Plant Molecular Biology **19**: 299–308, 1992.

Construction and characterisation of a yeast artificial chromosome library containing three haploid maize genome equivalents

Keith J. Edwards, Helen Thompson, David Edwards, Antoine de Saizieu, Caroline Sparks, John A. Thompson, Andrew J. Greenland, Mark Eyers and Wolfgang Schuch
Plant Biotechnology Section, I.C.I. Seeds, Jealotts Hill Research Station, Bracknell, Berkshire RG12 6EY, UK

Received 15 October 1991; accepted in revised form 21 January 1992

Key words: cloning in YACs, genome mapping, maize, PCR

Abstract

We have constructed a yeast artificial chromosome (YAC) library using high-molecular-weight DNA prepared from agarose-embedded leaf protoplasts of the maize inbred line UE95. This library contains 79000 clones with an average insert size of 145 kb and should therefore represent approximately three haploid genome equivalents. The library is organised as an ordered array in duplicate microtitre plates. Forty-one pools of DNA from 1920 individual clones have been prepared for rapid screening of the library by the polymerase chain reaction (PCR). Using this approach, together with conventional colony hybridisation, we have been able to identify between one and eight positive clones for every probe used.

Introduction

The recent developments in yeast artificial chromosome (YAC) systems capable of cloning DNA fragments several hundred kilobases in size, has for the first time opened up the possibility of linking the genetic map (measured in centimorgans, CM) and the physical map (measured in kb) [8]. A number of mammalian YAC libraries have been constructed for the characterisation of various genomic regions and individual genes [1, 7]. Complete YAC libraries prepared from plant DNA have so far been limited to those species having relatively small genomes. For instance, a number of YAC libraries have been constructed for the crucifer *Arabidopsis thaliana* [12, 24]. Unfortunately Arabidopsis is not typical of the agronomically important crop species. These species, typified by maize, have genomes at least ten times larger than *Arabidopsis*. This increase in genome size is mainly due to the presence of various types of repeat sequences [22]. Such sequences while representing only 25% of the *Arabidopsis* genome [15] may represent up to 80% of the maize genome [22]. Unfortunately, this means that if a cloning method is to be useful in chromosome walking it must be capable of bridging these large stretches of repetitive DNA. YAC libraries therefore seem to be an ideal tool for the representative cloning of maize DNA and for the development of a model for the organisation of larger plant genomes.

The difficulties in isolating and manipulating high molecular weight DNA from non-tissue culture derived protoplasts led us to apply the 'agarose embedding' techniques developed to produce human YAC libraries [2] to construct a maize YAC library. Based on a haploid genome size of 3.7×10^9 bp [5] this library should contain three haploid genome equivalents and represent 94% of the maize genome. Our initial efforts have centred upon characterising this library with respect to the abundance of both single, low-copy number and repeat sequences. We intend to use this library to carry out a number of chromosome walks with a view to developing a model for the organisation of the maize genome. In order to facilitate this process we have used the PCR and conventional hybridisation to identify a number of sequences representing transcribed genes, RFLP markers and highly repetitive sequences.

Materials and methods

Chemicals

Lyticase and casein amino acid hydrolysate were obtained from Sigma. All growth media were obtained from Difco. Media for the growth and selection of recombinant yeast was as described [2]. Restriction and modification enzymes were obtained from Pharmacia. AmpliTaq DNA polymerase was obtained from Perkin-Elmer/Cetus. Enzymes used in the isolation of leaf protoplasts were obtained from Yakult Honsha Co. Sea Plaque FMC agarose was obtained from Flowgen Instruments. Agarase was obtained from Calbiochem. Hybond N Nylon membrane and all radioactive chemicals were obtained from Amersham.

Yeast strain and culture conditions

Saccharomyces cerevisiae AB1380 (Matα, *ura3*, *trp1*, *ade2- 1*, *can1-100*, *lys2-1*, *his5*) and pYAC4 were prepared and used as described [8]. Yeast cultures were grown on either complete medium (YPD) or selective medium lacking uracil (single selection) or uracil and tryptophan (double selection) as described [2].

Contour-clamped homogeneous electrophoresis

Contour-clamped homogeneous electrophoresis (CHEF) was carried out using a BioRad CHEF apparatus. Gels were 1% agarose (Pharmacia) run in 0.5× TBE (1× TBE = 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) at 200 V. Gels were run using various switch times as indicated for individual experiments. Where gels were subsequently blotted on to Hybond N membrane they were first subjected to HCl depurination as described [23]. Hybridisation was carried out in 6× SSPE, 0.1% SDS and 0.25% Blotto [19] at 65 °C. Filters were washed in 0.2× SSC, 0.2% SDS at 65 °C.

