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Construction and characterisation of a yeast artificial chromosome library containing five haploid sugarbeet (*Beta vulgaris* L.) genome equivalents

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Abstract A yeast artificial chromosome (YAC) genomic library of *Beta vulgaris* was constructed in the pYAC4 vector. High-molecular-weight DNA was prepared from agarose-embedded leaf protoplasts from a triploid cultivar. The library was found to contain 33,500 clones in an ordered array of microtiter plates. Mean size of the inserts was estimated to be 135 kb, and the library should therefore represent the equivalent of five haploid genomes. The library was characterised for the presence of highly repetitive, chloroplast and single-copy sequences. In order to isolate single-copy sequences, 18 pools of DNA, each from 1920 individual YAC clones, were prepared for rapid screening of the library by the polymerase chain reaction. The results of these screenings showed that the number of isolated clones was at or near the frequency expected.

Key words *Beta vulgaris* · Polymerase chain reaction · Yeast artificial chromosome library · Genome mapping · Restriction fragment length polymorphism

Introduction

Until recently the analysis of complex eukaryotic genomes was a daunting task due to the relatively small inserts (20–45 kb) that could be cloned and maintained using bacteriophage or cosmid vectors in the bacterial host *E. coli*. The yeast artificial chromosome (YAC) technology has altered this situation by providing the possibility to construct clone-based physical maps of any genome. Several YAC libraries of higher plants have recently been constructed: e.g. *maize* (Edwards et al. 1992), *tomato* (Martin et al. 1992), *A. thaliana* (Grill and Somerville 1991) and *sugarbeet* (Eyers et al. 1992). However, the sizes of their inserts are much smaller than those obtained with mammalian genomes. These differences are problably due to problems unique to higher plants, such as the existence of a cell wall, the presence of polysaccharides and the large size of the plant cell. During the last few years a large amount of effort has been put into constructing restriction fragment length polymorphism (RFLP) maps of sugarbeet (Pillen et al. 1992; Barzen et al. 1992; Zeneca Seeds unpublished data). The existence of these RFLP maps together with the availability of a YAC bank will play an important role in the isolation and characterisation of economically important traits or genes on the basis of their known map position by chromosome walking and/or chromosome landing.

We report the construction and characterisation of a YAC library for sugarbeet. This library was constructed using agarose-embedded isolation and manipulation of high-molecular-weight DNA (Van Daelen et al. 1989) and contains 33 500 clones, which represents approximately five genome equivalents. We have evaluated it for the presence of repetitive, chloroplast and single-copy sequences and have screened it with a number of RFLP markers known to be tightly linked to one another and to a gene (R gene) that controls the production of red pigmentation in sugarbeet.

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Materials and methods

Beet line, yeast strain, plasmids and media

The YAC bank was constructed from a triploid beet cultivar, 'L122-13/OCDE' (*Beta vulgaris* L.), provided by Zeneca seeds. The cloning vector pYAC4 and the *Saccharomyces cerevisae* strain AB1380

(MATα, ura3, trp1, ade2-1, can 1–100, lys2-1, his5) were prepared and used as described by Burke et al. (1987). Yeast cultures were grown on either complete medium (YPD), single selection medium (lacking uracil) or double selection medium (lacking uracil and tryptophan) as described by Anand et al. (1989).

Preparation of DNA from individual YAC clones

Yeast DNA suitable for polymerase chain reaction (PCR) analysis and restriction digestion was prepared by resuspending yeast spheroplasts obtained from 5 ml of overnight culture into 400 µl yeast DNA extraction buffer (50 m*M* Tris-HCl pH 7.5, 25 m*M* EDTA, 250 m*M* NaCl, 1% SDS). After an incubation of 15 min at 65 °C, the sample was phenol and chloroform extracted and the DNA precipitated with ethanol. Total DNA was resuspended in 100 µl 1 × TE, and 1 µl of this mixture was subsequently used for PCR analysis.

Isolation of high-molecular-weight (HMW) DNA

High-molecular-weight DNA from yeast cells was prepared by embedding yeast spheroplasts obtained from 5 ml overnight of culture into 200 µl 1% low-melting agarose (Albertsen et al. 1990). High-molecular-weight DNA from beet was isolated from agarose-embedded leaf protoplasts obtained from 3-week-old, in vitro-grown plants (Shlangstedt et al. 1992). The protoplasts were resuspended in an equal volume of 2% Sea plaque low-gelling-temperature agarose at a final concentration of 4×10^7 protoplasts per milliliter. The molten agarose/protoplast suspension was gently poured into a mould and allowed to solidify at 4 °C. The plugs, each containing approximately 7 µg of HMW DNA, were incubated in ESP buffer (0.5 *M* Na_2EDTA pH 8, 1% sodium N-lauroylsarcosine, 1 mg/ml proteinase K) at 55 °C for 48 h. The plugs were finally stored at 4 °C in 50 m*M* Na_2EDTA.

