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Genetic Resources

Construction, Characterization, and Screening of a Transformation-Competent Artificial Chromosome Library of Peach

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Abstract. As a genome model of fruit trees, peach (*Prunus persica* [L.] Batch) has advantages for studying structural and functional genomics. Okubo, a traditional peach variety used as a parent in Asian peach breeding, displays economically valuable agronomic traits. To develop an efficient platform for peach gene cloning and genomic research, a large-insert genomic DNA library of Okubo was constructed in a transformation-competent artificial chromosome (TAC) vector, pYLTAC7, which can accept and stably maintain large genomic DNA fragments in both *Escherichia coli* and *Agrobacterium tumefaciens*. The TAC library contains 41,472 recombinant clones with an average insert size of approximately 42 kb, and it is equivalent to 6 haploid peach genomes. The TAC library was stored in 2 ways: one copy as frozen cultures in 108 pieces of 384-well plates and another copy as bulked pools in 36 pieces of 96-well plates, each well containing 12 individual clones. The lack of hybridization signal to chloroplast and mitochondrial genes indicated that the TAC library had no significant cytoplast organelle DNA contamination. TAC clones were stable in *E. coli* cells until generation 100 and stable in both *E. coli* and *A. tumefaciens*. Twenty-one clones containing the polygalacturonase-inhibiting protein (PGIP) gene were detected by using pooled PCR in the TAC library. Positive clones can be used for peach PGIP gene cloning and functional analysis. The library is well suited for gene cloning and genetic engineering in peach.

Key words: genomic library, Okubo, peach, *Prunus persica*, TAC, transformation-competent artificial chromosome

Abbreviations: BAC, bacterial artificial chromosome; CIAP, calf intestinal alkaline phosphatase; EDTA, ethylenediaminetetraacetic acid; HB, homogenization buffer; HMW, high molecular weight; LMP, low melting point; ORF, open reading frame; PFGE, pulsed field gel electrophoresis; PG, polygalacturonase; PGIP, polygalacturonase-inhibiting protein; PMSF, phenylmethyl sulfonyl fluoride; TBE, buffer of Tris-base, boric acid, and EDTA; TAC, transformation-competent artificial chromosome.

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Introduction

Prunus is an economically and biologically important genus. Currently, peach (*Prunus persica* [L.] Batch) is the best genetically characterized species in the genus and one of the best genetically characterized trees. As a genome model of fruit trees, peach is useful in studying structural and functional genomics because of its short juvenile period (2-3 y) and high self-compatibility (Scorza and Sherman, 1996). It is diploid, with $2n = 16$ (Jelenkovic and Harrington, 1972) and $2c = 5.8 \times 10^8$ bp (Baird et al., 1994). The peach genome is about twice the size of the *Arabidopsis thaliana* genome (Arumuganathan and Earle, 1991). Many genes controlling fundamentally important traits have been genetically described in peach.

Recent research of the peach genome has focused on structural and functional genomics. Main research goals are developing a complete physical genome map to which genetic maps of other *Rosaceae* species can be anchored, developing an extensive expressed sequence tag (EST) database, and integrating the unigene set into the physical and genetic maps (Abbott et al., 2003).

Molecular linkage maps of peach have been constructed, and genes controlling important traits (e.g., flower development; fruit development; growth habit; dormancy; cold, disease, and pest resistance) have been mapped (Belthoff et al., 1993; Chapparo et al., 1994; Dirlewanger et al., 1998; Lu et al., 1998; Sosinski et al., 1998; Viruel et al., 1998; Warburton et al., 1996; Rajapakse et al., 1995). A large insert genomic DNA library is necessary for map-based cloning and research of gene function. We constructed the first peach bacterial artificial chromosome (BAC) library of var. Jingyu in China (Wang et al., 2001). Okubo is another parent in Asian peach breeding that displays economically valuable agronomic traits. For direct transformation with *Agrobacterium*-mediated methods, a peach Okubo genomic DNA library was constructed by using a transformation-competent artificial chromosome (TAC) vector system. pYLTAC7 is a positive selection vector because expression of its *sacB* gene can kill *E. coli* cells that are grown in the presence of sucrose. Furthermore, it has *cis*-sequences required for *Agrobacterium*-mediated gene transfer into dicotyledon plants (Liu et al., 1999). Recently, new vectors pYLTAC17 and pYLTAC27 were developed on the basis of pYLTAC7 and were suitable for transforming monocotyledons and constructing TAC libraries (Liu et al., 1999, 2000, 2002; Fang et al., 2000; Wang et al., 2002). A TAC clone with a large DNA fragment has been successfully transformed into *Arabidopsis* and rice by means of *Agrobacterium*-mediated transformation (Liu et al., 1999, 2002).

