Melon bacterial artificial chromosome (BAC) library construction using improved methods and identification of clones linked to the locus conferring resistance to melon Fusarium wilt (*Fom-2*)

Meizhong Luo, Yi-Hong Wang, David Frisch, Tarek Joobeur, Rod A. Wing, and Ralph A. Dean

Abstract: Utilizing improved methods, two bacterial artificial chromosome (BAC) libraries were constructed for the multidisease-resistant line of melon MR-1. The *Hin*dIII library consists of 177 microtiter plates in a 384-well format, while the *Eco*RI library consists of 222 microtiter plates. Approximately 95.6% of the *Hin*dIII library clones contain nuclear DNA inserts with an average size of 118 kb, providing a coverage of 15.4 genome equivalents. Similarly, 96% of the *Eco*RI library clones contain nuclear DNA inserts with an average size of 114 kb, providing a coverage of 18.7 genome equivalents. Both libraries were evaluated for contamination with high-copy vector, empty pIndigoBac536 vector, and organellar DNA sequences. High-density filters were screened with two genetic markers FM and AM that cosegregate with *Fom-2*, a gene conferring resistance to races 0 and 1 of Fusarium wilt. Fourteen and 18 candidate BAC clones were identified for the FM and AM probes, respectively, from the *Hin*dIII library, while 34 were identified for the AM probe from filters A, B, and C of the *Eco*RI library.

Key words: bacterial artificial chromosome (BAC) library, Fusarium wilt, melon, pCUGIBAC1, resistant gene.

Résumé : À l'aide de méthodes améliorées, deux banques de chromosomes bactériennes artificielles, « bacterial artificial chromosome » (BAC), ont été produites à partir de la lignée de melon MR-1, laquelle est résistante à de nombreuses maladies. La banque *Hin*dIII compte 177 plaques de titration à 384 puits, tandis que la banque *Eco*RI comprend 222 plaques. Environ 95,6 % des clones *Hin*dIII contiennent des inserts d'ADN nucléaire d'une taille moyenne de 118 kb, ce qui procure une couverture équivalente à 15,4 génomes. De façon semblable, 96 % des clones de la banque *Eco*RI sont porteurs d'inserts constitués d'ADN nucléaire dont la taille moyenne est de 114 kb pour une couverture de 18,7 génomes. Les deux banques ont été évaluées pour le degré de contamination avec du vecteur en grand nombre de copies ou avec du vecteur pIndigoBac536 vide ou encore avec des séquences d'ADN provenant des organites. Des membranes à haute densité ont été criblées avec deux marqueurs moléculaires, FM et AM, qui montrent une coségrégation avec *Fom-2*, un gène qui confère la résistance aux races 0 et 1 de la flétrissure fusarienne. Quatorze et 18 clones BAC candidats ont été identifiés parmi la banque *Hin*dIII à l'aide des sondes FM et AM respectivement. Chez la banque *Eco*RI, 34 clones ont été identifiés parmi les membranes A, B et C à l'aide de la sonde AM.

Mots clés : banque de « bacterial artificial chromosome » BAC, flétrissure fusarienne, melon, pCUGIBAC1, gène de résistance.

[Traduit par la Rédaction]

Introduction

Libraries with large genomic DNA inserts are essential for physical mapping, positional cloning, and genome sequencing, particularly for higher eukaryotes (Hosoda et al. 1990; Shizuya et al. 1992; Tanksley et al. 1995; Zhang et al. 1994). The BAC (bacterial artificial chromosome) cloning system has become an invaluable tool in genomics studies

Received June 26, 2000. Accepted December 5, 2000. Published on the NRC Research Press Web site March 6, 2001.

Corresponding Editor: R.J. Kemble.

M. Luo,[1] D. Frisch, and R.A. Wing. Clemson University Genomics Institute (CUGI), 100 Jordan Hall, Clemson, SC 29634, U.S.A.
Y.-H. Wang,[2] T. Joobeur and R.A. Dean. Fungal Genomics Laboratory, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27606, U.S.A.

[1]Corresponding author (e-mail: mluo@clemson.edu).
[2]Present address: United States Department of Agriculture – Agriculture Research Services (USDA–ARS), U.S. Plant, Soil and Nutrition Lab, Cornell University, Tower Road, Ithaca, NY 14853, U.S.A.

 DOI: 10.1139/gen-44-2-154

because of its ability to stably maintain large DNA fragments and its ease of manipulation (Shizuya et al. 1992; Wang et al. 1995; Woo et al. 1994). However, although there have been recent advances in robotics and improvements in the methodology (Osoegawa et al. 1998), the process of BAC library construction remains relatively challenging and time consuming.

A good vector preparation is essential for the success in BAC library construction. However because of the low copy number of the BAC vectors (one to two copies per cell; Shizuya et al. 1992), it is difficult to obtain large quantities of high-quality plasmid DNA. The group of de Jong et al. (Frengen et al. 1999) produced a high-copy derivative of pBAC108L, which contained the lethal SacBII gene for positive selection. The low-copy pBAC108L SacBII derivative was ligated with the high-copy pUC vector to improve plasmid yield. Since the resulting high-copy vector (pBACe3.6) has the SacBII gene disrupted with a pUC plasmid, it was no longer possible to distinguish high-copy vector (pBACe3.6) colonies from recombinant BAC clones by selection. In order to eliminate contamination of the library with the high-copy vector clones, all undigested vector has to be carefully removed from the BAC vector preparation (Osoegawa et al. 1998). A vector propagating in a high-copy manner that can then be readily processed to yield the appropriate BAC cloning vector is highly desirable.

Another challenge in BAC library construction is the prevention of DNA degradation. Megabase DNA embedded in agarose plugs and size-selected high-molecular-weight (HMW) genomic DNA in agarose slices tend to shear when frozen. In our hands, restriction-digested and size-selected genomic DNA in agarose slices also deteriorate rapidly when stored at 4°C. After about 2 weeks of storage, the digested DNA is usually no longer ligatable. The instability of the size-selected insert DNA impedes library construction, because the DNA can degrade significantly in storage during the ~6 consecutive days required to go from DNA elution to checking of insert size in BAC clones of test ligations. Therefore, methods to stabilize HMW DNA fragments in agarose slices would be valuable. Also megabase DNA plugs are important resources. For those species whose materials are extremely precious or difficult to obtain, such as endangered species and mutants, long-term storage of the DNA is extremely important. High concentration of EDTA can be used to store DNA plugs and HMW DNA inserts in agarose slices at 4°C (Osoegawa et al. 1998) for up to half a year. However, thorough washes are required before each use because EDTA is a strong inhibitor of enzymes. Also slow deterioration of the DNA quality can still be observed, and longer storage by this way is not expected. Jacobs and Neilan (1995) and Laniel et al. (1997) stored DNA in agarose gels in 70% ethanol at 4°C or 25°C for 3–4 days to evaluate stability. We extended this method to store DNA plugs and HMW DNA inserts in agarose slices for BAC library construction.

