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Construction and characterization of a bacterial artificial chromosome library of peach

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Abstract A peach [*Prunus persica* (L.) Batch] bacterial artificial chromosome (BAC) library of var. Jingyu was constructed. Jingyu is a traditional variety, that displays many of the important agronomic characters of stone fruits. Since peach leaves are rich in polysaccharides, high-molecular-weight (HMW) DNA was extracted from leaf nuclei using a protocol adapted to peach. The HMW DNA embedded in agarose plugs was partially digested by *Hin*dIII. After size-selection by pulsed field gel electrophoresis, the selected DNA fragments were ligated to pBeloBAC11 and transformed into *E. coli* DH10B cells by electroporation. In total 20,736 recombinant clones were obtained. The BAC library has an average insert size of 95 kb and represents approximately 6.7 peach haploid genome equivalents. The BAC clones were stable in *E. coli* cell after 100 generations. The lack of hybridization to chloroplast and mitochondrial genes demonstrated that the library is predominantly composed of nuclear DNA. The library was screened with two molecular markers, W4 and P20, that are linked to white flesh and nectarine genes of peach, respectively. Ten positive clones were detected. Their fingerprints will be used to determine clone relationships and assemble contigs. This library should be well-suited for the map-based cloning of peach genes and genome physical mapping.

Key words Bacterial artificial chromosome · High-molecular-weight DNA · *Prunus persica* (peach)

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Introduction

Peach [*Prunus persica* (L.) Batch] is a member of the family *Rosaceae* and one of the most economically important fruit trees in the world. It is a diploid with 2n=16 (Jelenkovic and Harrington 1972) and 2C=580 Mb (Baird et al. 1994), which is only about twice the size of that of *Arabidopsis thaliana*. In addition, peach has a rather high self-compatibility and relatively short generation time (approx. 2–3 years for the juvenile stage) compared with most other fruit trees (Scorza and Sherman 1996). These properties have resulted in peach being the best genetically characterized species of *Pruncus* genus and a model species for identifying and isolating agricultural important genes in perennial tree fruit species (Baird et al. 1996)

Recent mapping studies using molecular markers have provided several linkage maps of peach (Belthptt et al. 1993; Chapparo et al.1994; Dirlewanger et al. 1998; Lu et al. 1998; Sosonski et al.1998), or ones based on interspecific crosses between peach and other *prunus* species (Foolad et al. 1995; Direwanger et al. 1996; Joobear et al. 1998). Many qualitative and quantitative agronomic characters have been mapped on them, such as weeping (Dirlewanger and Bodo 1994), pillar (Chaparro et al. 1994; Rajapakse et al. 1995), shape of the tree, red leaves (Chaparro et al. 1994), double flower (Chaparro et al. 1994; Rajapakse, et al. 1995), peach/nectarine (Chaparro et al. 1994), flesh color (Rajapakse et al. 1995; Warburton et al. 1996), stony hard flesh/free stone (Warburton et al. 1996), acid/non-acid, flat/round, pollen sterility (Dirlewanger et al. 1998) and disease resistance (Dirlewanger et al., 1996; Viruel et al. 1998; Lu et al. 1998). Closely linked DNA markers have been identified on both sides of some genes. These investigations have opened the door for map-based cloning (MBC) of peach genes.

Map-based cloning is becoming a valuable and efficient strategy for gene cloning, which requires molecular markers closely linked to the target gene and a high-quality library with large-insert DNAs. Until now, a large-insert peach genomic DNA library has not been

available in China. Two cloning systems have been widely used in plants for the construction of large-fragment genomic DNA libraries: the yeast artificial chromosome (YAC) (Burke et al. 1987) and the bacterial artificial chromosome (BAC) (Shizuya et al. 1992). There are some advantages of BAC over YAC, including lower levels of chimerism, ease of library generation and insert manipulation (Shizuya et al. 1992; Woo et al. 1994; Cai et al. 1995; Wang et al. 1995), which are very important for map-based cloning and genome analysis. As a result, BAC is becoming the predominant cloning system. Our peach BAC library was constructed from the variety Jingyu, an important parent in Chinese peach breeding. It has many economically valuable agronomic traits; for example, non-acid/acid, white/yellow flesh; peach/nectarine; separated kernel/ stinky kernel, etc. Thus the library will be suited for map-based cloning of these genes and for construction of a physical map of the peach genome.