Isolation of high-molecular-weight DNA from maize protoplasts

Leaf protoplasts were isolated from all but the first leaf of UE95 maize seedlings (ICI seeds) which had been grown at 25 °C in the light for 7 days followed by dark growth at 25 °C for 2 days. The seedlings were germinated from kernels which had been imbibed overnight in Knops medium (1 g/l $Ca(NO_3)_2 \cdot 4H_2O$, 250 mg/l each of KNO_3, KH_2PO_4 and $MgSO_4 \cdot {}_7H_2O$, 6.4 mg/l Fe^{2+} EDTA, pH 5.6 [14]). Imbibition was at 4 °C prior to growth on wire-mesh platforms in open glass jars containing liquid Knops medium. Longitudinal leaf slices were digested in an enzyme mixture (0.5% Cellulase RS, 0.25% Macerozyme R10, 375 mM mannitol, 50 mM $CaCl_2$, 0.2% MES pH 5.6) for 3 h, without shaking at 30 °C in the dark. The tissue was teased apart prior to passing through a 53 μm sieve. Two washes were carried out using a sorbitol medium (0.6 M sorbitol, 10 mM Hepes-KOH, pH 7.5, 2 mM $CaCl_2$) and centrifuging at 700 rpm for 3.5 min between each wash. The protoplasts were finally resus-

pended in 1% Sea Plaque low temperature gelling agarose in Kao's medium (kept at 42 °C [13]) at a final concentration of 5×10^6 protoplasts per 200 µl. The molten agarose/protoplast mixture was gently poured into a BioRad mould and allowed to solidify at 4 °C for 20 min. The plugs were then processed further as described [2]. Using this procedure it was possible to produce five 200 µl plugs from ca. 100 maize seedlings.

Preparation of Eco *RI partially digested DNA*

In order to establish the conditions for partial digestion we found that it was first necessary to remove the low-molecular-weight (LMW) DNA which would otherwise interfere with the subsequent partial digestion [9]. LMW DNA was removed by pre-electrophoresis of the intact plugs in a 1% agarose gel, using a pulse duration of 60 s for 6 h. Using these conditions genomic DNA greater than 3 Mb remained within the original plug. The plugs were then removed from the agarose gel and processed further for restriction digestion. Conditions for partial digestion were determined essentially as described [2] with the exception that digestion was allowed to proceed for 1 h at 37 °C. Following partial digestion LMW DNA fragments were removed by returning the agarose plug to a 1% agarose gel and subjecting them to a further electrophoresis using a pulse duration of 10 s at 200 V for 3 h. This procedure removed >90% of DNA fragments smaller than 90 kb without significantly reducing the amount of larger DNA fragments. The plugs were then re-isolated and used to construct the YAC library as described below.

Construction of the YAC library

For the construction of the YAC library, partially digested high-molecular-weight (HMW) DNA from five separate (but identical) partial digestions was liberated from the agarose plugs by digestion with agarase as described [2]. This procedure produced approximately 85 µg of digested and fractionated DNA in 1 ml of highly viscous solution (from 5 plugs). This was ligated to 170 µg (i.e. a 30-fold molar excess) of *Eco* RI cut and dephosphorylated pYAC4 in a total volume of 2 ml. 15 µl of this ligation mix (representing ca. 64 ng of maize DNA) was used to transform yeast spheroplasts which were then plated into single selection media [2]. Transformed colonies were grown at 30 °C for 3–4 days before being picked onto double selection media. For long term storage, colonies were transferred to microtitre plates containing 75 µl of single selection media plus 20% glycerol before being stored at −75 °C.

Preparation of DNA from individual YAC clones

Intact yeast chromosome DNA was prepared by embedding yeast spheroplasts produced from a 5 ml overnight culture in 200 µl 1% Sea Plaque agarose as described [1]. LMW yeast DNA suitable for restriction digestion and the PCR was prepared by resuspending yeast spheroplasts, produced from a 5 ml overnight culture, in 1 ml of a yeast DNA extraction buffer (50 mM Tris-HCl pH 7.5, 25 mM EDTA, 250 mM NaCl, 1% SDS). This was phenol- and chloroform-extracted and ethanol-precipitated. The total nucleic acids pellet was resuspended in 100 µl 1 × TE. 2.5 µl of this was sufficient for restriction digestion and analysis by Southern blot hybridisation.