Melon (*Cucumis melo* L.) is an important economic crop worldwide. It is a diploid plant with an estimated genome size of 4.5–5.0 × 10^8 bp, very similar to that of rice (Arumuganathan and Earle 1991). The genome contains about 30% of repeated sequences (Bendich and Anderson 1974). One of the most important diseases of melon is Fusarium wilt caused by *Fusarium oxysporum* f.sp. *melonis* (Martyn and Gordon 1996). Four races of the fungal pathogen 0, 1, 2, and 1,2 were identified by differential varieties. Two independent single dominant genes *Fom-1* and *Fom-2* control resistance to races 0 and 2 and races 0 and 1. Currently no genes have been identified to resist race 1,2 (Zink and Thomas 1990).

The melon breeding line MR-1 resists several melon diseases. It carries *Fom-1* and *Fom-2* as well as genes conferring resistance to downy and powdery mildew (Zink and Thomas 1990). Previously, Wechter et al. (1995, 1998) identified a randomly amplified polymorphic DNA (RAPD) marker tightly linked to the *Fom-2* gene. Wang et al. (2000) identified an amplified fragment length polymorphism (AFLP) marker also tightly linked to this gene. Both RAPD and AFLP markers were then converted to co-dominant markers by isolating the corresponding sequences from both MR-1 and the susceptible parent AY and renamed FM and AM, respectively. FM and AM appear to be closely linked to the *Fom-2* gene and co-segregated with the gene in a backcross population of 60 progeny (Wang 1999).

Many plant-resistant genes (R genes) have been cloned (Dixon et al. 1996; Martin et al. 1993; Meyers et al. 1998; Song et al. 1995) and they are usually organized in clusters containing DNA fragments related in either sequence or function. Usually, R genes function via a signal transduction pathway and contain motifs for receptors. According to the organization and combination of motifs, cloned plant R genes could be classified into four groups: (*i*) containing a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) region; (*ii*) containing an LRR region and a transmembrane (TM) domain; (*iii*) containing an LRR region and a protein kinase domain; and (*iv*) containing only a protein kinase domain but required an NBS-LRR-containing gene for its function. Most of the plant R genes cloned so far fall in the NBS-LRR group (reviewed in Michelmore and Meyers 1998).

In this paper, we describe methods for preparing large quantities of vector DNA and long-term storage of HMW DNA samples for BAC library construction. Also, we report the construction of two BAC libraries for a multidisease-resistant line MR-1 of melon using these methods. BAC libraries have been developed for many crop species, such as sorghum (Woo et al. 1994), rice (Wang et al. 1995; Zhang et al. 1996), soybean (Danesh et al. 1998; Marek and Shoemaker 1997; Tomkins et al. 1999), wheat (Lijavetzky et al. 1999), common bean (Vanhouten and MacKenzie 1999), and tomato (Budiman et al. 2000). However, to our knowledge, the BAC libraries presented in this paper are the first BAC libraries constructed from members of the Cucurbitaceae family. These libraries are facilitating the cloning of *Fom-2*. We describe the identification of candidate BACs in the *Fom-2* region identified by co-segregating markers FM and AM.

Materials and methods

Biological materials

Melon MR-1 seeds were from Dr. C.E. Thomas, USDA-ARS, U.S. Vegetable Laboratory, Charleston, S.C. Restriction enzyme *Not*I, MidRange I & II PFG molecular weight markers, Lambda

ladder PFG marker, 1-kb DNA ladder, calf intestinal alkaline phosphatase (CIAP) were obtained from New England Biolabs (Beverly, Mass.). All other restriction enzymes except for *Not*I, T4 DNA ligase, and the vector pGEM-4Z were obtained from Promega (Madison, Wis.). BAC vector pIndigoBac536 was a gift from H. Shizuya and M. Simon (Caltech, Calif.). DH10B competent cells were purchased from Life Technologies (Rockville, Md.).

High-copy BAC vector pCUGIBAC1 construction

High-copy BAC vector construction was performed following standard procedures (Sambrook et al. 1989). High-copy plasmid vector pGEM-4Z and low-copy BAC vector pIndigoBac536 were digested with *Hin*dIII or *Eco*RI. The linearized pIndigoBac536 vector was dephosphorylated, gel purified, and then ligated with pGEM-4Z linearized with the same enzyme. The ligated DNA was used to transform DH10B-competent cells. Transformants were selected on LB–X-gal–IPTG (X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; IPTG: isopropyl-β-thiogalactopyranoside) plates containing both 50 mg/L of ampicillin and 12.5 mg/L of chloramphenicol. DNA was prepared for analysis using an Autogen 740 according to the manufacturer's instruction (AutoGen, Framingham, Mass.).

BAC vector preparation

High-copy pCUGIBAC1 plasmid DNA was isolated using Qiagen plasmid midi kit (Qiagen, Valencia, Calif.) according to the manufacturer's instruction, except that after adding solution P2, the sample was incubated at room temperature for not more than 3 min instead of 5 min. The plasmid DNA was digested with *Hin*dIII or *Eco*RI at 37°C for 1 h. After adding 1 U/5 μg DNA (0.33 U/pmol ends) of CIAP phosphatase, the sample was incubated again at 37°C for 1 h. Enzymes were inactivated by heating the sample at 75°C for 15 min in the presence of 5 mM of EDTA. DNA was precipitated, self-ligated at 16°C overnight, and separated on 1% of agarose gel in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) as modified from Osoegawa et al. (1998). After electrophoresis, the two sides of the gel were stained with ethidium bromide and aligned to the center part to recover the pIndigoBac536 gel band of 7.5 kb. The linear, dephosphorylated pIndigoBac536 DNA was electroeluted in 1× TAE buffer and stored in 1:1 glycerol at –20°C (for frequent use) or –80°C (for long-term storage) in aliquots. The quality of the prepared vector was evaluated by cloning lambda DNA restriction fragments.