Plant polygalacturonase-inhibiting proteins (PGIPs) inhibit fungal endopolygalacturonases and are important for plant resistance to phytopathogenic fungi (De Lorenzo and Ferrari, 2002). Many PGIP genes have been cloned, but none have been cloned in peach. In this study, the TAC library was screened with pooled PCR by using conserved primers of the PGIP gene, and 21 positive clones containing the candidate peach PGIP gene were obtained.

The library constructed with TAC vector pYLTAC7 can be used to isolate clones containing target genes and for other genomic research in peach.

Material and Methods

Materials and reagents

Young peach (variety Okubo) leaves were collected from the Forestry and Fruit Research Institute, Academy of Agricultural and Forestry Sciences of Beijing, China.

E. coli strain DH10B and cloning vector pYLTAC7 were used to construct the TAC library. *Hin*d III restriction enzyme was purchased from TaKaRa (Japan). T4 DNA ligase and calf intestinal alkaline phosphatase (CIAP) were purchased from New England BioLabs.

Preparation of TAC vector

TAC vector pYLTAC7 was isolated by means of alkaline lysis and purified with a Qiagen kit (QIAGEN, Germany). The purified vector was completely digested with *Hin*d III and dephosphorylated with CIAP. Dephosphorylation efficiency was tested by means of ligation with λ DNA / *Hin*d III fragments and self-ligation. The 50 ng aliquots of prepared linear vector DNA were stored at –70°C.

High-molecular-weight DNA isolation

High-molecular-weight (HMW) DNA was prepared according to Wang et al. (2001) with some modifications. Approximately 10 g of young leaves was ground into a fine powder in liquid nitrogen with a cold mortar and pestle. The powder was transferred into an ice-cold beaker with 200 mL 1× HB nuclei extraction buffer containing 0.15% β-mercaptoethanol. The mixture was gently swirled with a magnetic stir bar for 15 min on ice, filtered through 2 layers of cheesecloth and 1 layer of miracloth, and centrifuged at 1800*g* at 4°C for 20 min. Nuclei were pelleted and washed 3× with 200 mL 1× HB nuclei washing buffer. Nuclei were resuspended in 2-3 mL 1× HB (β-mercaptoethanol free) and embedded in an equal volume of 1% low-melting-point (LMP) agarose plugs.

Plugs were incubated in 10× vol of lysis buffer containing 0.5 mg/mL proteinase K at 50°C for 48 h, changing the buffer and enzyme once. Plugs were transferred into 0.5 M EDTA (pH 9) at 50°C for 1 h and stored in 0.5 M EDTA (pH 8) at 4°C for later use. After being dipped in 0.5× TBE buffer for 1 h, plugs were electrophoresed by using pulsed field gel electrophoresis (PFGE) (CHEF, BioRad; agarose, 1%; buffer, 0.5× TBE; pulse time, 60-90 s; voltage, 6 v/cm; angle, 120°; running time, 24 h; temperature, 14°C). DNA fragments of about 2 Mb embedded in 1% LMP agarose were recovered and stored in 0.5 M EDTA (pH 8) at 4°C for later use in cloning.

Partial digestion of DNA and size selection

Plugs were dialyzed twice for 1 h on ice against 50× vol of 1× TE (pH 8) and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and then washed twice for 1 h on ice with 50× vol of 1× TE (pH 8) without PMSF. HMW DNA was partially digested with 2, 4, 6, 8, 10, or 12 U of *Hin*d III at 37°C for 5 or 8 min. Digested fragments were size-fractionated by means of PFGE (CHEF, BioRad; agarose, 1%; buffer, 0.5× TBE; pulse time, 1-40 s; voltage, 6 V/cm; angle, 120°; running

time, 18 h; temperature, 12°C). The region of the gel between 30 and 90 kb was excised with a glass slide cover and divided horizontally into 3 sections: 30-50 kb, 50-70 kb, and 70-90 kb. Selected DNA in each gel slice was electroeluted into a dialysis tube by means of PFGE (CHEF, BioRad; agarose, 1%; buffer, 0.5× TBE; pulse time, 30-30s; voltage, 6 V/cm; angle, 12°; running time, 4 h; temperature, 120°C) for ligation with the TAC vector after concentration quantification.

TAC library construction

Ligation was performed in a 100-μL solution containing 80 ng of partially digested HMW DNA, 100 ng of the linearized and dephosphorylated pYLTAC7 vector, and 160 U (2.4 Weiss U) of T4 DNA ligase. The reaction was performed in a thermocycler as follows: 30 cycles of 10°C for 3 min, ramping to 16°C in 3 min, 16°C for 3 min, and 18°C for 1 min and a final cycle of 65°C for 10 min, according to Lund et al. (1996), with some modifications. The ligation mixture was desalted and concentrated on a Millipore filter (TYPE VS 0.025 μm) against sterile distilled water at 4°C for 2 h.