Partial *Eco*RI digestion of the HMW plant DNA

Preparation of *Eco*RI partially digested DNA was essentially as described by Edwards et al. (1992), with the modification that the size fractionation was carried out in 1% agarose gel with a pulse duration of 10 s at 200 V for 2–4 h. Contour-clamped homogeneous electrophoresis (CHEF) was carried out using a BioRad CHEF apparatus.

Construction of the YAC library

For the construction of the YAC library, partially digested HMW DNA plugs were first washed three times in 1 × TE and 100 m*M* NaCl. Polyamines (1 m*M*: 0.75 m*M* spermidine and 0.3 m*M* spermine) were added, and the plugs were then melted by heating at 65 °C for 15 min. (Larin et al. 1991). After cooling to 37 °C, 200 units/ml agarase (Calbiochem) was added, and the mixture was further incubated at 37 °C for 6 h. After heat inactivation (15 min at 65 °C) of the agarase, the mixture was ligated to a 30-fold molar excess of *Eco*RI/*Bam*HI-cut and-dephosphorylated pYAC4 vector, and 6 µl of this latter mixture (i.e. approximately 50 ng of DNA) was used to transform 200 µl yeast spheroplasts as described by Burgers and Percival (1987).

Transformants were selected on solidified single-selection media (Anand et al. 1989). Transformed colonies were grown at 30 °C for 3–4 days before being transferred onto double-selection media supplemented with 0.5 µg/ml adenine hemisulphate (Sigma) to screen for inactivation of the *sup4* gene. After 2–3 days of growth, red colonies were scraped into 96 microtitre plates containing 100 µl of single-selection media. After 2 days of growth at 30 °C, 20% glycerol was added, and the plates were stored at −70 °C.

Screening of the library for chloroplast, repetitive and single-copy sequences

Screening of the library for chloroplast and repetitive sequences was carried out using conventional colony hybridisation (Brownstein et al. 1989), with the exception that lyticase was used instead of Zymolase 100T. Radioactive probes were prepared by the random primer labelling method (Feinberg and Vogelstein 1983). Screening of the beet YAC library for single-copy sequences was carried out by the PCR screening method (Green and Olsen 1990). DNA was extracted from 5 × 96 YAC clones grown on single-selection medium (secondary pools, 480 colonies). Primary pools were constructed by mixing the DNA of 4 secondary DNA pools (1920 colonies), resulting in 18 primary pools for the entire library. Screening was first carried out on the primary pools. Positive primary pools were divided in the four subsequent secondary pools. Finally, positive secondary pools from this screening were used to make filters which were screened using colony hybridisation in order to identify individual positive clones.

Results

Construction of the Yac library

DNA prepared from agarose-embedded leaf protoplasts was subjected to partial *Eco*RI digestion. The best partial digestion conditions were 60 units *Eco*RI per milliliter protoplasts for 1 h, as judged by CHEF (data not shown). After partial digestion, a size-fractionation step is critical for the removal of low-molecular-weight DNA (LMW) in order to obtain reasonable insert sizes of the YAC clones. Our experiments showed that size fractionation by electrophoresis using a switch time of 10 s for 4 h gave the best result in obtaining good average insert sizes (150 kb); the transformation efficiency using this DNA was quite low (about 500 transformants/µg DNA). A size fractionation of 2 h, however, gave a better transformation efficiency (about 4000 transformants/µg DNA), but the average insert size was smaller (120 kb). To overcome these problems we preferred to use, for the final construction of the YAC library, a combination of size-fractionated DNA pools to obtain a good transformation efficiency (2000–3000 transformants/µg) and YACs with reasonable (135 kb) insert sizes. We used three size-fractionation times, i.e. 2, 3 and 4 h with a pulse time of 10 s.