Preparation of high-molecular-weight insert DNA

Melon plants were grown in the greenhouse. Young leaves were collected and stored at –80°C. DNA plugs were prepared by embedding nuclei into 0.5% of low-melting agarose followed by a series of treatments as described by Zhang et al. (1995). Washed DNA plugs were stored in Tris-EDTA (TE) buffer at 4°C (for frequent use) or in 70% of ethanol (about 20 volumes) at –20°C (for long-term storage). Before partial digestion, TE-stored plugs were soaked in distilled H_2O for 30 min, while ethanol-stored plugs were transferred to TE buffer or directly in distilled H_2O (about 20 volumes) at 4°C the previous day. Partial digestions and two size selections were as described by Budiman et al. (2000) and Tomkins et al. (1999) with modifications. A series of pilot partial digestions at 37°C were performed for a fixed time using different enzyme concentrations. The results guided the scaled up partial digestions used for library construction. Each half plug (about 50 μL) was chopped to fine pieces separately and transferred into an eppendorf tube containing (in μL): 24.5 H_2O, 9.5 of 10× restriction buffer, 10 of 40 mM spermidine, and 1 of 10 mg/mL BSA. After 30 min incubation on ice, 5 μL of 0.9 U/μL *Hin*dIII or 1.4 U/μL *Eco*RI diluted in 1× buffer was added, and a further 30 min of incubation on ice was performed. Digestion was performed at 37°C for 20 min and stopped by adding 10 μL of 0.5 M EDTA. The partially digested DNA fragments were separated on a 1% agarose CHEF (Bio-Rad, Hercules, Calif.) gel with 1- to 50-s linear ramp at 6 V/cm at 14°C in 0.5× TBE buffer for 20 h using a lambda ladder as the marker. Two fractions between 120 and 250 kb were cut out and subjected to the second size selection in 1% low-melting agarose gel with 4 s constant time at 6 V/cm at 14°C in 0.5× TBE buffer for 20 h. Gel fractions were recovered and used immediately or stored at –20°C in 70% ethanol (about 20 volumes). Gel slices were allowed to stand in 70% ethanol at room temperature for about 30 min or at 4°C overnight before being transferred to –20°C to avoid freezing the center part of the gel slices.

BAC library construction

HMW insert DNA was electroeluted from the gel slices as described by Strong et al. (1997). Ethanol-stored gel fractions were soaked in 1× TAE buffer for about 3 h or overnight before electroelution. Ligations were performed as described by Budiman et al. (2000) except that they were stopped at 65°C for 15 min and desalted in 0.1 M glucose : 1% agarose cones for 1.5 h on ice as described by Atrazhev and Elliott (1996) or on Millipore filters floating on 5% PEG8000 in Petri dishes set on ice for 1.5 h as modified from Osoegawa et al. (1998). Transformations were performed as described by Tomkins et al. (1999). Transformants were selected on LB–X-gal–IPTG plates containing 12.5 mg/L of chloramphenicol. White colonies were picked using a Genetix Q-bot (Genetix Ltd., Christchurch, Dorset, U.K.) and stored in 384-well microtiter plates (Genetix Ltd.) at –80°C. We found that light blue color colonies did not contain inserts and the Genetix Q-bot could not distinguish the light blue color from the white color. Light blue colonies were destroyed manually before robotic picking.

BAC insert sizing

DNA was prepared as described above for vector colony analysis, digested with *Not*I and separated on 1% agarose CHEF (Bio-Rad) gels with a 5- to 15-s linear ramp time at 6 V/cm and 14°C in 0.5× TBE buffer for 16 h.

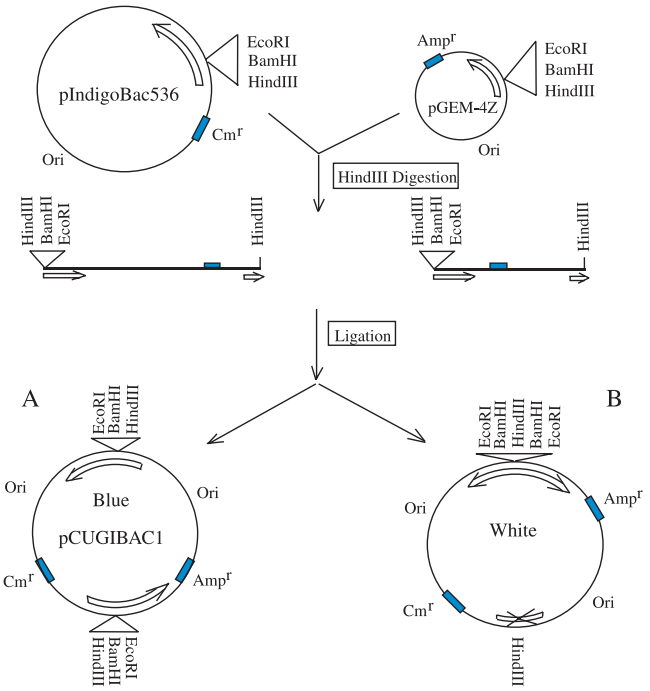
BAC library evaluation

Colonies from random 384-well plates were replicated on both LB–X-gal–IPTG plates containing 50 mg/L of ampicillin and LB–X-gal–IPTG plates containing 12.5 mg/L of chloramphenicol using a 384-pin hand replicator (Fisher Scientific Co., Pittsburgh, Pa.). Colonies that grew on both ampicillin and chloramphenicol plates were classified as high-copy pCUGIBAC1 contamination. Blue and light blue colonies that grew on chloramphenicol plates were classified as empty vector contamination. Evaluation of the library for contamination by organellar DNA clones was performed by hybridization of the libraries with organellar DNA probes.

BAC library screening

Colonies were doubly spotted in high density onto Hybond N^+ filters in a 4 × 4 pattern with the Genetix Q-bot as described by Budiman et al. (2000) and Tomkins et al. (1999). Each 22.5 × 22.5 cm filter contained 18 432 clones in its six fields (6 fields × 8 plates/field × 384 wells/plate; also see our web site <www.genome.clemson.edu>). Hybridization was performed as described by Budiman et al. (2000). Three barley chloroplast probes *ndhA*, *rbcL*, and *psbA*, which are equally spaced around the 133-kb barley chloroplast genome, were originally from Dr. J. Mullet (Texas A&M University, College Station, Tex.). Four rice mitochondria probes *atpA*, *cob*, *atp9*, and *coxI* were from T. Sasaki (Rice Genome Research Program, Institute of Society for Techno-innovation of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan). A 352-bp of melon *Hin*dIII repeat sequence (Brennicke and Hemleben 1983) was reconstructed (Y.H. Wang, C.E. Thomas, and R.A. Dean, un-

Fig. 1. Schematic outline for the construction of the plasmid pCUGIBAC1. The BAC vector pIndigoBac536 has the *lacZ* gene and multiple cloning site region identical to those of the high-copy cloning vector pGEM-4Z. When the two vectors were digested with *Hin*dIII and ligated with each other, two ligation products were expected (A and B). However only the ligation product A (pCUGIBAC1) that contained two reconstituted *lacZ* genes and multiple cloning site regions was observed. On double antibiotics (12.5 mg/L of chloramphenicol and 50 mg/L of ampicillin) and X-gal–IPTG-containing plates, only blue transformants were found.