Screening of the library for chloroplast and repeat sequences

Screening of the library for chloroplast and repeat sequences was carried out by conventional yeast colony hybridisation [7], with the exception that lyticase at 50 units per ml was used to lyse the colonies instead of Zymolyase 100T. ^{32}P-labelled probes were prepared using the random primer labelling method [10]. Autoradiography was carried out for 1–4 days at −70 °C with a single intensifying screen.

Screening of the library for low- or single-copy sequences

Screening of the maize YAC library for low- or single-copy sequences by colony hybridisation was impractical due to the large number of individual clones involved. To screen the library for such sequences we therefore adopted the PCR screening procedure [11]. Screening of the library for such sequences was carried out in three stages. Firstly, PCR was carried out on DNA pools prepared from 1920 individual colonies (41 pools for the entire library) grown on single selection agar for two days. Secondly, positive pools were further divided into four smaller pools each consisting of 480 individual colonies for a second round of the PCR. Finally, positive pools from this screening were used to make conventional filters which were screened using colony hybridisation to identify individual positive clones.

Results

Preparation and partial digestion of high-molecular-weight maize DNA

DNA prepared from agarose embedded protoplasts, routinely consisted of approximately 50% LMW (1–300 kb) and 50% HMW (>3 Mb) DNA as judged by CHEF. Following pre-electrophoresis the plugs contained only high molecular weight genomic DNA which was a suitable substrate for partial *Eco* RI digestion. Surprisingly, the pre-electrophoresis did not significantly reduce the amount of chloroplast DNA within the original plug despite the fact that the chloroplast genome is only 137 kb [4]. Presumably this is due to the apparent inability of intact circular chloroplast DNA to migrate from the original plug under the CHEF conditions used, such an abnormal migration pattern within CHEF has previously been described for other circular DNAs [6]. DNA prepared from agarose embedded protoplasts was found to be very sensitive to *Eco* RI per digestion (Fig. 1), as judged by the observation that digestion with 1 unit of *Eco* RI per ml for 1 h was sufficient to produce DNA fragments in the 100–500 kb range.

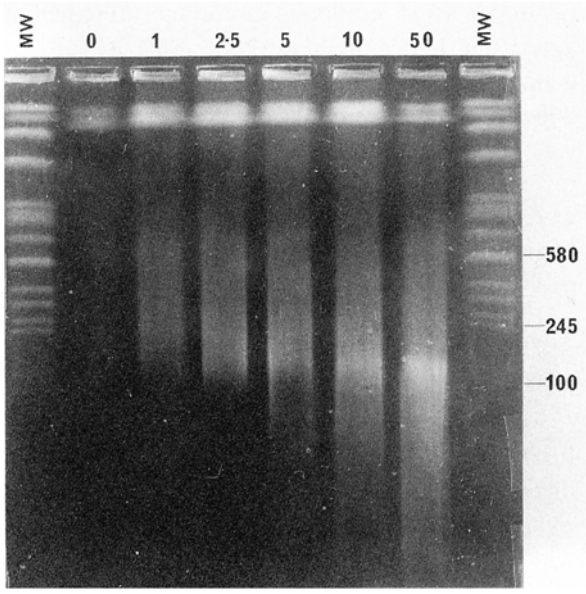

Fig. 1. Agarose-embedded genomic DNA was subjected to digestion with the indicated number of units of *Eco* RI (per ml) for 1 h at 37 °C and then subjected to CHEF using a switch time of 60 s. The molecular weight markers (MW) were yeast chromosomes prepared from the strain AB1380. All sizes are in kb.

Construction of the YAC library

Electrophoresis of the partially digested, embedded DNA using a switch time of 10 s was found to be successful in removing LMW (<90 kb) DNA. Attempts to increase the stringency of this size fraction as described [2] were unsuccessful. Our most stringent fractionation while resulting in larger inserts (200–500 kb) also resulted in a 100 fold reduction in the subsequent transformation frequency as described [1]. The fractionated DNA was then ligated to a 30-fold molar excess of pYAC4 vector arms.

Transformation of AB1380 spheroplasts with 15 μl of the ligation mix (in 10 separate transformations) resulted in between 50 and 250 transformants appearing on the urea plates after 3–4 days. This represented a transformation fre-

quency of between 1000 and 5000 transformants per μg of maize DNA. Approximately 95% of these transformants continued to grow on the double selection media.