Materials and methods

Materials and chemicals

Leaves were collected from young shoots of adult trees of Chinese peach variety Jingyu from the Experimental Station of Forestry and Fruit Research Institute, Academy of Agricultural and Forestry Sciences of Beijing City (China).

*Hin*dIII and T_4 DNA ligase were purchased from Boehringer Mannheim (Germany). HK phosphoralase were purchased from Epicentre Technologies (USA). Other chemicals were purchased from Sigma (USA).

BAC vector preparation

pBeloBAC11 (Kim et al.1996), which was used as the cloning vector for constructing the peach BAC library, was isolated from a 1-l overnight culture using the alkaline-lysis method (Sambrook et al. 1989). After electrophoresis, the surpercoiled vector DNA was cut and electroeluted from the gel. The recovered vector DNA (1 µg) was then completely digested by *Hin*dIII and dephosphoralated with HK phosphoralase. The 25-ng aliquots of linear dephosphoralated vector DNA were stored at –70°C until needed.

Isolation of high-molecular-weight (HMW) DNA from peach leaf nuclei

Nuclei were prepared from peach leaves according to Fu et al. (1998) with some modifications. About 10 g of young leaves was ground into fine powder in liquid nitrogen with a mortar and pestle. The powder was gently mixed in 200 ml cold 1 × HB nuclei extraction buffer (10 m*M* Tris, 80 m*M* KCl, 10 m*M* EDTA, 1 m*M* spermidine, 1 m*M* spermine, 0.5 *M* sucrose, 2% β-mercaptoethanol, 0.5% triton X-100, pH 9.3) for 10 min on ice. The mixture was filtered through two layers of cheesecloth and centrifuged at 3,000 *g* for 20 min. The pellet was resuspended in 200 ml 1 × HB nuclei washing buffer using a paintbrush and then centrifuged at 60 *g* for 2 min. The supernatant was transferred to a new tube and pelleted at 2,200 *g* for 20 min. The pellet was washed twice with washing buffer, with each washing step being followed by centrifugation at 1,500 *g* for 20 min. The nuclei were then resuspended and embedded in low-melting point (LMP) agarose plugs. The plugs were incubated in a 5× volume of lysis buffer (0.5 *M* EDTA, 1% sodium lauryl sulfate, 1 mg/ml proteinase K, pH 9.3) for 24 h at 50°C. After being washed with 0.5 *M* EDTA (pH 9.3) twice, the plugs were stored in 0.05 *M* EDTA (pH 8.0) at 40°C. Finally, the DNA fragments above 2 Mb embedded in 1% LMP agarose were recovered through pulsed field gel electrophoresis (PFGE) (CHEF; Bio-Rad; agarose: 1%; buffer: 0.5 × TBE; pulse time: 60–90 s; voltage: 4.5 V/cm; angle: 120°; running time: 18 h; temperature: 11°C) and then stored in TE (100 m*M* Tris-HCl, 2 m*M* EDTA, pH8.0) at 4°C.

Partial digestion of HMW DNA and recovery of the digested fragments

The HMW DNA of peach was partially digested by varying units of *Hin*dIII at 37°C over varying periods of time. The digested fragments were size-fractionated by PFGE (CHEF; Bio-Rad; agarose: 1%; buffer: 0.5 × TBE; pulse time: 60–90 s; voltage: 4.5 V/cm, angle: 120°; running time: 18 h; temperature: 110°C). The region of the gel between 100 kb and approximately 500 kb was cut into a number of slices perpendicular to the direction of migration. The DNA was electroeluted from each gel slice as described by Strong (1997) and used for ligations with the BAC vector after their concentrations were quantified.

Construction of the BAC library

The linearized and dephosphoralated pBeloBAC11 DNA was mixed with the size-selected peach DNA on a series molar ratio and then ligated with T_4 DNA ligase (2 U/100µl) at 4°C for 20 h. The ligation s were immediately used to transform the competent cells of *E. coli* strain DH10B by electroporation using an ECM600 electroporater machine (BTX, San Diego, Calif.) at the following settings (1-mm gap cuvette): choose-mode of 2.5 kV, set-resistance of 129 Ω, charge-voltage at 1.3 kV. The transformed cells were grown at 37°C in 1 ml SOC medium (Sambrook et al. 1989) for 50 min to enable cell recovery, then plated on LB agar containing 12.5 µg/ml chloramphenical, 50 µg/ml X-gal and 25 µg/ml IPTG (IsoPropyl β ThioGala-otopyranoside). The plates were incubated at 37°C for at least 24 h to allow a blue and white color to develop completely.