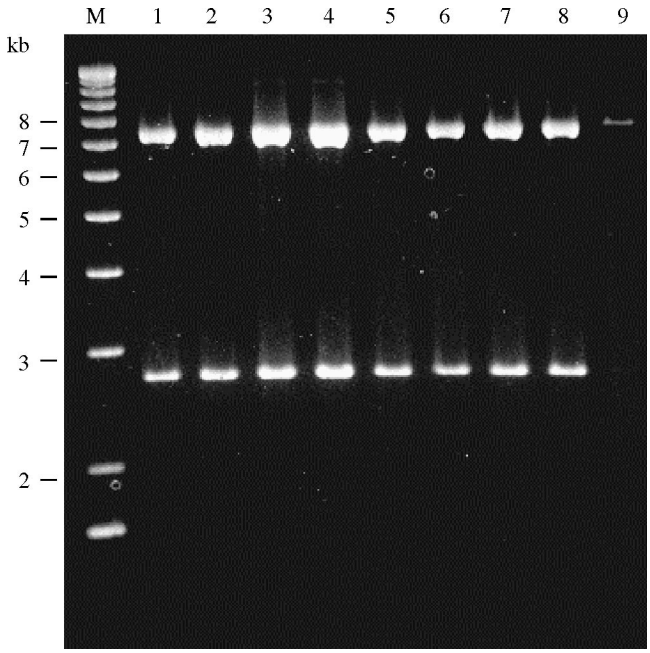
published data). Two melon genetic markers FM and AM co-segregating with the resistant gene *Fom-2* were available from the work of Wechter et al. (1998) and Wang et al. (2000).

Results

High-copy vector construction for BAC vector preparation

We converted the low-copy BAC vector into a high-copy vector to facilitate vector preparation. Upon digestion and isolation, the low-copy BAC vector is reconstituted for use in library construction. The low-copy BAC vector pIndigoBac536 and the high-copy cloning vector pGEM-4Z were digested with *Hin*dIII and ligated. To prevent the possible co-transformation of the two self-ligated plasmids, pIndigoBac536 was dephosphorylated before ligation. The pIndigoBac536 vector is a derivative of pBeloBAC11, whose *lac*Z gene and multiple cloning site regions were derived from pGEM-3Z/4Z (Wang et al. 1997). Ligation of pIndigoBac536 with pGEM-4Z could result in two possible products, and the transformants were expected to have about equal blue and white colonies on X-gal–IPTG containing selection medium (Fig. 1). To our surprise, the results showed that only the ligation product containing two reconstituted *lacZ* genes and multiple cloning site sequences in a head-to-tail orientation, i.e., in tandem, was able to replicate. On

Fig. 2. DNA analysis of randomly picked colonies transformed with pIndigoBac536 (lane 9) and with pIndigoBac536-pGEM-4Z ligated at *Hin*dIII site (lanes 1–8). DNA samples were prepared from 3 mL overnight cultures by an Autogen 740 robot and re-suspended in 30 μL of TE. Aliquots of 4 μL (lanes 1–8) or 10 μL (lane 9) were digested with *Hin*dIII and loaded on 1% agarose gel. M is a 1-kb ladder marker (New England Biolabs).



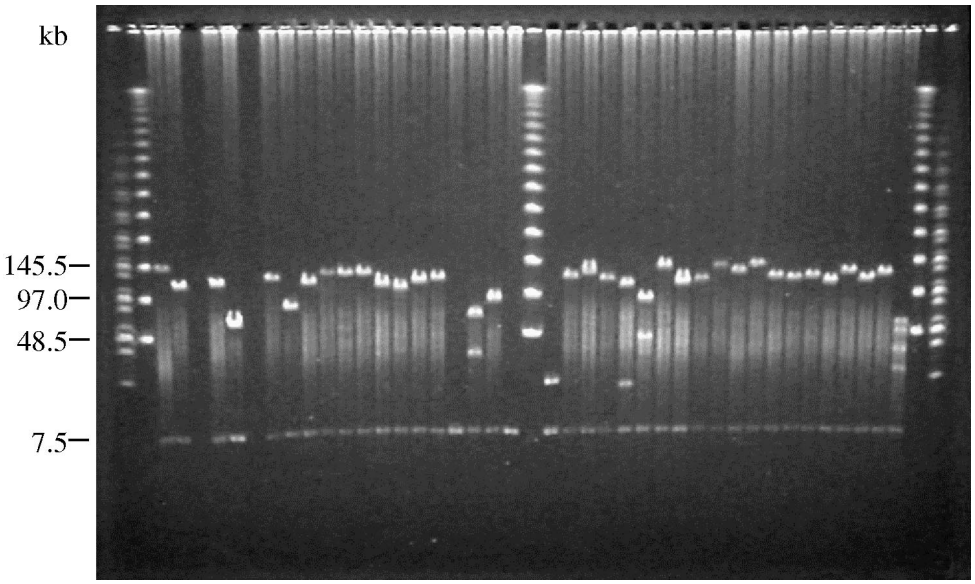
double antibiotics (12.5 mg/L of chloramphenicol and 50 mg/L of ampicillin) and X-gal–IPTG-containing selection medium, all transformants were dark blue in color (data not shown). DNA analysis of 20 randomly picked single colonies indicated that DNA was replicated in high-copy number. Digestion of these DNA samples with *Hin*dIII, *Eco*RI, and *Bam*HI released fragments corresponding to the sizes of pIndigoBac536 and pGEM-4Z. DNA from eight randomly chosen clones digested with *Hin*dIII is presented in Fig. 2.

We also ligated pIndigoBac536 with pGEM-4Z at the *Eco*RI site. As expected in Fig. 1, it resulted in the same plasmid. DNA analysis of six randomly picked colonies showed the same characteristics as clones constructed using *Hin*dIII (data not shown).

The new vector, designated pCUGIBAC1, retained all three cloning sites of the original pIndigoBac536 (*Hin*dIII, *Eco*RI, and *Bam*HI) and had a deep blue color on X-gal–IPTG medium.

