of the elements required for replication and proper segregation in *S. cerevisiae* (centromere, telomere, and ARS elements) (31). The ligation mixture was used to transform competent yeast spheroplasts (24) and *Ura+*, *Trp+* colonies were selected. Of approximately 16,000 carrot DNA-containing yeast transformants, 200 colonies were selected and 198 were found to be resistant to canavanine; the phenotype expected for a DNA insertion in the *SUP* 4 gene of pYAC4. Yeast chromosomal DNAs were prepared from putative tranformants and separated by PFGE. In addition to the normal complement of yeast chromosomal DNAs, all of the transformants contained additional chromosomal-size DNAs (YACs) which were visualized in UV light after staining with ethidium bromide (Fig. 5A). YACs ranged in size from 75 to 260 kb; the average size was about 150 kb. After Southern blotting, the separated yeast chromosomal DNAs were hybridized to radiolabeled pBR322, which contains sequences homologous to pYAC4, but not to yeast or carrot DNA. YACs prepared from cells grown under non-selective conditions showed similar hybridization intensities (Fig. 5C). Control samples of chromosomal DNAs from the parental yeast strain AB1380, which contained only the normal complement of yeast chromosomes, showed no hybridization to pBR322 sequences (data not shown). The blot was then stripped and reprobed with radiolabeled total carrot genomic DNA. Strong hybridization to several of the YACs was evident (Fig. 5E). Under the conditions employed, hybridization to the total carrot genomic DNA probe was expected to involve primarily repeated sequences in the probe and the cloned segments of the carrot genome. Upon several fold longer exposure, most of the carrot YACs showed hybridization to the carrot genomic probe (data not shown). These results are not unexpected since the carrot genome is comprised of approximately 40 % repetitive DNA sequences (32). In addition, significant cross-hybridization of rDNA sequences in the total carrot DNA probe to the rDNA-containing yeast chromosome (XII) was evident (Fig. 5E).

Similar cloning experiments were carried out using high MW *Arabidopsis* DNA in solution. In a single ligation/transformation experiment, approximately 10,000 yeast transformants were obtained and 40 were picked and streaked for isolation of single colonies. Yeast plugs were prepared and chromosomal DNAs were separated by PFGE (Fig. 5B) Ethidium bromide staining of the gel indicated that the average size *Arabidopsis* DNA-containing YAC was about 75 kb. Blot hy-

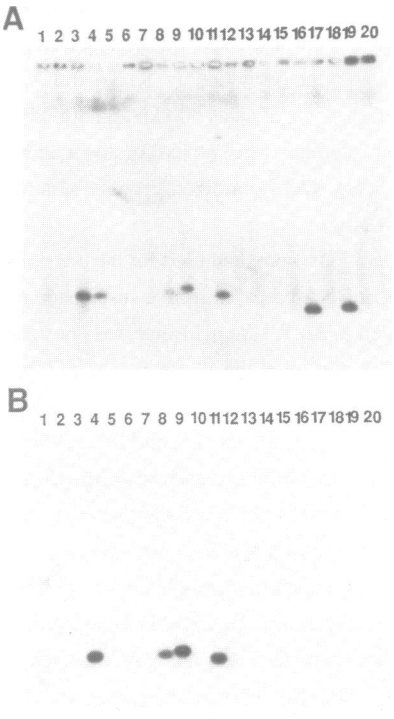

Figure 6. Representation of chloroplast and repetitive DNA sequences in the *Arabidopsis* YAC library. Chromosomal DNA from 20 randomly chosen yeast transformants was prepared, separated by PFGE, transferred to nylon, and hybridized with either (**A**) total *Arabidopsis* genomic DNA or (**B**) total chloroplast DNA probes.

bridization of PFG-separated DNAs to radiolabeled pBR322 DNA indicated that all of the putative yeast transformants contained YACs and that the hybridization signal for each YAC was about equal (Fig. 5D). Reprobing of this blot with total *Arabidopsis* genomic DNA revealed hybridization of this complex probe with several YACs (Fig. 5F).

