Theor Appl Genet (2000) 100:112–117

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Construction of a yeast artificial chromosome library of pepper (*Capsicum annuum* L.) and identification of clones from the *Bs2* resistance locus

Received: 15 January 1999 / Accepted: 11 May 1999

Abstract A yeast artificial chromosome (YAC) library was constructed using high-molecular-weight DNA isolated from pepper (*Capsicum annuum* L.) leaf protoplasts. Insert DNA was prepared by partial digestion using *Eco*RI and subjected to electrophoretic fractionation before in-gel ligation to the pJS97/98 YAC vector. Prior to transformation of yeast spheroplasts, ligation products were subjected to a second electrophoretic size selection. The library consists of about 19 000 clones with an average insert size of 500 kb, thus representing approximately three haploid genome equivalents. Three PCR-based markers tightly linked to the pepper *Bs2* resistance gene were used to assess the utility of this library for positional cloning. Three YAC clones containing pepper genomic DNA from the *Bs2* resistance locus were isolated from the library. The clones ranged in size from 270 kb to 1.2 Mb and should prove useful for the cloning of the *Bs2* gene.

Key words Pepper · YAC library · Positional cloning · Resistance gene · *Bs2*

Introduction

The isolation of genes of interest via map-based or positional cloning is a well-established strategy that has become more feasible with the advent of large-insert cloning systems. Since the development of the yeast artificial chromosome (YAC) cloning system, several YAC libraries have been constructed for various plant species (Ward and Jen 1990; Grill and Somerville 1991; Martin et al. 1992; Edwards et al. 1992). YAC libraries have facilitated the positional cloning of a number of resistance genes (Martin et al. 1993; Bent et al. 1994; Yoshimura et al. 1996). The power and utility of YAC libraries is particularly evident in studies aimed at isolating genes of interest from agronomically important crop species that tend to have large genomes. YAC clones make chromosome walking in these species practical by reducing the number of "steps" that must be taken as well as potentially spanning large stretches of repetitive DNA that might confound walking efforts.

Although pepper is a leading vegetable crop, there has been relatively little reported with regard to development of tools for gene isolation and genome analysis such as a high-density molecular genetic map (Prince et al. 1992). The development of randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analysis has circumvented the need for the construction of molecular genetic maps of entire genomes (Welsh and McClelland 1990; Williams et al. 1990; Vos et al. 1995). However, positional cloning in many crop species still requires the development of large-insert genomic libraries.

The pepper genome is about three times that of tomato and twenty times that of *Arabidopsis* (Arumuganathan and Earle 1991), thus making positional cloning a daunting if not impossible task without the aid of a large-insert genomic DNA library. In order to facilitate the isolation of genes from pepper by positional cloning, we have constructed a YAC library consisting of about 19 000 clones. Based on a haploid genome size of about 3×10^9 base pairs (Arumuganathan and Earle 1991), the library should theoretically contain three haploid genome equivalents and represent at least 95% of the pepper genome. Three molecular markers, tightly linked to the pepper Bs2 resistance gene, were used to screen YAC DNA pools representing approximately 95% of the clones. At least 1 clone was identified for each marker. This library is currently being used for the positional cloning of the *Bs2* resistance gene. Moreover, the avail-

Communicated by J. Dvòràk

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ability of this library will facilitate the cloning of other pepper genes and aid in the physical characterization of the pepper genome.

Materials and methods

Chemicals and enzymes

All chemicals and components for growth media were obtained from Sigma (St. Louis, Mo.) or Fisher Scientific (Pittsburgh, Pa.) unless otherwise noted. Restriction and modifying enzymes were obtained from New England Biolabs (Beverly, Mass.) and US Biochemicals (Cleveland, Ohio). Proteinase K was from Boehringer Mannheim (Indianapolis, Ind.). Agarase and enzymes for protoplast isolation were obtained from Calbiochem (La Jolla, Calif.) except pectolyase which was from Seishin Pharmaceutical (Tokyo, Japan). Zymolyase was from Seikagaku America (Rockville, Md.) and lyticase was from Sigma. Low-melting-point agarose was from FMC Bioproducts (Rockland, Me.).