Ligation products were used to transform competent *E. coli* DH10B cells by means of electroporation with a Gibco BRL Electroporator-voltage booster system (4 kΩ resistance, 340 V, 330 μF, fast charge, low ohm impedence). After incubation in 1 mL SOC at 37°C for 60 min, the SOC medium with the cells was plated on LB solid medium containing 5% sucrose and 25 μg/mL kanamycin and incubated at 37°C for 18-20 h. Only clones with inserts grew because of positive selection by the *sacB* gene. Individual colonies were put into 96-well plates, with 600 μL LB medium in each well. Colonies were cultured at 37°C for 16-18 h, transferred to 384-well plates containing 75 μL LB freezing medium, incubated at 37°C for 10 h, and stored at –80°C. Additionally, 12 colonies as a bulked pool were collected into 96-well plates.

TAC library characterization

To estimate clone insert size and library capacity, 60 TAC clones were selected randomly from the library and analyzed by means of restriction digestion with *Hin*d III.

To determine cpDNA and mtDNA contamination, a 384-well plate containing TAC clones was selected randomly. TAC clones were transferred onto a piece of Hybond filter (12 × 8 cm) (Amersham Life Science) by using a 384-replicator. The filter was processed and hybridized with standard techniques (Sambrook et al., 1989). The plasmid containing the sorghum chloroplast gene *psbA* and the maize mitochondrial gene *atp6* were then spotted on the filter. Mixed probes from *psbA* and *atp6* were used to assess contamination of the TAC library.

TAC clone stability was checked by culturing 3 randomly selected clones. Samples were taken at generation times of 0, 50, and 100, and *Hin*d III restriction patterns were analyzed.

To investigate structural stability of TAC clones in *E. coli* and *A. tumefaciens* strains, the plasmids of 3 TAC clones selected randomly from the library were isolated. Plasmid DNA was electroporated into *A. tumefaciens* strain EHA105, reisolated from the *Agrobacterium* transformations, and transferred

back into *E. coli* DH10B. Restriction analyses of these plasmids with *Hin*d III were performed to determine TAC clone stability.

Screening the TAC library

PGIPs are ubiquitous plant cell wall proteins that play a key role in the response to fungal infection (De Lorenzo and Ferrari, 2002). To evaluate the potential of the TAC library for identifying genomic regions associated with the peach PGIP gene, specific primers were designed and synthesized according to the conservative region of the PGIP gene in GenBank (www.ncbi.nlm.nih.gov). PGIP-1 was 5′-CGTTCACCCGCAATCACATTTCTTATCC-3′; PGIP-2 was 5′-TGGCCG-TGGGAATTATTTGCAGC-3′.

By using the primers, pooled PCR was carried out on the pooled TAC library for the first screening. Stock (10 μL) from the well of every 9 pieces of 96-well plates was pooled into a new 96-well plate containing 1 mL of LB medium and 25 μg/mL kanamycin; this resulted in the preparation of 4 new 96-well plates containing the pooled library. After cultivating *E. coli* in the 4 new 96-well plates overnight at 37°C, plasmid DNA was isolated and dissolved in 50 μL 1× TE buffer (pH 8). Consequently, 2 μL of pooled plasmid DNA was used for the first PCR screening of the peach PGIP gene fragment. The PCR reaction was performed in a volume of 20 μL containing 10 mM of Tris-HCl (pH 8.4), 1.5 mM of $MgCl_2$, 50 mM of KCl, 0.25 mM of each dNTP, 0.2 μM of each primer, 1 U of *Taq* DNA polymerase, and template DNA, as follows: 94°C for 2 min; 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min; and a final extension of 72°C for 10 min. A 10-μL amplification product was examined by means of 1% agarose gel electrophoresis containing 0.1 μg/mL of ethidium bromide. When a positive pool was identified, plasmid DNAs were isolated from the 9 wells corresponding to the positive pool in the original plates and served as templates for the second screening. The corresponding 12 single clones in another copy of the TAC library (384-well plate) containing the positive clone were amplified for the third screening. In the third screening, the positive single colony was obtained.

Results

Preparing partially digested peach HMW DNA

Isolating high-quality HMW peach DNA is difficult because peach leaves are rich in polysaccharides. To yield high-quality and stable HMW DNA, nuclei were purified by washing 3× with a washing buffer. Nuclei were embedded in an LMP agarose gel plug, cell-lysed, DNA purified, and partially digested to protect from physical shearing during preparation. HMW DNA obtained was greater than 2 Mb, contained no phenol products, and had little cytoplast organelle DNA contamination.