The average insert size of each ligation was determined by analyzing 10 individual BAC clones, as described by Zhang et al. (1996). The ligations chosen for the construction of the library were further characterized by analyzing about 100 clones. The recombinant white clones were then picked out individually and put into 384-well microtiter plates containing 60 µl LB freezing medium (Wang et al. 1995) with 12.5 µg/ml of chloramphenicol (CM). After incubation at 37°C overnight to allow sufficient bacterial growth, the plates were stored at –70°C.

Preparation of the high-density filter

A Beckman 2000 Automated Workstation was used to spot BAC clones of peach onto Hybond N^+ filters (12 cm × 8 cm) (Amersham Life Science). Each filter contained 3 × 384 clones (each clone with three duplications). The entire library was inoculated onto 18 filters. The filters were transferred to the trays containing LB agar with 12.5 µg/ml CM and were incubated at 37°C overnight until the clones were about 1–1.5 mm in diameter. They were processed according to Nezetic (1989), baked at 80°C for 2 h and then stored at 4°C.

BAC library screening

Total genomic DNA from var. Jingyu was extracted from leaves using a modified sodium dodecyl sulphate (SDS) protocol (Tai et al. 1990). The probes for the chloroplast (cp) and mitochondrial (mt) sequences were sorghum chloroplast *rbs*L and maize mitochondrial *apt*6 and *18s+5s* genes. Two molecular markers, W4 and P20, which are linked to the white fresh and nectarine genes at 9.3 cM and 5.6 cM, respectively, were used for identifying the library rep-

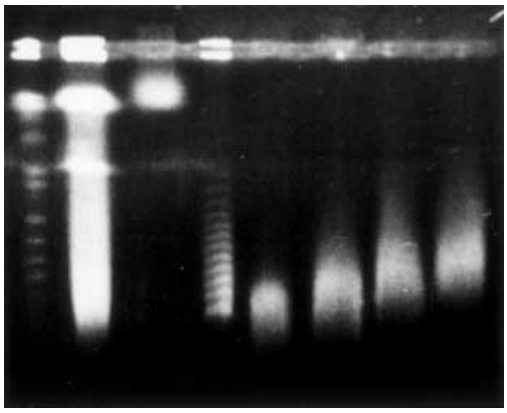

Fig. 1 PFGE analysis of HMW DNA of peach and its partial digestion with *Hin*dIII. *Lanes*: M1 yeast chromosome PF marker (Bio-Rad), *1* HMW DNA of peach embedded in agarose plug, *2* HMW DNA of peach purified by PFGE *3–6*: partial digestion of peach HMW DNA with 3 and 2 unit(s) *Hin*dIII for 10, 5 and 5 min, respectively, M2 Lambda concatamer (48.5 kb) (Bio-Rad)

resentation. Probes were radioactively labeled using the random primer extension method (Feinberg and Vogelstein 1983). The 18 filters of the library were pre-hybridized at 65°C for 10 h in a plastic box containing 150 ml of hybridization solution (5 × SSC, 5 × Denhart's, 0.5% SDS, 5 mg/ml salmon DNA), then the labeled probes were added and hybridized at 65°C for approximately 13–16 h. Two 5-min post-hybridization washings were carried out in 2 × SSC, 0.1% (w/v) SDS at 65°C. The hybridized filters were autoradiographed for 4–7 days depending on the strength of their hybridization signals.

Stability tests of peach BAC clones

Three BAC clones with insert DNA of about 100 kb, 130 kb and 150 kb, respectively, were inoculated into LB medium (containing 12.5 µg/ml CM) and grown at 37°C for approximately 100 generations (in total, a culture of about 5 days, with a transfer to new medium every 24 h). The DNA of plasmids isolated from day 1 and day 5 was digested with *Hin*dIII and subjected to electrophoresis to check their restriction patterns.

Results

Preparation and partial digestion of peach HMW DNA

A large amount of high-quality HMW DNA is required to construct a large insert genomic DNA library. Since peach leaves are rich in polysaccharides, we moderately modified the protocol of Fu et al. (1998) to yield HMW DNA above 2 Mb in size. There were no LMW DNA, phenol products or cell debris contamination, which made this HMW DNA readily accessible by restriction enzymes.