Plant material, yeast strain, and growth and culture conditions

Seeds from pepper cultivar 'Early Calwonder-123R' (ECW-123R; *Bs1/Bs1, Bs2/Bs2, Bs3/Bs3*; provided by R. Stall, University of Florida) were germinated under sterile conditions on media containing 1 × MS salts (Gibco BRL, Gaithersburg, Md.), 3% sucrose and 0.8% agar noble (pH 5.7 using KOH) and grown for 4–8 weeks before protoplast isolation. *Saccharomyces cerevisiae* strain YPH252 (Matα, ura3–52, lys2–801^{amber}, ade2–101^{ochre}, trp1-Δ1, his3-Δ200, leu2-Δ1) cultures were grown on complete media (YPD media; 1% yeast extract, 2% bactopeptone, 2% dextrose). After transformation, yeast cells were grown on selective media (minimal media lacking uracil and tryptophan).

Pulsed field gel electrophoresis (PFGE)

High-molecular-weight pepper DNA and yeast chromosome preparations were analyzed by contour-clamped homogeneous electrophoresis (CHEF) using a CHEF DR II apparatus (BioRad, Richmond, Calif.). Typical electrophoretic conditions for plant DNA were: 200 V, 5- to 120-s ramped switch time, 24- to 35-h run, at 10° to 14°C . For the analysis of YACs, the conditions were: 200 V, 60-s constant switch time for 15 h, followed by a 90-s constant switch time for 6 to 8 h, at 10° to 14°C . Gels consisted of 1% agarose and were electrophoresed in 0.5 × TBE buffer.

Isolation of high-molecular-weight DNA from pepper protoplasts

Abaxial sides of fully expanded leaves were abraded with 1% celite in 50 m*M* monobasic potassium phosphate buffer (pH 7.0) and placed in a petri dish with protoplast buffer (10% mannitol, 5 *mM* MES, pH 5.6–5.7) containing 0.1% bovine serum albumin, 1% Cellulysin, 0.05% macerase and 0.01% pectolyase. Leaves were digested for 4–5 h at 25° to 30°C with gentle shaking (50–60 rpm). Protoplasts were collected by filtering through nylon mesh (40 to 80 μm) and centrifuging at 600–700 rpm for 6 min. Pelleted protoplasts were suspended in 10 ml of protoplast buffer and underlaid with 3 ml of 20% sucrose. Following another centrifugation, protoplasts were collected from the interface, diluted with 3–4 volumes of protoplast buffer and then centrifuged again. The final pellet of protoplasts was suspended in 1–2 ml of protoplast buffer, and the concentration of protoplasts was determined using a hemocytometer. The concentration was adjusted to between 2 and 4 × 107 protoplasts per microliter. Protoplasts were warmed to 37°C and combined with an equal volume of 1–1.2% Seaplaque GTG agarose being held at 42°C. The evenly mixed suspension was transferred to plug molds (BioRad, Richmond, Calif.) and placed at 4°C for 10 min. Solidified plugs were processed according to Ganal and Tanksley (1989) and then stored in TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8) at 4°C. The final concentration of embedded high-molecular-weight DNA was estimated to be 10 μg per 100 μl of agarose.