Characterisation of a random selection of clones by CHEF showed that about 10% of the clones contained two YACs. In the five cases which we have examined so far (by Southern blotting and hybridisation to specific probes) these have been shown to result from two independent YAC clones being picked together, presumably when the clones were transferred from the single to the double selection plates. Therefore before further analysis, all YAC clones were streaked to single colonies.

Clone size distribution

CHEF of six randomly chosen yeast clones showed that each contained an extra chromosome (Fig. 2). These were between 100–200 kb in size. Southern blotting [20] and hybridisation of with either ^{32}P-labelled pAT153 (specific for the pYAC4 vector, [21]) or total maize genomic DNA showed that all the clones contained maize DNA cloned into the YAC vector (Fig. 2B and C respectively).

Steps were also taken to ensure that the average insert size throughout the library was maintained above 90 kb. To show that this had been achieved, 10 agarose plugs were prepared as de-

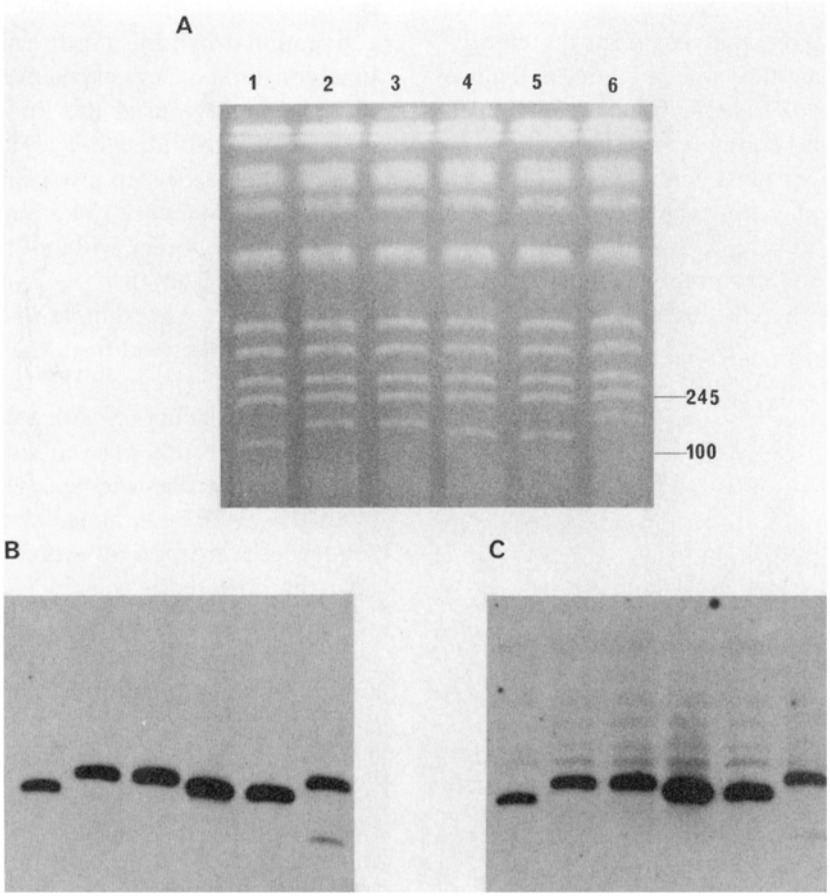

Fig. 2. CHEF of chromosomes from six yeast cultures carrying YACs. A. After staining with ethidium bromide. B. After Southern analysis and hybridisation to ^{32}P-labelled pAT 153. C. After hybridisation to ^{32}P-labelled total maize genomic DNA. The extra faint band below the main YAC band in lane 6B and 6C was found to be due to second YAC clone. All sizes are in kb.

scribed [3], each plug consisting of 96 individual clones taken at random from throughout the library, i.e. 960 clones in total. These multi-clone plugs were subjected to CHEF and blotted onto Hybond N membrane. Following hybridisation to ^{32}P-labelled pAT153 and autoradiography, the hybridisation pattern was analysed by an LKB scanning densitometer and plotted as an histogram (Fig. 3). These results show that the library had an average insert size of ca. 145 kb (155 kb minus the 10 kb vector arms) with only 12.5% of clones having an insert size of less than 100 kb.

Extent of chloroplast and other high-copy-number sequences

Hybridisation of 960 randomly chosen YAC clones with a 500 bp fragment from the chloroplast gene encoding the large subunit of ribulose bisphosphate carboxylase (Rubisco) [16] showed that 43 of the clones contained chloroplast DNA. Assuming the chloroplast genome is 137 kb [4], then this represents approximately 4.5% of the library.