We tested whether the pIndigoBac536 vector released from pCUGIBAC1 by restriction enzyme digestion maintained the blue or white selection phenotype. When pCUGIBAC1 was digested with *Hin*dIII, *Eco*RI, or *Bam*HI and the digested products were religated without fragment separation, less than 2% white colonies were found in each transformation on medium containing chloramphenicol (12.5 mg/L) and X-gal–IPTG (data not shown). DNA analysis showed that the white colonies contained damaged pIndigoBac536 vector or pIndigoBac536 vector with unidentified inserts, while the blue clones contained self-ligated pIndigoBac536 or undigested (or) religated pCUGIBAC1.

Fig. 3. DNA analysis of random BAC clones from the melon *Hin*dIII BAC library by pulse-field gel electrophoresis. Autogen-prepared DNA samples were digested with *Not*I and separated on 1% agarose gel with ramp pulse time of 5–15 s at 6 V/cm at 14°C in 0.5× TBE buffer for 16 h. Markers used are midrange I (outside lanes) and lambda ladder (New England Biolabs). The 7.5-kb common band is the vector pIndigoBac536.



No cells harboring self-ligated pGEM-4Z alone were detected because they could not grow on chloramphenicol-containing medium. We also did not detect co-transformed colonies on this single antibiotic selection medium. Cells containing pCUGIBAC1 (containing two *lacZ* genes) were darker blue in color than the self-ligated pIndigoBac536 (data not shown) and grew more slowly. The results indicated that pIndigoBac536 was unchanged during construction. With the new high-copy pCUGIBAC1 vector, 100 μg of pure plasmid was obtained from 100 mL of culture (37°C for 20 h) following purification on a Qiagen midi column. To obtain 100 μg of DNA from the low-copy BAC vectors requires isolating DNA from more than 8 L of culture. Large quantities of vector were prepared for BAC library construction by digesting pCUGIBAC1 followed by dephosphorylation and gel purification.

Improvements on DNA storage for BAC library construction

Improvements in the storage of vector preparations and HMW DNA samples could save time and costs. During storage, HMW DNA should be prevented from both shearing and degrading. Freezing HMW DNA without a protectant results in DNA shearing and should be avoided. We found that size-selected HMW genomic DNA fragments in agarose slices could be stored in 70% ethanol at –20°C for long periods of time without change of qualities. DNA stored in agarose slices for long periods of time can be eluted and cloned. Sometimes the quality even improved possibly due to diffusing-out of salts, trapped small DNA fragments, and inhibitors during the storage (data not shown). We also extended this method to store megabase DNA plugs. Size-selected HMW DNA fragments in agarose slices and DNA plugs that have been stored in this manner for many months are routinely used for BAC library construction in our laboratory. Ethanol can be used to preserve DNA for long-term storage (Sambrook et al. 1989) and is a clear solution that can be easily removed.

Even though the BAC vector is only 7.5 kb in size, freeze–thaw cycles still need to be avoided. We stored the prepared vector at –20°C (for frequent use) or –80°C (for long-term storage) in 40–50% of glycerol in aliquots and effectively avoided deterioration of the prepared vector during freeze–thaw cycles.

Construction and insert sizing of two melon BAC libraries

With the goal of isolating melon disease-resistant genes, we constructed two BAC libraries (*Hin*dIII and *Eco*RI) for a multidisease-resistant line MR-1 of melon using our improved BAC library construction methods.

The *Hin*dIII library consisted of 177 × 384 well microtiter plates. DNA of 530 clones randomly picked from the library was analyzed. Figure 3 shows the *Not*I restriction pattern of 38 randomly picked clones. The pIndigoBac536 vector maintained the two *Not*I restriction sites of its ancestor pBeloBAC11 at the flanks of the multiple cloning site to facilitate releasing the inserts. Analysis indicated that about 97% of clones contained inserts with an average insert size of 118 kb.

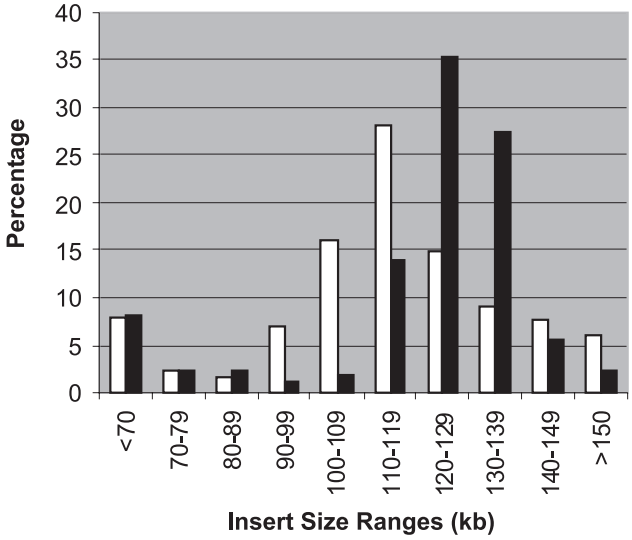
The *Eco*RI library consisted of 222 × 384 well microtiter plates. A total of 422 randomly picked clones were checked by *Not*I digestion (data not shown). Approximately 97% of clones were recombinant with an average insert size of 114 kb.

The insert size data for the 530 *Hin*dIII BACs and 422 *Eco*RI BACs above are summarized in Fig. 4. The data shows that 84.6% of *Hin*dIII BACs (black columns) contained inserts greater than 110 kb; 81.6% of *Eco*RI BACs (white columns) contain inserts greater than 100 kb.

Evaluation of the melon *Hin*dIII and *Eco*RI BAC libraries

The two melon libraries were constructed with vector pre-

Fig. 4. Insert size distributions of the melon MR-1 *Hin*dIII (black columns) and *Eco*RI (white columns) BAC libraries. DNA samples of 530 clones randomly picked from the melon *Hin*dIII BAC library and 422 clones randomly picked from the melon *Eco*RI BAC library were analyzed (see Materials and methods) and grouped.



pared from our high-copy vector pCUGIBAC1 and picked by a Genetix Q-bot. To determine whether the libraries picked were of high quality and were not contaminated with the high-copy vector, colonies from 12 randomly selected 384-well microtiter plates (4608 colonies) of each library were replicated on both LB plates containing ampicillin–X-gal–IPTG and LB plates containing chloramphenicol–X-gal–IPTG with a 384-pin hand replicator (see Materials and methods). Colonies harboring pCUGIBAC1 should grow on both chloramphenicol- and ampicillin-containing plates, while those harboring pIndigoBac536, with or without insert, could grow only on chloramphenicol-containing plates. No colonies harboring pGEM-4Z vector were expected on ampicillin-containing plates, because the libraries were from chloramphenicol selection.