Partial digestion and size selection of insert DNA

Partial restriction enzyme digests were performed on the embedded high-molecular-weight DNA using *Eco*RI. Two methods were used to generate DNA for the library. In the first method, plugs, which had been equilibrated with restriction enzyme buffer, were incubated on ice in the presence of enzyme (2.5–5 U per microgram of DNA) for 15 min and then transferred to 4°C for another 15 minutes. The plugs were then placed at 37°C for 10–15 min before terminating the digestion by adding EDTA to 50 m*M* and placing on ice. The second method employed a competition strategy involving *Eco*RI and *Eco*RI methylase and was performed essentially as previously described (Larin et al. 1991; Smith et al. 1990). Immediately following partial digestion, the samples were subjected to size fractionation using CHEF gel electrophoresis. Plugs were transferred to a 1% Seaplaque GTG agarose gel and electrophoresed in 0.5 × TBE buffer under the following conditions:150 V, 20-s constant switch time, 16–18 h, at 10 to 14°C. Fragments larger than 300–400 kb were compressed into a zone of limiting mobility (Anand et al. 1989; Albertsen et al. 1990; Imai and Olson 1990; Bentley et al. 1990), which was then excised from the gel and used immediately in ligation reactions or stored in T50E buffer (10 m*M* Tris, 50 m*M* EDTA, pH 7.5) at 4°C.

Preparation of YAC vector arms, ligation of insert DNA and size selection of YAC constructs

The pJS97/98 YAC vector system (McCormick et al. 1989; Shero et al. 1991) was chosen for the generation of the ECW-123R YAC library. Plasmid DNA was isolated by the alkaline lysis method and purified twice using cesium chloride/ethidium bromide banding (Sambrook et al. 1989). The pJS97 plasmid was digested with *Cla*I and the pJS98 plasmid was digested with *Sal*I. Linearized plasmids (i.e. vector arms) were treated with shrimp alkaline phosphatase according to the manufacturer's instructions. Dephosphorylation was checked by ligation and gel electrophoresis. Each arm was subjected to digestion with *Eco*RI, ethanol-precipitated and suspended in TE buffer. The arms were then combined in equimolar amounts to a final concentration of 1 μg/μl. For each ligation, a part of the excised agarose which contained partially digested insert DNA (typically 20–40 μg in 0.5–1 g of agarose) was cut into small blocks (2–3 mm thick) and equilibrated in 50 m*M* Tris-HCl (pH 7.8), 10 m*M* MgCl2, 30 m*M* NaCl, and 1 × polyamines (0.75 m*M* spermidine trihydrochloride, 0.3 m*M* spermine tetrahydrochloride) at room temperature. The agarose-embedded insert DNA was transferred to a 2-ml centrifuge tube, and an equal amount of vector DNA was added. Samples were then melted at 65°C for 15 min and cooled to 42°C for a few minutes before the addition of bovine serum albumin (50 μg/ml), ATP (1 m*M*) and DTT (20 m*M*). T4 DNA ligase (4000–8000 units) was diluted in 1× ligase buffer and warmed briefly to 37°C before being added to the molten mixture of insert and vector DNA. Mixing was accomplished by gently swirling the pipet tip as the ligase was added. Ligations were allowed to proceed at 37°C for 15–30 min before transferring to 16°C or room temperature for 16–24 h. The ligation products were subjected to a second size fractionation using CHEF gel electrophoresis as described earlier.

Preparation and transformation of yeast spheroplasts with YAC clones

Spheroplasts were prepared from the yeast strain YPH 252 essentially as described (Burgers and Percival 1987). Zymolyase was