To investigate the occurrence of highly repetitive sequences within the library, a further 960

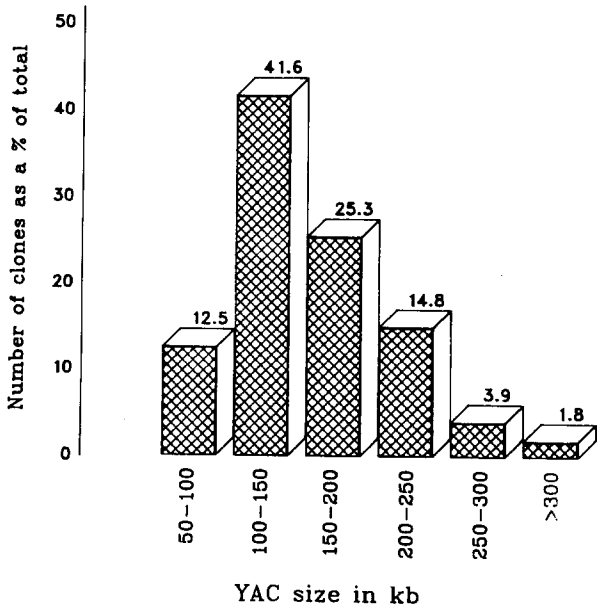

Fig. 3. Size distribution of 960 randomly chosen YAC clones. The calculated mean YAC size is 155 kb.

randomly chosen YACs were probed with a ^{32}P-labelled oligonucleotide homologous to 30 base pairs of the knob repeat sequence [17]. Colony hybridisation revealed that 3 clones (0.3%) contained sequences homologous to knob DNA. Preliminary characterisation of one of these clones has revealed that despite having approximately 550 copies of the knob repeat sequence the clone appears to be completely stable (A. de Saizieu, unpublished data).

Isolation of single-copy sequences

The usefulness of any cloning system depends upon the ability to quickly identify and isolate clones carrying sequences of interest. Because of the size and format of the maize YAC library conventional hybridisation techniques would be time-consuming and expensive. To circumvent this problem we used the PCR in combination with colony hybridisation to identify individual clones. This three-step procedure was capable of confirming whether or not a particular clone was present in the library within 4 h. Individual positive clones could then be isolated within four working days. An example of such a screening using primers derived from the alcohol dehydrogenase 1 (Adh1) gene [18] is shown in Fig. 4. Screening of the library with a further 9 randomly chosen primer sets derived from publicly available RFLP markers and gene sequences obtained from the EMBL database showed that all the sequences were present within the library at or near the expected frequency (Table 1).

Characterisation of the Adh1 YAC clones

Numerous studies have been carried out in mammalian and plant systems to investigate the YAC cloning system. These studies have indicated that in a minority of YAC clones the insert appears to be unstable and prone to deletions. As the maize genome is known to contain large numbers of repeat sequences organised into extensive arrays [22], we were interested in confirming the authen-