Our results showed that no colonies were found on ampicillin-containing plates for both *Hin*dIII and *Eco*RI libraries indicating no contamination of the high-copy vector pCUGIBAC1 in these libraries (data not shown). On chloramphenicol-containing plates, 17 (0.37%) pin spots from the *Hin*dIII library and 23 (0.50%) from the *Eco*RI library did not grow colonies. Among the colonies that grew, 51 (1.11%) from the *Hin*dIII library and 95 (2.07%) from the *Eco*RI library were blue or light blue in color and did not contain inserts.

To estimate the representation of organellar DNA and repeat sequences in the libraries, high-density colony filters were prepared and screened with respective probes. Highly conserved barley chloroplast genes and rice mitochondrial genes (see Methods) are usually used as the probes to roughly estimate the representation of chloroplast and mitochondrial DNA inserts in plant BAC libraries, respectively. With the probe mixture of three barley chloroplast genes, 2.78% and 1.17% of chloroplast DNA clones were detected from the *Hin*dIII and *Eco*RI libraries, respectively. With the probe mixture of four rice mitochondrial genes, 0.18% and 0.20% of mitochondrial DNA clones were detected in the *Hin*dIII and *Eco*RI libraries, respectively. With a melon *Hin*dIII repeat sequence as probe, 2.80% and 3.19% of repeat sequence clones were identified from the *Hin*dIII and *Eco*RI libraries, respectively.

It is reported that the melon haploid genome size is 5×10^8 bp (Arumuganathan and Earle 1991). After subtracting the number of empty wells, blue and light blue clones and organellar DNA clones, the *Hin*dIII library contains 64 961 nuclear DNA-containing clones (118 kb in average) and provides a 15.4 genome coverage. The *Eco*RI library contains 81 904 nuclear DNA-containing clones (114 kb in average) and provides 18.7 genome coverage.

Isolation of *Fom*-2 candidate BAC clones

High-density colony filters for the two melon BAC libraries were screened with two melon genetic markers, FM and AM, that co-segregate with the *Fom-2* gene. When the whole *Hin*dIII library was probed with the two markers, 14 and 18 positive BACs were identified for FM and AM, respectively (data not shown). The marker AM identified 34 positive BACs from filters A, B, and C (containing 65% of the clones of the library) of the *Eco*RI library. Figure 5 shows the hybridization image of the filter C of the *Eco*RI library using the marker AM as probe. Positive BACs from the *Hin*dIII library were fingerprinted and end-sequenced. R gene homologues containing NBS or LRR motifs were found in the BAC end sequences (Wang 1999).

The hybridization data demonstrate the utility of our libraries for facilitating the isolation of disease resistance genes as well as the utility of our modifications to vector preparation and DNA storage for library construction.

Discussion

BAC libraries provide a necessary tool for physical mapping, genome sequencing, and positional cloning. However BAC library construction is still a time-consuming endeavor. Good preparations of vector and size-selected HMW DNA inserts share in the success of BAC library construction. While ligation, transformation, insert size checking, etc. are technically routine, most of the time is spent on repeatedly optimizing the preparation of vector and HMW inserts. Improvements in methodologies facilitating the preparation of the vector and storage of HMW DNA could save time and costs.

The BAC vector replicates in one to two copies per cell (Shizuya et al. 1992). To obtain sufficient plasmid DNA for optimizing the vector preparation and constructing BAC libraries, a large volume of culture is required. Vector DNA purified from large volumes of culture using a Qiagen kit was usually contaminated with bacterial genomic DNA, and further purification with CsCl centrifugation was needed. We cloned the low-copy BAC vector in a conventional high-copy plasmid vector pGEM-4Z. The resultant plasmid, named as pCUGIBAC1, replicates as a high-copy plasmid. From 100 mL of culture, 100 μg of plasmid DNA can be easily purified using a Qiagen kit. An important feature of the modified high-copy vector is that it retains the ability to produce blue color on X-gal–IPTG selection plates. This allows for the discrimination of contaminating pCUGIBAC1

from the recombinant clones (white color). It is interesting that the replication is unidirectional and only the ligation product containing two reconstituted *lazZ* genes and multiple cloning site sequences can be replicated. This provides a second level of protection. Even if the linearized and dephosphorylated pIndigoBac536 is contaminated with poorly dephosphorylated pGEM-4Z, the transformants of the ligation will reproduce pCUGIBAC1 which produces blue colonies on selective medium and can be excluded from picking (self-ligated pGEM-4Z would not grow on chloramphenicol-containing plates). Furthermore, pCUGIBAC1 facilitated the purification of the vector while retaining all the properties of the BAC vector pIndigoBac536. This method can also be used to prepare other BAC vectors such as pBAC108L, pBeloBAC11, and their derivatives.

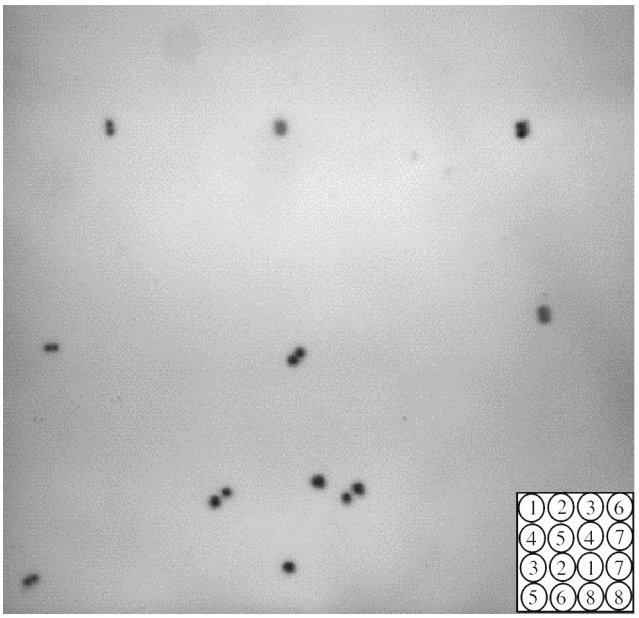
Even though pCUGIBAC1 contains a duplicated region (*lacZ* gene and the multiple cloning site region), it appears to be very stable in the cell line DH10B. We have maintained this clone for multiple generations and used it successfully for nearly 2 years. We have not observed any rearrangements. It was reported that DH10B can stably maintain high-copy plasmid DNA (Tao and Zhang 1998).