We also tested the effect of polyamines (Larin et al. 1991) on the insert size of the YAC clones obtained. Experiments showed that there was no significant increase in insert size when polyamines were used. These results are in accordance with those reported by Larin et al. (1991), but not with the data reported by Connelly et al. (1991). The only effect we could observe when the polyamines were used at a 1 m*M* concentration throughout the transformation process was a drastic decrease in the transformation efficiency without an increase in insert size. When polyamines were added prior to transformation, particularly when a heating step at 65 °C was involved, there was an increase in transformation efficiency (10–20%) without a significant increase in the insert size of the YAC clones.

Clone size distribution

To obtain an idea about the average size of the inserts of the YAC library, ten agarose plugs were prepared, each plug containing 96 individual YAC clones taken at random throughout the library. These plugs were subjected to pulse-field gel electrophoresis and blotted onto hybond N membranes (Amersham). The blot was hybridised with [^{32}P]-labelled pBR328. The patterns of the autoradiographs were analysed by an LKB scanning densitometer and plotted as an histogram (Fig. 1). The results show that the library has an average insert size of 135 kb (145 kb minus 10 kb from the YAC vector) with 82% of the clones having an insert size bigger than 100 kb.

Presence of chloroplast, repetitive and single-copy sequences in the library

In order to investigate the percentage of clones containing repetitive elements and chloroplast sequences we screened 960 randomly chosen YAC clones by colony hybridisation. The percentage of YACs containing chloroplast-derived sequences was determined by probing the 960 YAC clones with a PCR-amplified DNA fragment for the large unit of ribulose biphosphate carboxylase (McIntosh et al. 1980). Out of 960 clones 9 contained chloroplast DNA, which represents approximately 1% of the library.

To evaluate the occurrence of highly repetitive elements, 960 randomly chosen YAC clones were hybridised with the pBV1 monomer of the *Bam*HI sequence family (Schmidt and Metzlaff 1991). Forty-five positive clones were found, which represents approximately 4.5% of the total YAC library.

For the isolation of single-copy sequences we used the three-stage PCR screening method, which makes it possible to tell within 4 h whether or not a particular clone is present in the library. Individual positive clones can be isolated within 5 working days. An example of such a screening is the isolation of the betaine aldehyde dehydrogenase gene (BADH) (McCue et al. 1992), as shown in Fig. 2. The appearance of two PCR bands with the genomic DNA was most interesting. These bands were also present in the positive YAC clones and most likely represent the two allelic forms of the gene described by McCue et al. (1992). Screening of the library with a set of primers form five RFLP markers (Zeneca Seeds) showed that all of these sequences were present in the YAC bank at or near the expected frequency (Table 1). Of these markers three are RFLPs for the R gene: PBVR1 and PBVR2 for the right side of the gene and PBVR3 for the left side.

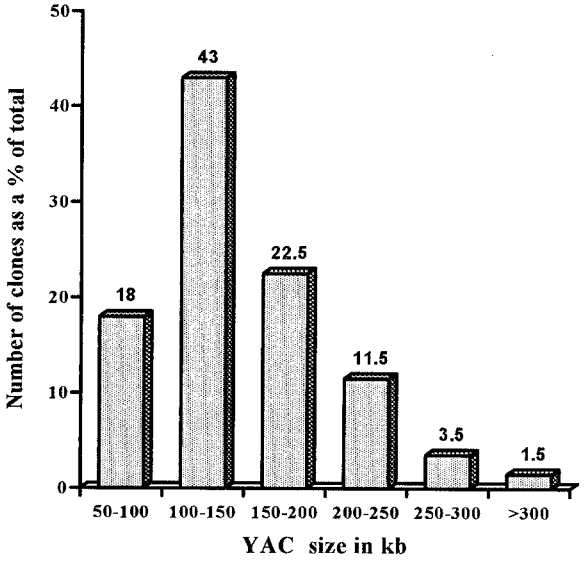

Fig. 1 Size distribution of 960 randomly chosen YAC clones. The calculated mean YAC size is 135 kb

Discussion

Like those of all other higher eukaryotes, the beet genome has a complex organisation that consists of unique or low-copy number sequences surrounded by

Fig. 2A, B Identification of YAC clones containing the beet BADH gene. **Panel A** Eighteen secondary pools of the sugarbeet YAC library (A–R) were analysed by PCR for the presence of the BADH gene sequence. **Panel B** Pools A, L and N were divided into four corresponding primary pools (i.e. 480 clones) and re-analysed by PCR. In panel A and B the molecular weight markers are lamba DNA cut with *Pst*I (*M*). Sizes are in kilobases (kb). *B* corresponds with the PCR product of 20 ng of genomic beet DNA. The position of the expected fragment is marked (0.7 kb)