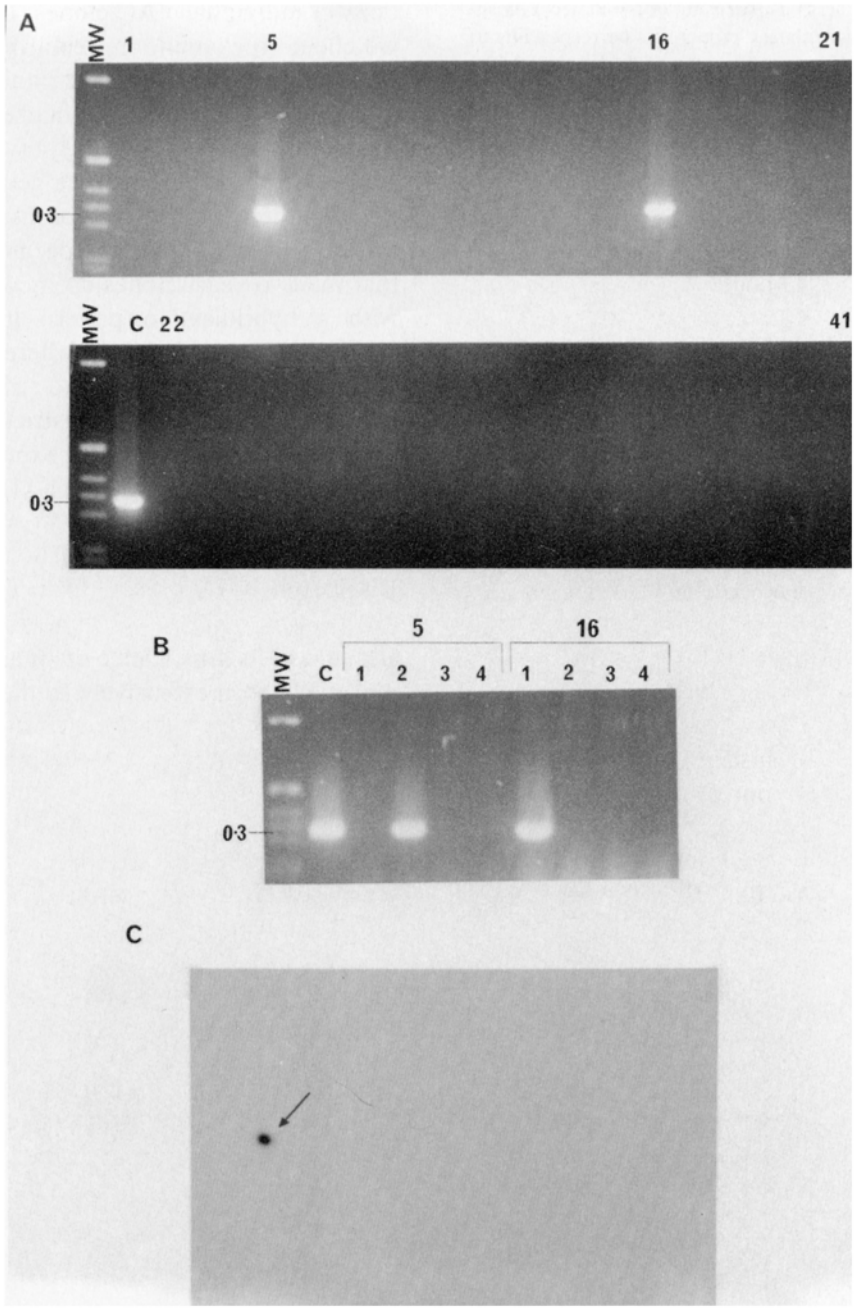

Fig. 4. Identification of a YAC clone containing the maize Adh1 gene. A. 41 pools of maize YAC clones (each pool containing DNA from 1960 clones) were analysed via the PCR for the presence of Adh1 gene sequences. Lane c contained the PCR product from 50 ng of maize genomic DNA. B. Pools 5 and 16 were divided into 4 further sub-pools (i.e. 480 clones) and re-analysed by the PCR. Lane c contained the PCR product from 50 ng of maize genomic DNA. In A and B the molecular weight markers (MW) are the 1 kb ladder from BRL. Sizes are in kb. The position of the expected fragment (0.3 kb) is marked. C. Colony hybridisation using an Adh1-specific probe was carried out on sub-pool 2 from primary pool 5 to localize the individual clone.

Table 1. Results of the PCR screening of the maize YAC library with 10 randomly chosen primer sets corresponding to either RFLP markers or sequences obtained from the EMBL database.

Source of PRC primers[1]	Chromosome location	Numer of positives
NPI447	1L	3
Adh1	1L	2
NPI298	2L	4
UMC27	5L	4
BNL6.29	6S	8
UMC85	6S	1
UMC48	8L	2
Bronze1	9S	3
NPI264	10L	1
NPI437	10L	2

[1] Where applicable, the RFLP markers are classified according to either the Native Plant Industries (NPI), the University of Missouri (UMC) or the Brookhaven National Laboratory (BNL) convention.

ticity of individual YAC clones. For this purpose we chose to examine in detail the two independent clones identified as containing Adh1 sequences (Fig. 4). Yeast and maize genomic DNA (50 ng and 20 μg respectively) was subjected to restriction digestion, agarose gel electrophoresis and Southern blotting. Hybridisation of the resulting blot with an Adh1-specific probe showed that the two yeast clones each contained a YAC with a hybridisation pattern indistinguishable from maize genomic DNA, whereas a yeast clone chosen at random showed no such hybridisation (Fig. 5). Furthermore, this restriction pattern corresponded to the pattern expected from the Adh1-1F allele gene sequence [18].