In order to examine the representation of chloroplast DNA sequences present in the *Arabidopsis* YAC library, chromosomal DNAs from 20 YAC containing yeast were prepared and separated. The gel was blotted and hybridized with total *Arabidopsis* DNA which contained both nuclear and chloroplast DNA (Fig. 6A). Seven out of twenty YACs hybridized strongly to this complex probe. Several more YACs showed weak hybridization upon longer exposure of the blot (data not shown). The filter was then stripped and reprobed with radiolabeled chloroplast DNA (Fig. 6B). Four out of the original seven YACs hybridized strongly to purified chloroplast DNA. No additional YACs showing hybridization to the chloroplast probe were observed even after several fold longer exposure (data not shown). Further analysis of an additional 300 randomly selected

Arabidopsis DNA-containing YACs (about 0.3 *Arabidopsis* genome equivalents) showed essentially identical results.

DISCUSSION

Procedures developed for the preparation of large DNA molecules from yeast, parasites and mammalian cells have been modified for extracting large DNA from plant cells. Conventional methods for the preparation of DNA from *Arabidopsis* cells by CsCl equilibrium banding usually result in DNA molecules of only 100-150 kb in size. These size DNA molecules are not suitable for long range restriction mapping studies using infrequent cutting restriction enzymes and pulsed-field gel electrophoresis (cf. 22). Standard methods of DNA preparation fail to yield sufficient amounts of large *Arabidopsis* DNA for two reasons. First, because of its small genome size (0.14 pg DNA/cell), a very large number of *Arabidopsis* leaf cell protoplasts are needed to obtain sufficient quantities of DNA for restriction enzyme digestion or molecular cloning studies (~ 7×10^7 protoplasts/10μg DNA). And second, standard methods for DNA preparation from leaf or root tissues result in DNA breakage due to extreme shear forces required to break the cell wall.

We have developed methods for preparing large amounts of *Arabidopsis* cell protoplasts which yield sufficient quantities of very large DNA molecules. Unbroken chromosome-sized DNA molecules from plants can be prepared by first suspending plant cell protoplasts in low melting temperature agarose. Protoplasts embedded in agarose blocks are then lysed by diffusing in proteinase in the presence of detergent and high concentrations of EDTA. The final sample consists of high MW DNA within a cavity in the agarose "plug" where a plant protoplast once resided. Alternatively, similar results can be obtained by directly embedding plant cells in agarose followed by treatment of the plug with cell wall removing enzymes.

Large plant DNA embedded in "solid" agarose plugs is suitable for restriction enzyme mapping using infrequent cutting enzymes such as Not I and Sfi I; restriction enzymes readily diffuse through the gel matrix (22). However, DNA embedded in agarose is not as useful for cloning studies which are more conveniently carried out using DNA in solution. We, therefore, developed methods for the preparation of plant DNA which are based on those first used for preparing chromosome size yeast DNA molecules in solution (23). These crude plant DNA preparations contain DNA molecules larger that 12.6 Mb in size and are suitable for restriction enzyme analysis and cloning in yeast.

A high-capacity yeast cloning system (20), that is based on the *in vitro* construction of large linear DNA molecules as yeast artificial chromosomes, was utilized to construct YAC libraries of several plant genomes. Briefly summarized, analysis of plant DNA-containing yeast artificial chromosomes demonstrated that: (1) transformants contained YACs large enough to be directly visualized by ethidium bromide staining of PFGE gels; the average size of insert was about 150 kb for carrot and 75 kb for *Arabidopsis*; (2) the number of transformants with inserts (10,000-16,000) was well over that needed to obtain a complete *Arabidopsis* genomic library; and (3) the

absence of minor bands in blots of plant DNA-containing YACs suggests that both unique and repetitive DNA inserts were stable. The inclusion of a DNA size selection step should allow the construction of YAC libraries of plant DNA with a significantly larger insert size.