Partial digestion of the HMW DNA was performed using different units of *Hin*dIII for varying periods of time. We found that most fragments were in the desirable size range of approximately 100–500 kb when the HMW DNA in one plug was digested by 2 units of *Hin*dIII for 5 min (Fig. 1). The result was readily reproducible.

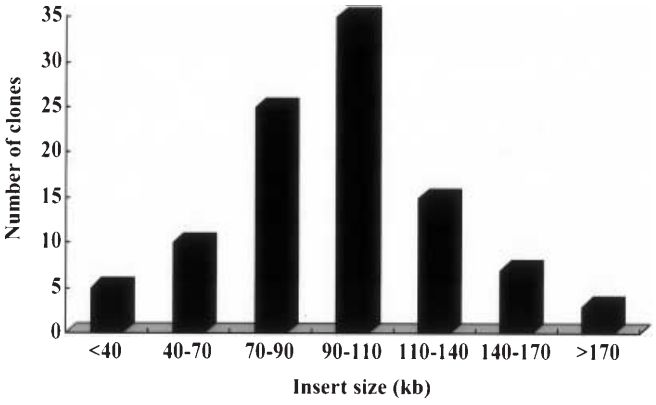

Fig. 2 Insert size distribution of peach BAC clones. The data are calculated based on 100 randomly selected clones. The average insert size of these clones is 95 kb

Characterization of the peach BAC library

To test the utility of the BAC library for map-based cloning, genome analysis and physical mapping, we characterized the library based on insert size distribution, clone representation, stability and levels of cpDNA and mtDNA content.

We extracted DNA of 100 random BAC clones from the peach library. The BAC DNAs were digested with *Not*I and analyzed by PFGE. As shown in Fig. 2, the insert size of clones in this library ranged from approximately 40 to 180 kb, with over 60% of the clones having inserts larger than 90 kb. The peach BAC library with an average insert size of 95 kb, was equivalent to 7× the haploid genome of peach. Theoretically, the probability of any peach gene being found in this library should be higher than 99%.

In order to confirm the reality of the insert DNA of BAC clones, we digested 18 randomly selected clones using *Not*I and subjected the digestion products to PFGE. The digested BAC DNA on the gel was Southern-blotted and hybridized with total genomic DNA of Jingyu. All 18 BAC inserts gave hybridization signals, as shown in Fig. 3A,B.

We screened the library with the mixed probes from the sorghum chloroplast *rbsL* and maize mitochondrial *apt*6 and *18s+5s* genes. No hybridized signal was detected (Fig. 4). This indicated that the preparation procedure of peach HMW DNA effectively eliminated the cpDNA and mtDNA and that the library was composed predominantly of nuclear DNA.

To examine the stability of peach BAC clones in *E. coli*, we analyzed the *Hin*dIII restriction digestion patterns of 3 large BAC clones in the 0 and 100 generations. No visible change was found (Fig. 5), which proved that the BAC cloning vector can stably maintain a large DNA fragment in *E. coli* up to 100 generations of culture and is suitable for map-based cloning, in contrast to YAC vector.

The library was screened with two molecular markers, W4 and P20, which are linked to the white flesh and