The pCUGIBAC1 vector has been used for constructing BAC libraries for many agriculturally important crops such as rice (*Eco*RI libray, M. Luo and M.A. Budiman, Clemson University; *Hin*dIII library, Y.-H. Wang, Chinese Academy of Sciences), wheat (*Hin*dIII library, C.E. Thomas, Zurich University, Switzerland), corn (*Hin*dIII library, J.P. Tomkins; *Eco*RI library, M. Luo, Clemson University), sorghum (*Hin*dIII library, D. Begum, Clemson University), soybean (*Eco*RI library, J.P. Tomkins, Clemson University), cotton (*Hin*dIII library, D. Peterson, Georgia University), and melon (*Hin*dIII and *Eco*RI libraries, this paper). The vector has been also used recently for constructing a mouse BAC library (M. Luo, unpublished data). We did not find any problems related to the use of this vector. Sampling colonies from 12 × 384 well microtiter plates from each of the melon *Hin*dIII and *Eco*RI libraries on ampicillin (50 mg/L) containing medium did not detect any contamination of this high-copy vector. Osoegawa et al. (1998) observed that the contaminating clones of their high-copy pBACe3.6 vector in the BAC library turned out as strong signals on autoradiograms of the high-density BAC library filters. One clone with a strong hybridization signal was found in the melon *Hin*dIII library. However, when this clone was transferred onto the X-gal–IPTG-containing plate, it had a blue color, indicating that it was incorrectly picked by the robot.

The quality of the size-selected HMW DNA inserts is also critical in BAC library construction, and the DNA must be maintained in a condition that prevents shearing and degradation. Jacobs and Neilan (1995) and Laniel et al. (1997) stored DNA in agarose gels in 70% ethanol at 4°C or 25°C for 3–4 days to evaluate stability. We found that the size-selected HMW DNA inserts in agarose slices can be stored in 70% ethanol at –20°C for extended periods of time without a decreased ligation efficiency. We also adopted this method to store megabase DNA plugs. This simple method for storage of high molecular weight DNA facilitates the construction of BAC libraries.

We constructed two BAC libraries for the multidisease-resistant line MR-1 of melon. About 95.6% of the *Hin*dIII library and 96% of the *Eco*RI library contained nuclear DNA inserts with the average insert sizes of 118 and 114 kb, respectively. The two libraries together provide total of 34 genome equivalents.

Fig. 5. Hybridization image of filter C of the melon *Eco*RI BAC library using as probe the marker AM that co-segregated with the melon Fusarium wilt resistant gene *Fom-2*. Colonies were doubly spotted in high density with the Genetix Q-bot on a 22.5 × 22.5 cm filter in a 4 × 4 pattern (inset). Each filter contains 18 432 clones.



Melon is a member of Cucurbitaceae family and an important economic crop. Genome studies of the Cucurbitaceae family have lagged behind those of other crops. We constructed the first two BAC libraries in this family. These libraries can be used for comparative studies of cucurbits, including homologous gene cloning, because homology among the species within the family at the DNA level has been reported to be over 70% (Pasha and Sen 1998). It also has been shown that the FM primer can amplify cucumber DNA and that the PCR products are highly homologous, if not identical, based on hybridization (Wang 1999). This homology is reasonable, because melon and cucumber are members of the same subtribe (Robinson and Decker-Walters 1997). Being able to exploit this homologous relationship among the cucurbits is advantageous considering that the cucurbits are understudied despite their economic value.

We screened the libraries for candidate clones potentially containing the *Fom-2* gene using the linked markers FM and AM as probes. The marker FM hybridized to 14 BACs from the *Hin*dIII library, while the marker AM hybridized to 18 BACs from the *Hin*dIII library and 34 BACs from filters A, B, and C of the *Eco*RI library. Many cloned R genes are organized in clusters and contain an NBS motif and an LRR region (reviewed in Michelmore and Meyers 1998). The ends of the positive BACs from the *Hin*dIII library were sequenced. The preliminary BAC end sequence data showed that 4 out of the 14 BACs identified by marker FM had an identical end sequence with LRR homology and that 5 out of

18 BACs identified by marker AM had an identical end sequence with NBS homology (Wang 1999). We are characterizing these clones further to find a fragment that contains both NBS and LRR motifs. The clones identified by the two genetic markers linked to *Fom-2* also found homologues to retroelements and cytochrome P450. Retroelements are associated with plant R gene diversification (Ronald 1998) and cytochrome P450 has also been shown to be related to R gene functioning (Oh et al. 1999).

When a 352-bp of melon *Hin*dIII repeat sequence (Brennicke and Hemleben 1983) was used to screen the libraries, about 3% of clones were detected in both libraries. The *Hin*dIII repeat sequence does not contain an *Eco*RI site. The data from the *Eco*RI library indicated the dispersed distribution of this repeat sequence. It was reported that melon contained about 30% of repeat sequences (Bendich and Anderson 1974). The *Hin*dIII repeat sequence probe detected only about 1/10 of them. It is possible that most of the dispersed repeat sequences do not contain the *Hin*dIII and *Eco*RI sites for cloning or melon contains different repeat sequences that could not be detected by the probe we used.

Acknowledgements

We thank Michael Atkins, Kerry Wilson, and John Bishop for their technical assistance for the library replication and filter production. The work was supported by an award from Seminis Vegetable Seeds to R.A.D., NSF MRI grant 9724557 to R.A.W. and R.A.D., and the Coker Endowed Chair to R.A.W.

References

Arumuganathan, K., and Earle, E.D. 1991. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. **9**: 208–218.

Atrazhev, A.M., and Elliott, J.F. 1996. Simplified desalting of ligation reactions immediately prior to electroporation into *E. coli*. BioTechniques, **21**: 1024.

Bendich, A.J., and Anderson, R.S. 1974. Novel properties of satellite DNA from muskmelon. Proc. Natl. Acad. Sci. U.S.A. **71**: 1511–1515.

Brennicke, A., and Hemleben, V. 1983. Sequence analysis of the cloned *Cucumis melo* highly repetitive satellite DNA. Z. Naturforsch. **38**: 1062–1065.

Budiman, M.A., Mao, L., Wood, T.C., and Wing, R.A. 2000. A deep-coverage tomato BAC library and prospects toward development of an STC framework for genome sequencing. Genome Res. **10**: 129–136.

Danesh, D., Penuela, S., Mudge, J., Denny, R.L., Nordstrom, H., Martinez, J.P., and Young, N.D. 1998. A bacterial artificial chromosome library for soybean and identification of clones near a major cyst nematode resistance gene. Theor. Appl. Genet. **96**: 196–202.

Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K., and Jones, J.D. 1996. The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell, **84**: 451–459.

Frengen, E., Weichenhan, D., Zhao, B., Osoegawa, K., van Geel, M., de Jong, P.J. 1999. A modular positive selection bacterial artificial chromosome vector with mutiple cloning sites. Genomics, **58**: 250–253.

Hosoda, F., Nishimura, S., Uchida, H., and Ohki, M. 1990. An F factor based cloning system for large DNA fragments. Nucleic Acids Res. **18**: 3863–3869.