The ability to clone and stably propagate large segments of DNA in a simple genetic backgrounc has several several important implications for gene isolation from *Arabidopsis*. The recent development of an RFLP map for *Arabidopsis* (7) should allow the isolation of many genes using the RFLP probes as start points for chromosome walking. However, because of the relatively large distance between RFLPs (750 kb on average) and because it is likely that certain DNA sequences (such as centromeric DNA) may be inherently unstable in *E. coli* (34), the isolation of many *Arabidopsis* genes via cosmid chromosome walking methods may be very difficult. It may, however, be possible to cover much larger distances by identifying overlapping YACs. Although it is still to early to predict the stability of certain plant DNA sequences in yeast, it is likely that such sequences (if they exist) would not be identical to those which are unstable in *E. coli*. In addition, YACs-containing several hundred kilobase inserts of *Arabidopsis* DNA should also be useful for filling in the gaps in the *Arabidopsis* contig map (35). In fact, construction of a low resolution physical map via isolation of a complete overlapping set of *Arabidopsis* DNA-containing YACs may also be feasible.

In view of the ease with which specific gene mutations can be identified (36) and because of its small genome size (3), *Arabidopsis* should be ideally suited for cloning genes by complementation. One important future application of large DNA methods for *Arabidopsis* is the transfer of these large segments of DNA back into *Arabidopsis*. The most direct method of transferring YACs-containing *Arabidopsis* DNA would be to fuse yeast spheroplast with *Arabidopsis* protoplasts and select for the appropriate plant transformation markers built into the YAC vector. This type of methodology has been successful for transferring DNA from yeast to mammalian cells in culture. Recently, Allshire *et al.* (37) have shown that transformation occurs when an *S. pombe* chromosome bearing a selectable marker is transferred into mouse cells by fusion. One important obstacle in developing this type of strategy for *Arabidopsis*, the regeneration of a fertile plant from a protoplast, has recently been overcome. Damm and Willmitzer (38) have described methods which allow the regeneration of fertile plants from leaf protoplast of *Arabidopsis*. Alternatively, because chromosome-size DNA in solution can be prepared from yeast, transformation of YACs into *Arabidopsis* protoplasts via one of several direct DNA transfer methods (39,40) may also be possible.