used to generate the protoplasts, and the amount used generated 95–100% spheroplasts following a 20-min incubation at 30°C. Prior to transformation, the agarose-embedded ligation products were equilibrated in 10 m*M* TRIS-HCl (pH 7.5), 1 m*M* EDTA, 30 m*M* NaCl and 3 m*M* spermidine (S. Foote, personal communication). Ligation products were liberated from the agarose by melting at 65°C for 15 min, cooling to 42°C, adding β-agarase (40–50 U/ml), and incubating at 42°C for 40—60-min. Transformations were performed as described by Burgers and Percival (1987) with the following modifications. Typically, 500 µl of spheroplasts was transformed with 50–75 µl of agarase-treated YAC DNA (about 50–100 ng) in a 50-ml conical tube. Transformed spheroplasts were rescued by the addition of 750 µl of SOS media (1 *M* sorbitol, 6.5 m*M* $CaCl_2$, 0.25% yeast extract, 0.5% bactopeptone) and incubation at 30°C for 20–30 min. Following the transformation procedure, spheroplasts were plated by adding 25–30 ml of TOP-UraTrp media (0.9 *M* sorbitol, 0.67% yeast nitrogen base without amino acids, 3% dextrose, 0.8 g/l of UraTrp dropout supplement, 2% agar noble), gently mixing and pouring onto plates of SORB-UraTrp media [1 *M* sorbitol, 0.67% yeast nitrogen base without amino acids, 2% dextrose, 2% agar noble, and 0.8 g/l of UraTrp dropout supplement (Bio101, LaJolla, Calif.)]. Plates were incubated at 30°C for 6–7 days. Transformants were transferred to KIWI agar plates (2% dextrose, 0.67% yeast nitrogen base without amino acids, 1.1% casamino acids, 1.3 g/l KIWI dropout powder, 2.2% Bacto-agar, pH 7.0; R. Schmidt, personal communication), and permanent glycerol stocks were made by transferring cells to 96-well microtiter plates containing KIWI broth with 20% glycerol and storing at –80°C.

Analysis of individual YAC transformants

Yeast chromosomal DNA plugs were prepared using a miniprep procedure as described by Burke (1989). The yeast chromosomes were analyzed by CHEF gel electrophoresis through 1% agarose gels in 0.5× TBE buffer. YACs were visualized by staining with ethidium bromide and Southern hybridization as described (Sambrook et al. 1989). Vector-specific DNA (pUC 19) was radiolabeled by random-hexamer labeling and used to probe the blots of the CHEF gels.

Screening of the library for chloroplast sequences

In order to determine what percentage of the library contains chloroplast-derived sequences, a 1.2-kb clone of the rbcL gene from spinach (a gift from M. Roell) was used to probe colony lifts of 698 independent YAC transformants. Colony lifts were performed according to R. Schmidt (personal communication).

Polymerase chain reaction (PCR) and hybridization-based screening of the library

To facilitate screening of the library, we isolated yeast genomic DNAs from 47 pools consisting of 384 transformants each (representing about 95% of the library). Colonies were arrayed (16×24) on filters using a 96-prong replicating device and grown on KIWI media for 48–72 h. Arrays were also made directly on growth media. Cells were scraped off the filter, and DNA was prepared as described (Green and Olson 1990). Arrays that were grown directly on media were stored at 4°C. The YAC pools were screened using PCR. Primer sequences for AFLP markers A2, B3 and F1 were as reported in Tai et al. (1999). Pools identified by PCR screening were analyzed by colony hybridization of lifts made from the arrays stored at 4°C. Lifts and hybridizations were performed as described above.

Results

Construction of the YAC library

A yeast artificial chromosome library was constructed using high-molecular-weight DNA from the pepper cultivar ‘ECW-123R’. High-molecular-weight DNA was isolated from agarose-embedded leaf protoplasts. Insert DNA was prepared from the high-molecular-weight DNA by partial digestion achieved by limited incubation with *Eco*RI or by *Eco*RI/*Eco*RI methylase competition reactions. Insert DNA fragments of more than 300 kb were size-selected by fractionation using CHEF gel electrophoresis and then ligated to pJS97/98 YAC vector arms. A second CHEF gel size selection to increase the stringency of selection for high-molecular-weight clones was performed prior to the transformation of the haploid yeast strain YPH 252 with these ligation products.

Transformations were performed using yeast spheroplasts that yielded 10^5–10^6 transformants per microgram of vector DNA (pJS97) in control experiments. Transformation efficiencies with YAC ligations ranged from about 300 to 2000 transformants per microgram of ligated DNA. Spheroplasts were plated directly on media lacking uracil and tryptophan, thus immediately selecting for transformants containing each vector arm and presumably an authentic intact YAC clone.