Discussion

Because of its importance as an agricultural crop, maize has been extensively studied by both clas-

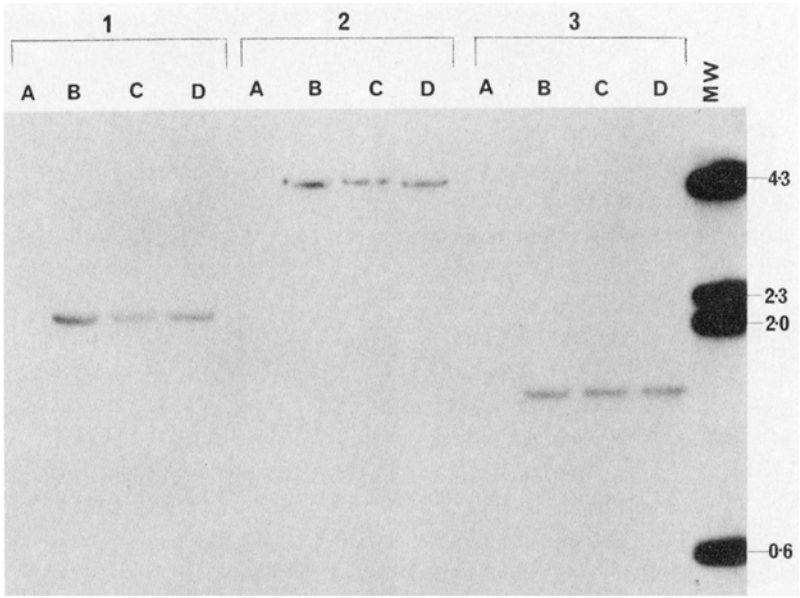

Fig. 5. Southern blot of a restriction digest of DNA from yeast containing YACs (lanes A, B, and C) and maize genomic DNA (lane D). Lane A contains a yeast clone chosen at random. Lanes B and C contain the two yeast clones identified as containing Adh1 sequence. Lanes 1A–D were digested with *Pst* I. Lanes 2A–D and 3A–D were digested with *Bgl* II and *Bgl* II plus *Pst* I respectively. Approximately 20 μg of maize genomic DNA and 50 ng of yeast DNA was digested. The Southern blot was hybridised to a ^{32}P-labelled Adh1-specific probe and subjected to autoradiography with an intensifying screen for 2 days. The molecular weight markers (MW) are ^{32}P-labelled lambda DNA digested with *Hin*d III. Sizes are in kb.

sical geneticists and molecular biologists. Whilst these studies have resulted in a highly developed genetic linkage map, they have also shown that the maize genome has a complex organisation consisting of unique or low-copy-number sequences surrounded by large arrays of highly repetitive DNA which may be up to 100 kb in length [22]. Unfortunately, such an organisation makes chromosome walking or general mapping using lambda or cosmid-based vectors virtually impossible. To overcome these difficulties we decided to use a YAC-based cloning system to clone HMW maize DNA which had been partially digested with *Eco* RI.

The library constructed consists of 79000 clones with an average insert size of 145 kb. This is considerably smaller than that described for the numerous mammalian YAC libraries which have been constructed using this or similar methods. However, the insert size is favourable when compared with other plant YAC libraries [12, 24]. It is likely that this difference between plant and mammalian library insert size is primarily due to the increased problems associated with extracting HMW DNA from plant cells.

Screening a small proportion of the library with either chloroplast or knob repeat specific probes showed that both were present within the library. However, whereas the chloroplast clones were present at or near the expected frequency, only 0.3% of clones appeared to contain knob repeat sequences. This was below the lower end of the expected range (0.5–5%) and may be due to the lack of *Eco* RI restriction sites within the knob repeat clusters [17]. Unfortunately this requirement for the presence of *Eco* RI restriction sites could introduce variations in the representative nature of the library. For instance, regions of the genome devoid of *Eco* RI sites would be underrepresented in the library whereas regions containing large numbers of *Eco* RI sites may be over represented. One way to eliminate this problem would be to use the random shearing method [24] which eliminates the effects of clustering due to any particular restriction site.

The ability of YACs to clone very large DNA fragments means that there is a finite chance that multiple inserts are cloned within a single YAC. Characterisation of other YAC libraries has shown that this can be a significant problem which only becomes apparent during the construction of large contiguous maps. The use of DNA depleted of low molecular weight fragments and a 30-fold molar excess of vector DNA used in constructing the maize library should have minimised this problem. Chromosome walks at present being undertaken with this library will further help to confirm its representative nature.

Using the PCR approach, we have so far screened the library with 10 sets of PCR primers derived from RFLP markers and sequences taken from the EMBL database. In all cases we have identified between 1 and 8 positive clones (average 3) for each probe used. Detailed characterisation of a limited number of the clones has so far failed to reveal any differences between the DNA cloned into YACs and maize genomic DNA. We therefore believe that this library will, together with other maize clone banks, provide a useful tool for the further characterisation of the maize genome.