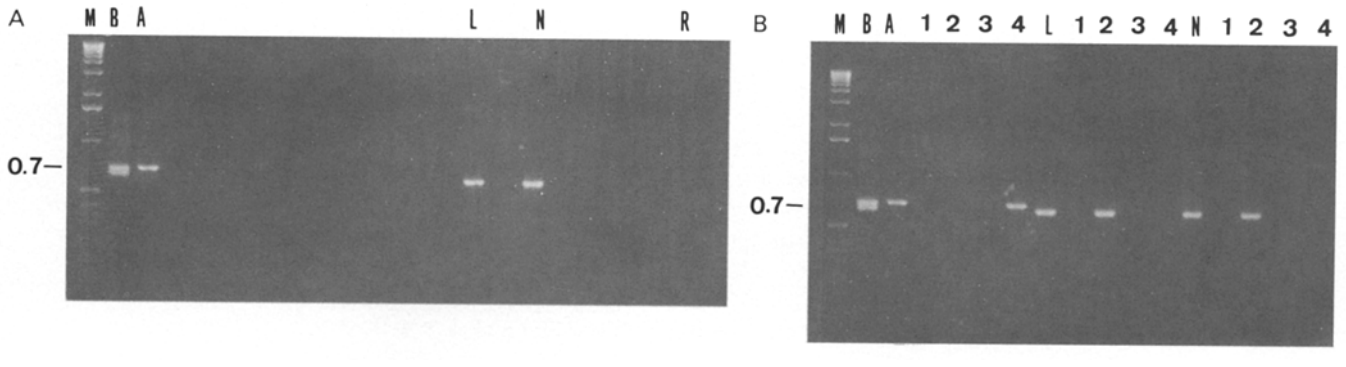

Table 1 Results of PCR screening of the beet YAC library with six sets of primers

Source of PCR primers	Number of positives
pBVR3	12
Ea332	1
Oa26	2
pBVR1	9
pBVR2	4
BADH	3

large arrays of highly repetitive DNA (Vedel and Delseny 1987). Such an organisation makes physical mapping using lambda or cosmid clones virtually impossible. Since YACs are able to bridge these large stretches of repetitive DNA it seems to be an ideal tool for the representative cloning of sugarbeet DNA.

We constructed a YAC library from high-molecular-weight DNA that had been partially digested with *Eco*RI. This partially digested HMW DNA was size fractionated to remove LMW (< 90 kb) DNA in order to obtain a reasonable average insert size of the YAC library. While constructing the YAC library we found that by adding polyamines immediately prior to heating the transformation efficiency increased by 20% without a significant increase in average insert size of the YAC clones. This increase is probably due to the protective effect of the polyamines on the degradation of HMW DNA (Larin et al. 1991). The final library consists of 33 500 clones with an average insert size of 135 kb and therefore should provide a 95% probability of containing any specific single-copy sequence.

Screening of the library with pBV1 repeat and chloroplast-specific probes showed that both were present within the library. The chloroplast clones were present at a much lower frequency than expected. Published data on plant YAC libraries show that between 2.5% (Eyers et al. 1992) and 10% (Martin et al. 1992) of the total YAC clones contain chloroplast sequences. In our library only 1% of the YACs contain chloroplast sequences. This is probably due to the extensive size fractionation that was used prior to the ligation of the YAC arms with the partially digested HMW DNA.

Using the PCR approach, we screened the library with six sets of PCR primers derived from RFLP markers and one sequence (BADH) taken from the EMBL database. Between 1 and 12 positive clones (average of 5) for each set of primers were found. We therefore believe that this sugarbeet library will enable together with the recently developed RFLP maps (Pillen et al. 1992; Barzen et al. 1992; Zeneca Seeds, unpublished data), the isolation of genes responsible for important agronomic traits using linkage to RFLP markers. Such RFLP markers that flank traits of interest can be used as a starting point for chromosome walking and will enable the isolation and characterisation of such traits from the YAC library. To test this hypothesis we started to screen our library with RFLP markers surrounding the R gene from sugarbeet. This R gene is responsible for the red colour in sugarbeets. So far, we have isolated the YAC clones containing each one of three RFLP markers for the R gene. Two of these (PBVR1 and PBVR2) are situated on the right site of the gene, at approximately 3 cM. On the left side, we have isolated the positive YAC clones with the corresponding RFLP marker (PBVR3), which is about 5 cM removed from the R gene.

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