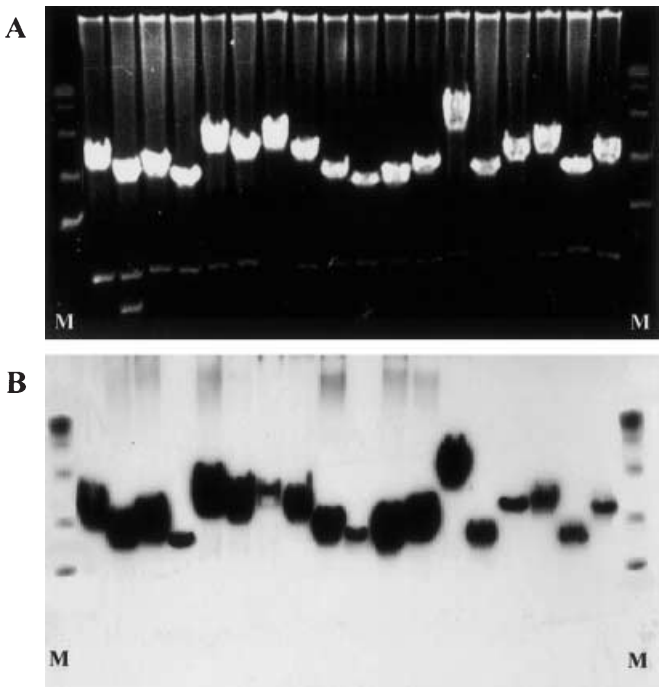

Fig. 3A, B Analysis of 18 randomly selected peach BAC clones. **A** Ethidium bromide-stained gel containing the *Not*I-digested DNA fragments after separation by PFGE (5 V/cm, 5 – to 15-s switch time, 13 h). **B** Autoradiograph of gel in **A** after Southern transfer and probing with total peach genome DNA. M Lambda concatamer (48.5 kb) (Bio-Rad)

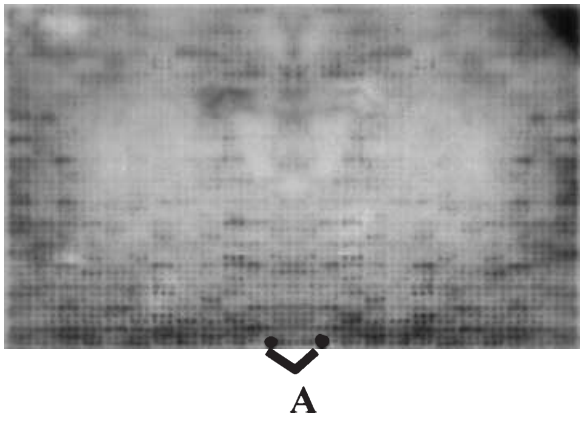

Fig. 4 Hybridization analysis of the contamination of ctDNA and mtDNA in peach BAC clones. *A* Two positive controls were the mixture of the chloroplast *rbs*L, mitochondria *apt*6 and *18s+5s* genes

nectarine genes, respectively. Ten positive clones were identified (Fig. 6). Consequently, the library represents the peach genome very well and is very suitable for map-based cloning and physical mapping of peach.

Discussion

Many investigations have demonstrated that BACs are well-suited for map-based cloning, physical mapping and genome sequencing. A number of human, animal, plant BAC libraries have been constructed (Zhang et al. 1995; Wang et al. 1995; Zhang et al. 1996; Cai et al. 1995; Kim et al. 1996; Frijters et al., 1997; Woo et al. 1994), and at the same time the methods of BAC library construction have been improved. We have developed an optimal procedure for constructing the peach library rapidly.

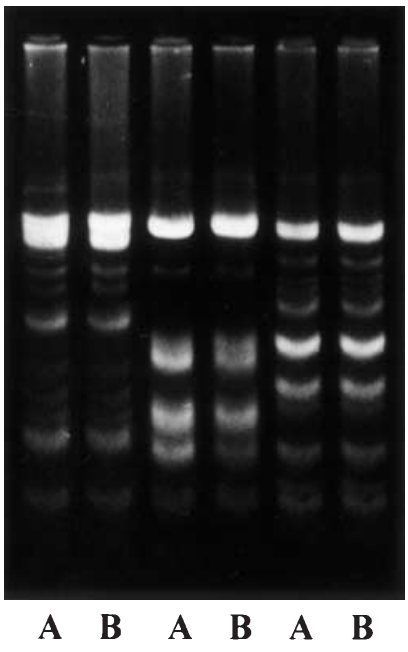

Fig. 5 Comparison of the *Hin*dIII digestion pattern of 3 BAC clones in generations 0 (*A*) and 100 (*B*)

Firstly, some improvements in vector preparation were made. To recover the linearized and dephosphorated vector by electroelution, we effectively reduced the background of transformation; the ratio of vector and insert DNA in ligation was also optimized and well-controlled. It is no longer necessary to carry out supercentrifugation to purify the vector DNA. This makes the preparation of vector DNA more convenient.

Secondly, we modified the protocol of HMW DNA preparation and made it suitable for peach species. The HMW DNAs are of high quality and large quantity (about 10 µg/plug), with no inhibitor, no cpDNA or mtDNA and very little small fragment contamination.