Acknowledgements

We thank A. Daly for providing sequence data for the generation of PCR primers. We further thank P. Bell for synthesising the PCR primers. Suggestions from Gunther Neuhaus (ETH, Zurich) on the leaf protoplast isolation are gratefully acknowledged. K.J.E would like to thank R. Anand for his continued advice and support during this work. M.E. was supported by the D. Collen Research Foundation. I.C.I. Seeds in the UK is part of Imperial Chemicals Industries PLC.

References

1. Albertsen HM, Abderrahim H, Cann HM, Dausset J, Paslier DL, Cohen D: Construction and characterisation of a yeast artificial chromosome library containing seven haploid human gene equivalents. Proc Natl Acad Sci USA 87: 4256–4260 (1990).
2. Anand R, Villasante A, Tyler-Smith C: Construction of

yeast artificial chromosome libraries with large inserts using fractionation by pulsed-field gel electrophoresis. Nucl Acids Res 17: 3425–3432 (1989).
3. Anand R, Riley JH, Butler R, Smith JC, Markham AF: A 3.5 genome equivalent multi access YAC library: construction, characterisation, screening and storage. Nucl Acids Res 18: 1951–1956 (1990).
4. Bedbrook JR, Coen DM, Beaton AR, Bogorad L, Rich A: Location of the single gene for the large subunit of ribulose bisphosphate carboxylase on the maize chloroplast chromosome. J Biol Chem 254: 905–910 (1979).
5. Bennett MD, Smith JB: Nuclear DNA amounts in Angiosperms. Trans Royal Society 274: 227–274 (1976).
6. Beverley SM: Characterisation of the unusual mobility of large circular DNA's in pulsed field-gradient electrophoresis. Nucleic Acids Res 16: 925–939 (1988).
7. Brownstein BH, Silverman GA, Little RD, Burke T, Korsmeyer SJ, Schlessinger D, Olson MV: Isolation of single-copy human genes from a library of yeast artificial chromosome clones. Science 244: 1348–1351 (1989).
8. Burke DT, Carle GF, Olson MV: Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236: 806–812 (1987).
9. Collins FS: Chromosome jumping. In: Davies KE (ed.) Genome Analysis, pp. 78–79. IRL Press, Oxford.
10. Freinberg AP, Vogelstein B: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13 (1983).
11. Green ED, Olsen MV: Systematic screening of yeast artificial-chromosome libraries by use of the polymerase chain reaction. Proc Natl Acad Sci USA 87: 1213–1217 (1990).
12. Grill E, Somerville C: Construction and characterisation of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. Mol Gen Genet 226: 484–490 (1991).
13. Kao KN, Michayluk MR: Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very population density in liquid media. Planta 126: 105–110 (1975).
14. Knop W: Quantitative Untersuchungen über die Ernahrungsprozesse der Pflanzen. Landwirtsch Vers Stn 7: 93–107 (1865).
15. Martinez-Zapater JM, Estelle MA, Somerville CR: A highly repeated DNA sequence in *Arabidopsis thaliana*. Mol Gen Genet 204: 417–423 (1986).
16. McIntosh L, Poulsen C, Bogorad L: Chloroplast gene sequence for the large subunit of ribulose bisphosphate-carboxylase of maize. Nature 288: 556–560 (1980).
17. Peacock WJ, Dennis ES, Rhoades MM, Pryor AJ: Highly repeated DNA sequence limited to knob heterochromatin in maize. Proc Natl Acad Sci USA 78: 4490–4494 (1981).
18. Sachs MM, Dennis ES, Gerlach WL, Peacock WJ: Two alleles of maize Alcohol dehydrogenase 1 have 3′ structural and poly(A) addition polymorphisms. Genetics 113: 449–467 (1986).
19. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
20. Southern EM: Detection of specific sequences among DNA fragments separated gel electrophoresis. J Mol Biol 98: 503–517 (1975).
21. Twigg AJ, Sherratt D: Trans-complementable copy number mutants of plasmid *col*E1. Nature 283: 216–218 (1980).
22. Verdel F, Delseny M: Repetitivity and variability of higher plant genomes. Plant Physiol Biochem 25: 191–210 (1987).
23. Wahl GM, Stern M, Stark GR: Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridisation using dextran sulfate. Proc Natl Acad Sci USA 76: 3683–3688 (1979).
24. Ward ER, Jen GC: Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly sheared *Arabidopsis thaliana* DNA. Plant Mol Biol 14: 561–568 (1990).