Jacobs, D., and Neilan, B.A. 1995. Long-term preservation of DNA in agarose gels using 70% ethanol. BioTechniques, **19**: 892–894.

Laniel, M.-A., EI-Amine, M., Boire, G., and Menard, H.-A. 1997. Southern blotting of long-term preserved DNA. BioTechniques, **22**: 595–596.

Lijavetzky, D., Muzzi, G., Wicker, T., Keller, B., Wing, R.A., and Dubcovsky, J. 1999. Construction and characterization of a bacterial artificial chromosome (BAC) library for the A genome of wheat. Genome, **42**: 1176–1182.

Marek, L.F., and Shoemaker, R.C. 1997. BAC contig development by fingerprint analysis in soybean. Genome, **40**: 420–427.

Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earleand, E.D., and Tanksley, S.D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science (Washington, D.C.), **262**: 1432–1436.

Martyn, R.D., and Gordon, T.R. 1996. Fusarium wilt of melon. *In* Compendium of cucurbit diseases. *Edited by* T.A. Zitter, D.L. Hopkins, and C.E. Thomas. APS Press, St. Paul, Minn. pp. 14–15.

Meyers, B.C., Shen, K.A., Rohani, P., Gaut, B.S., and Michelmore, R.W. 1998. Receptor-like genes in the major resistance locus of lettuce are subject to divergent selection. Plant Cell, **10**: 1833–1846.

Michelmore, R.W., and Meyers, B.C. 1998. Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res. **8**: 1113–1130.

Oh, B.-J., Ko, M.K., Kim, Y.S., Kim, K.S., Kostenyuk, I., and Kee, H.K. 1999. A cytochrome P450 gene is differentially expressed in compatible and incompatible interactions between pepper (*Capsicum annuum*) and the anthracnose fungus, *Colletotrichum gloeosporioides*. Mol. Plant-Microbe Interact. **12**: 1044–1052.

Osoegawa, K., Woon, P.Y., Zhao, B., Frengen, E., Tateno, M., Catanese, J.J., and de Jong, P.J. 1998. An improved approach for construction of bacterial artificial chromosome libraries. Genomics, **52**: 1–8.

Pasha, M.K., and Sen, S.P. 1998. Numerical and chemical approaches to taxonomy and relatiohships. *In* Cucurbits. *Edited by* N.M. Nayar and T.A. More. Science Publishers, Enfield, N.H. pp. 19–32.

Robinson, R.W., and Decker-Walters, D.S. 1997. Cucurbits. CAB International, New York.

Ronald, P.C. 1998. Resistance gene evolution. Curr. Opin. Plant Biol. **1**: 294–298.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiiri, Y., and Simon, M. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. U.S.A. **89**: 8794–8797.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauguet, C., and Ronald, P.C. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science (Washington, D.C.), **270**: 1804–1806.

Strong, S.J., Ohta, Y., Litman, G.W., and Amemiya, C.T. 1997. Marked improvement of PAC and BAC cloning is achieved using electroelution of pulsed-field gel-separated partial digests of genomic DNA. Nucleic Acids Res. **25**: 3959–3961.

Tanksley, S.D., Ganal, M.W., and Martin, G.B. 1995. Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet. **11**: 63–68.

Tao, Q., and Zhang, H.-B. 1998. Cloning and stable maintenance of DNA fragments over 300 kb in *Escherichia coli* with conventional plasmid-based vectors. Nucleic Acids Res. **26**: 4901–4909.

Tomkins, J.P., Mahalingam, R., Smith, H., Goicoechea, J.L., Knap, H.T., and Wing, R.A. 1999. A bacterial artificial chromosome library for soybean PI 437654 and identification of clones associated with cyst nematode resistance. Plant Mol. Biol. **41**: 25–32.

Vanhouten, W., and MacKenzie, S. 1999. Construction and characterization of a common bean bacterial artificial chromosome library. Plant Mol. Biol. **40**: 977–983.

Wang, G.L., Holsten, T.E., Song, W.Y., Wang, H.P., and Ronald, P.C. 1995. Construction of a rice bacterial artificial chromosome library and identification of clones linked to the Xa-21 disease resistance locus. Plant J. **7**: 525–533.

Wang, K., Boysen, C., Shizuya, H., Simon, M.I., and Hood, L. 1997. Complete nucleotide sequence of two generations of a bacterial artificial chromosome cloning vector. BioTechniques, **23**: 992–994.

Wang, Y.-H. 1999. Marker development and genome mapping for localizing disease resistance genes in melon (*Cucumis melo* L.). Ph.D dissertation, Clemson University, Clemson, S.C.

Wang, Y.-H., Thomas, C.E., and Dean, R.A. 2000. Genetic mapping of Fusarium wilt resistance gene *Fom-2* in melon (*Cucumis melo* L.). Mol. Breed. **6**: 379–389.

Wechter, W.P., Whitehead, M.P., Thomas, C.E., and Dean, R.A. 1995. Identification of a randomly amplified polymorphic DNA marker linked to the Fom-2 Fusarium wilt resistance gene in muskmelon Mr-1. Phytopathology, **85**: 1245–1249.

Wechter, W.P., Dean, R.A., and Thomas, C.E. 1998. Development of sequence-specific primers that amplify a 1.5-kb DNA marker for race 1 Fusarium wilt resistance in *Cucumis melo* L. HortScience, **33**: 291–292.

Woo, S.S., Jiang, J., Gill, B.S., Paterson, A.H., and Wing, R.A. 1994. Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor.* Nucleic Acids Res. **22**: 4922–4931.

Zhang, H.B., Martin, G.B., Tanksley, S.D., and Wing, R.A. 1994. Map-based cloning in crop plants: tomato as a model system II. Isolation and characterization of a set of overlapping yeast artificial chromosomes encompassing the jointless locus. Mol. Gen. Genet. **244**: 613–621.

Zhang, H.-B., Zhao, X., Ding, X., Paterson, A.H., and Wing, R.A. 1995. Preparation of megabase-size DNA from plant nuclei. Plant J. **7**: 175–184.

Zhang, H.B., Choi, S.D., Woo, S.-S., Li, Z., and Wing, R.A. 1996. Construction and characterization of two rice bacterial artificial chromosome libraries from the parents of a permanent recombinant inbred mapping population. Mol. Breed. **2**: 11–24.

Zink, F.W., and Thomas, C.E. 1990. Genetics of resistance to *Fusarium oxysporum* f.sp. *melonis* races 0, 1, and 2 in muskmelon line MR-1. Phytopathology, **80**: 1230–1232.