Thirdly, we only performed one round of size-fractionation of the digested DNA fragments. By reducing the loading DNA on PFGE and precisely separating the gel slices into size ranges, we obtained high-quality ligations. For some small fragments entrapped in the library, the average size of the insert was relatively small, but a high ligation and transformation efficieny enabled us to construct the library rapidly.

Moreover, the size-fractionated DNA fragments were recovered by electroelution rather than by the common method of melting gel slices followed by gelase or agarase treatment. In agreement with Strong (1997), the

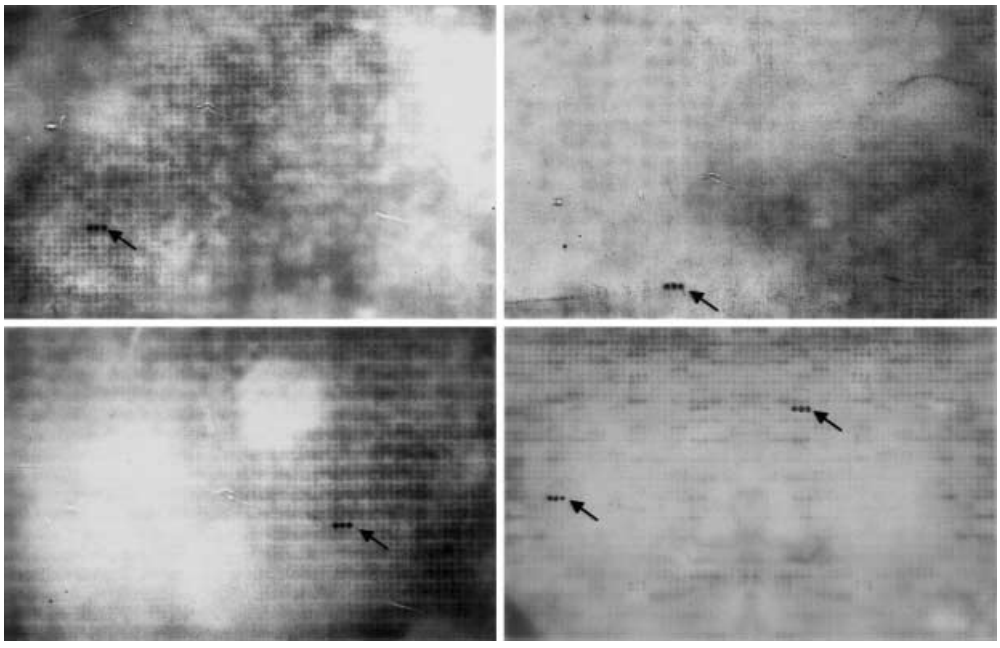

Fig. 6 Autoradiograph of 5 positive BAC clones screened with W4 and P20 as probes. *Arrows* show the positive clones. Each set of three parallel *strong points* represent three copies of 1 BAC clone

yields DNA were comparatively less degraded and more amenable to ligation.

Finally, for a high DNA concentration in ligation could lead to chimeric clones (Frijters et al. 1997), we used a 5:1 ratio of vector to insert DNA. Our results demonstrated that a lower voltage could increase the average insert size of the clones. We selected 1.3 kV/cm for electroporation, and the efficiency of transformation was about 1.5×10^6 cfu/μg ligation. For the procedures of HMW DNA and size-fractionation of digested DNA fragments it may be possible to make a library bias to peach total genome; the library was constructed in approximately 30 separated transformations involving different batches of three ligations.

There are two basic methods to screen a BAC library which are hybridization and the polymerase chain reaction (PCR). One advantage of hybridization is the ability to combine probes for screening the BACs derived from an entire region and identifying them in a single experiment. To allow efficient hybridization screening, the BAC library was spotted at 3×384 onto high-density nylon filters, on which every clone has three duplications; this facilitated real positive clone identification. Ten positive clones were obtained from screening the peach library with two probes, and these proved to represent the library.

Peach is the best representative of *Prunus*. With a small genome size and relatively short juvenile period, peach is a model plant for studying the molecular genetics of fruit trees. Construction of the peach BAC library will benefit these studies. Jingyu is a main parental variety in Chinese peach breeding, and in addition to our target characters (white fresh, nectarine) there are some other important agronomic traits in this peach. So the library can be suitable for the screening of many peach genes, physical mapping and genome sequencing.

In conclusion, with its large insert size, high stability and good genomic coverage, the peach BAC library should serve as an invaluable tool in peach genomic studies at all levels.

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