Characterization of the library

The ‘ECW-123R’ YAC library consists of approximately 19 000 clones obtained from several ligations and transformations (Tai 1995). To determine the size of the clones, we prepared yeast chromosomal DNAs from 110 randomly selected transformants (10–20 per set of transformations). These DNAs were examined by CHEF gel electrophoresis and Southern hybridization with vector-specific radiolabeled probes (Fig. 1). More than 95% of the transformants for each transformation set contained single YACs based on Southern hybridization. The remaining transformants belonged to one of two classes. One class consisted of transformants containing more than 1 YAC. This suggests that the DNA was prepared from mixed colonies. Members of the second class displayed an identical hybridization pattern made up of five diffuse bands ranging in size from several hundred kilobases to fewer than 50 kb. These transformants were not characterized further. The YAC clones analyzed by CHEF gel electrophoresis ranged from 50 kb to about 1.2 Mb. The average clone size was approximately 500 kb, with most clones ranging from 300–600 kb (Fig. 2).

The extent of chloroplast sequences contained in the librarywas determined using a clone of the chloroplast rbcL gene to probe colony lifts of 698 transformants (about 4% of the library). A total of 10 positives were detected, indicating that less than 2% of the library contains chloroplast-derived sequences.

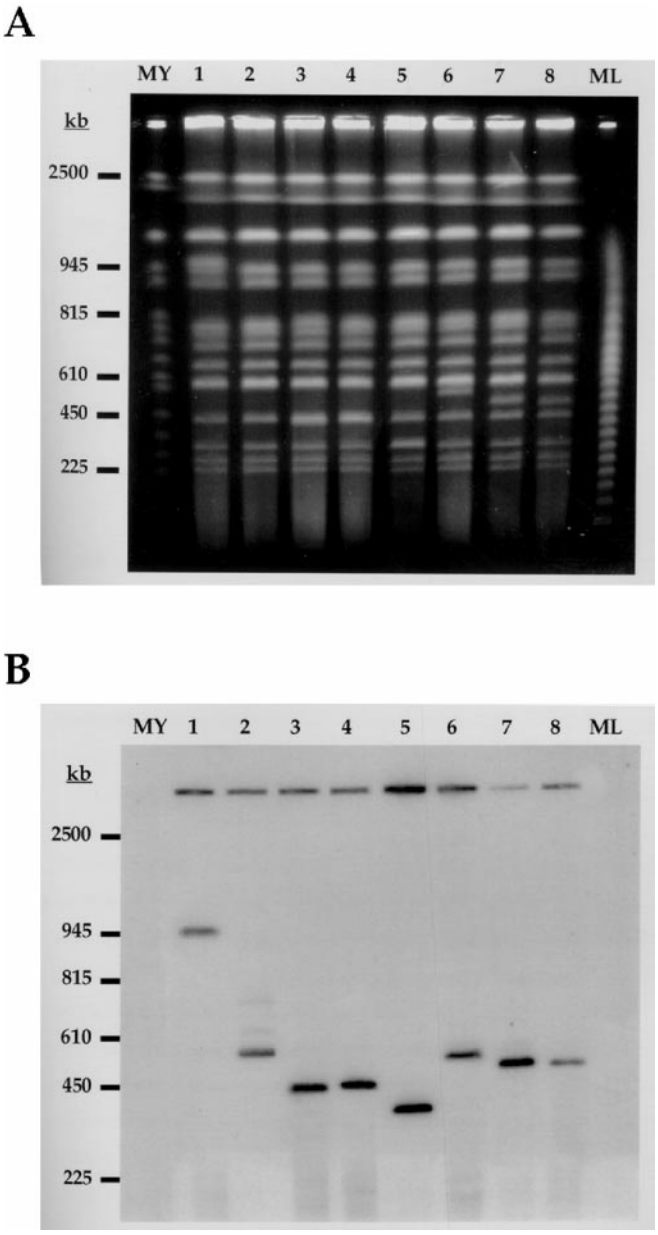

Fig. 1A B Pulsed field gel electrophoresis of randomly selected YAC transformants. **A** CHEF gel stained with ethidium bromide. *Lanes 1–8* are randomly chosen YAC transformants, *MY* yeast chromosomal DNA marker, *ML* lambda ladder DNA marker (New England Biolabs, Beverly, Mass.). **B** Southern blot of CHEF gel probed with radiolabeled vector specific DNA (pUC19)