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REFERENCES

1. King, P.J. (1988) *Trends Genet.* **4**: 157-162.
2. Pryor, T. (1987) *Trends Genet.* **3**: 157-161.
3. Haughn, G.W. and Somerville, C.R. (1988) *Dev. Genet.* **9**:73-89.
4. Meyerowitz, E.M. (1987) *Ann. Rev. Genet.* **21**:93-111.
5. Botstein, D., White, R., Skolnick, M. and Davis, R.W. (1980) *Am. J. Hum. Genet.* 32:314 331.
6. Bender, W., Spierer, P. and Hogness, D.S. (1983) J. Mol. Biol. **168**:17-33.
7. Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S. and Meyerowitz, E.M. (1988) *Proc. Natl. Acad. Sci. USA* **85**:6856-6860.
8. Helentjaris, T. (1987) *Trends Genet.* **3**:217-221.
9. Landry, B.S., Kesseli, R.V., Farrara, B. and Michelmore, R.W. (1987) *Genetics* **116**:331-337.
10. Bernatzky, R. and Tanksley, S.D. (1986) *Genetics* **112**:887-898.
11. Collins, F.S. and Weissman, S.M. (1984) *Proc. Natl. Acad. Sci. USA* **81**:6816-6818.
12. Poustka, A. and Lehrach, H. (1986) *Trends Genet.* **2**:174-178.
13. Rappold, G.A. and Lehrach, H. (1988) *Nucl. Acids. Res.* **16**:5361-5377.
14. Karch, F., Weiffenbach, B., Peifer, M., Bender, W., Duncan, I., Celniken, S., Crosby, M. and Lewis, E.B. (1985) *Cell* **43**:81-96.
15. Kuner, J.M., Nakanishi, M., Ali, Z., Drees, B., Gustauson, E., Theis, J., Kauvar, L., Kornberg, T and O'Farrell, P.H. (1985) *Cell* **42**:309-316.
16. Monaco, A.P., Neve, R.L., Collette-Feener, C., Bertelson, C.J. Kurnit, D.M. and Kunkel (1986). *Nature* **323**:646-650.
17. Schwartz, D.C., and Cantor, C.R. (1984) *Cell* **37**:67-75.
18. Carle, G.F., and Olson, M.V. (1984). *Nucl. Acids Res.* **12**:5647-5664.
19. Orbach, M.J., Vollrath, D., Davis, R.W., and Yanofsky, C. (1988). *Mol. Cell Biol.* **8**:1469-1473.
20. Burke, D.T., Carle, G.F., and Olson, M.V. (1987). *Science* **236**:806-812.
21. Ford, K., Sowell, E. and Sumpter, C. (1987) Abstract # 137, *Third International Meeting on Arabidopsis,* Michigan State University.
22. Smith, C.L., Matsumoto, T., Niwa, O., Klco, S., Fan, J.-B., Yanagida, M. and Cantor, C. R. (1987) *Nucl. Acids Res.* **14**:4481-4489.
23. Olson, M.V., Loughney, K. and Hall, B.D. (1979) *J. Mol. Biol.* **132**:387-410.
24. Ecker, J.R. and Davis, R.W. (1986) *Proc. Natl. Acad. Sci. USA* **83**:5372-5376.
25. Burgers, P.M.J. and Percival, K.J. (1987) *Anal. Biochem.* **163**:391-397.
26. Chu, G., Vollrath, D., and Davis, R.W. (1986). *Science* **234**:1582-1585.
27. Vollrath, D. and Davis, R. W. (1987) Nucl. Acids. Res. **19**:7865-7876.
28. Davis, R.W., Botstein, D. and Roth, J.R. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
29. Vollrath, D., Davis, R.W., Connelly, C. and Hieter, P. (1988) *Proc. Natl. Acad. Sci. USA,* **85**:6027-6031.
30. Feinberg, A.P. and Vogelstein, B. (1983) *Analyt. Biochem.* **132**:6-13.
31. Blackburn, E.M. and Szostak,, J.W. (1984) *Ann. Rev. Biochem.* **53**:163-194.
32. Duhrssen, E., Saavedra, E. and Neumann, K.-H. (1980) *Plant System. Evol.* **136**:267-273.
33. Smith, C.L., Lawrance, S.K., Gillespie, G.A., Cantor, C.R., Weissman, S.M. and Collins, F.S. (1987) in *Methods in Enzymology* (ed. by M. Gottesman) Academic Press, Orlando, Vol. **151**:461-489.
34. Fishel, B., Amstutz, H., Baum, M., Carbon, J. and Clarke, L. (1988) *Mol. Cell. Biol* **8**:754-763.
35. Goodman, H.M., den Boer, B., Fritze, C., Hauge, B., Lazar, G., Loos, B., Nam, H-G., Paek, K-H. and Yett, D. (1988) *J. Cell. Biochem.* **12C**:135
36. Estelle, M.A. and Somerville, C.R. (1986) *Trends Genet.* **2**:89-93.
37. Allshire, R.C., Cranston, G., Gosden, J.R., Maule, CJ.C., Hastie, N.D., and Fantes, P.A. (1987) *Cell* **50**:391-403.
38. Damm, B. and Willmitzer, L. (1988) *Mol. Gen. Genet.* **213**:15-20.
39. Fromm, M., Taylor, L. and Walbot, V. (1986) *Nature* **319**: 791-793.
40. Schillito, R.D., Saul, M., Paszkawski, J., Miller, M. and Potrykus, I. (1985)*Bio/Technology* **3**:1099-1103.