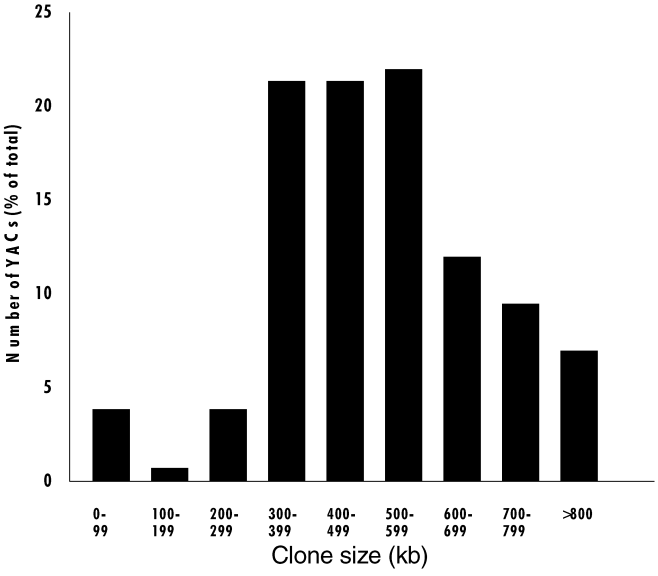

Fig. 2 Size distribution of YAC clones. The size distribution of 110 randomly selected YAC clones is shown. Approximately 60% of the clones range in size from 300 to 600 kb. The average clone size is about 500 kb

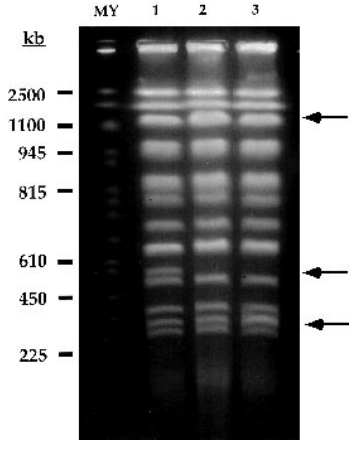

Fig. 3 CHEF gel of YACs from the *Bs2* region. YAC clones identified by screening the library with PCR primers specific to the A2, B3, and F1 markers. *Lanes: 1* YCA22D8 contains markers A2 and F1–550 kb, *2* YCA80H11 contains markers A2 and F1–1.2 Mb, *3* YCA164C12, which co-migrates with second smallest yeast chromosome, contains marker B3–270 kb. *MY* Yeast chromosomal DNA marker (New England Biolabs, Beverly, MA)

Based on these data, this YAC library represents about three haploid genome equivalents of pepper. Thus, there is a 95% probability of identifying any given sequence within the library (Clarke and Carbon 1976). The YAC clones are maintained as glycerol stocks in 96-well microtiter plates as described above. DNA pools made from the library to facilitate PCR-based screening are available upon request.

Identification of clones containing sequences in the *Bs2* region

Previously, a high-resolution linkage map of the *Bs2* locus was developed using RAPD and AFLP methods (Tai et al. 1999). The AFLP marker A2 was shown to cosegregate with the *Bs2* gene. PCR primers for the A2 marker were used to screen 47 YAC DNA pools, each of which consisted of 384 transformants. Two pools produced the A2 PCR product. Subsequent colony hybridizations of the 384 transformants of each pool resulted in the isolation of 2 clones, YCA22D8 and YCA80H11,

which hybridized to the A2 marker. Further analysis revealed that both YACs also contained the F1 marker which had previously been shown to be ≤ 150 kb distant from the A2 marker (Tai et al. 1999). A third YAC, YCA164C12, was identified by screening the library with primers for the AFLP marker B3, which maps to the other side of the A2 marker. YCA164C12 did not contain either the A2 or F1 markers. CHEF gel electrophoresis analysis of yeast chromosomal DNA from the 3 transformants indicated that the 3 YACs range in size from 270 kb to 1.2 Mb (Fig. 3).

Discussion

Positional or map-based cloning provides one of the most effective methods for isolating genes of unknown function, such as plant disease resistance genes. This approach is of particular importance in plant species where efficient transformation has not been achieved, thus precluding the deployment of transposon tagging or other insertional mutagenesis strategies. In the case of pepper, positional cloning is a plausible strategy for the isolation of biologically and agronomically important genes. Given the genome size of pepper, it is necessary to construct large-insert genomic libraries to expedite the chromosome walking component of positional cloning. In order to facilitate the isolation of pepper genes of interest and analysis of the pepper genome, we have constructed a YAC library.

Due to the size and relatively uncharacterized nature of the pepper genome, it was necessary to generate fairly large clones to make chromosome walking or landing practical. The library consists of approximately 19 000 YAC clones with an average insert size of 500 kb. The average insert size is much larger than earlier plant libraries (Ward and Jen 1990; Grill and Somerville 1991; Martin et al. 1992; Edwards et al. 1992) and is comparable to the sizes of recently reported YAC libraries made from *Arabidopsis* and tomato (Creusot et al. 1995, Bonnema et al. 1996). The generation of large YAC clones was greatly facilitated by the use of CHEF gel electrophoresis-based size selection of partially digested high-molecular-weight DNA and subsequent ligation products (Anand et al. 1989; Albertsen et al. 1990; Imai and Olson 1990; Larin et al. 1991).

In constructing this library, a relatively new YAC cloning vector system was employed. Most YAC libraries have been constructed using the pYAC4 vector system (Burke et al. 1987); however, several new cloning vectors have been available for some time (Smith et al. 1990; Shero et al. 1991). For this library, the pJS97/98 cloning system was chosen. In this system, the two arms of the YAC vector are maintained as separate plasmids and, thus, plasmid end rescue of both ends of a YAC clone is facilitated. More importantly, with regard to library construction, enhancements in the vector allow for immediate double selection of transformants, which increases the efficiency of library construction (Shero et al. 1991). Simultaneous selection of both arms in a YAC clone eliminates spurious transformants that result from the integration of clones containing a single arm into native yeast chromosomes through recombination.

Molecular characterization of the library indicates that it should represent at least 95% of the pepper genome. Screening of the library with molecular markers linked to the pepper *Bs2* resistance gene (Tai et al. 1999) resulted in the detection of at least 1 positive clone for each marker examined. Two YACs, YCA22D8 and YCA80H11, contain both A2, a marker which co-segregates with the *Bs2* gene, and F1, a tightly linked flanking marker previously shown to be within 150 kb of A2 (Tai et al. 1999). The identification of multiple YAC clones containing DNA from the *Bs2* locus provides a strong foundation for positional cloning of this gene. Subsequent characterization of these clones has shown that they are not chimeric and should faithfully represent the genomic region containing the *Bs2* gene (T. Tai and B. Staskawicz, manuscript in preparation).

Although the advent of bacterial artificial chromosome (BAC) cloning (Shizuya et al. 1992; Woo et al. 1994) has diminished the need for the construction of large-insert libraries using the YAC system, the possibility of generating clones that are two to three times larger on average and the use of an alternative host remain important features of YAC cloning. Ultimately, the utility of any genomic library is based on the presence of desired sequences. The results reported here suggest that this library should be a useful tool for positional cloning and genome analysis of pepper.

Acknowledgements We thank Romela Pasion for technical assistance in storage of the YAC library, and Maureen Whalen and Mary Beth Mudgett for the critical reading of and useful comments concerning this manuscript. This work was supported by a grant from Diatech Ltd. to B.J.S. T.H.T. was supported by a NSF Predoctoral Fellowship and a Chancellor's Opportunity Fellowship from the University of California.

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