Optimal partial digestion conditions were determined by using different amounts of *Hin*d III (2, 4, 6, 8, 10, and 12 U) for varying periods. Most fragments were of the desired size (i.e., 30-90 kb) when HMW DNA was digested with 10 μL *Hin*d III for 8 min at 37°C. We used 10 μL of *Hin*d III for large-scale digestion of HMW DNA.

Ligation and transformation

To optimize ligation, we tested molar ratios of vector–to–HMW DNA ranging from 8:1 to 1:5. Ligation with a 4:1 ratio generated the most clones. Sterile distilled water was used as a dialysis buffer to desalt and concentrate the ligation mixture, resulting in higher transformation efficiency. Recovering all the original volume of the ligation mixture was not possible after dialysis, but a 3- to 5-fold increase in electroporation efficiency was observed. We obtained optimal ligation by using 40 ng HMW DNA, 50 ng vector, and 80 U ligase in a 50-μL reaction volume, in which 1 μL ligation produced 1000 clones on an LB plate containing 5% sucrose.

To obtain higher transformation efficiency, various ligation reaction conditions and electroporation settings were tested. A ligation reaction using cyclic temperatures for a short time (~5 h) had 30-80% higher transformation efficiency than that of conventional incubation at 16°C or 4°C overnight. In this study, ligation was carried out in a programmable thermal controller PTC-100, and an optimal setting was obtained when using a Gibco BRL Electropon-Voltage Booster System (see "Materials and Methods").

Library characterization

The TAC library contains 41,472 recombinant clones and was stored both as frozen cultures in 108 pieces of 384-well plates and as bulked pools in 36 pieces of 96-well plates. Each well contained 12 individual clones.

To determine library quality, we analyzed insert size distribution, contamination of cpDNA and mtDNA, and clone stability.

Plasmid DNA was isolated from 60 randomly selected TAC clones from the library, completely digested by means of *Hin*d III, and run on 0.8% agarose gel. All TAC clones contained insert DNA fragments. Clone insert size ranged from 10-80 kb, with more than 60% of clones containing inserts larger than 40 kb (Figures 1-2).

With an average insert size of 42 kb, the TAC library was equivalent to 6× the haploid genome of peach, providing a 99% probability of isolating a single-copy sequence of peach in the library.

Contamination by cytoplast organelle DNA can decrease library capacity and cause chromosome walking errors (Zhang et al., 1996). We used PFGE to separate HMW DNA and remove most cytoplast organelle DNA. Filter binding clones were probed with mixed probes from the sorghum chloroplast gene *psbA* and the maize mitochondrial gene *atp6*; no hybridization signal was detected except in controls (Figure 3). This screening proved the method to be suitable for eliminating cpDNA and mtDNA and the library to be qualified for cloning desirable genomic DNA sequences.

TAC clone stability in *E. coli* was assessed by analyzing *Hin*d III restriction digestion pattens of 3 randomly selected TAC clones at generations 0, 50, and 100. Electrophoretic banding patterns of plasmid DNA had no visible absences or recombinations, indicating that TAC clones are stable in *E. coli* for up to 100 generations (Figure 4). To investigate structural stability of TAC clones in *E. coli* and *A. tumefaciens* strains, plasmids were isolated from 3 randomly selected *E. coli*

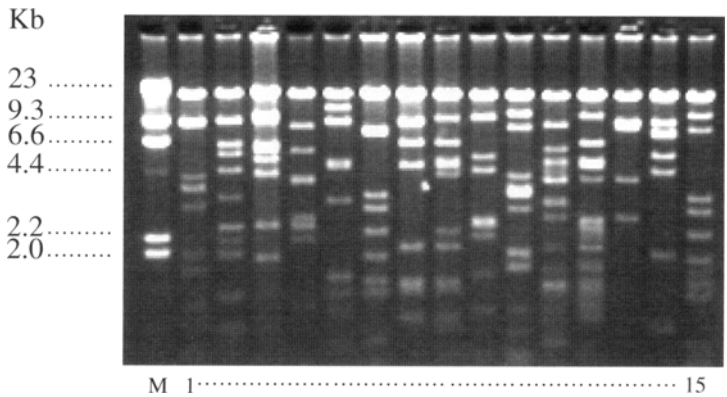

Figure 1. Analysis of 15 peach transformation-competent artificial chromosome (TAC) clones randomly selected from the library. Plasmid DNAs of TAC clones were digested completely with *Hind* III and separated on 0.8% agarose gel. M, molecular weight marker λ DNA / *Hind* III; Lanes 1-15, individual TAC clones. The upper band of each lane is a vector.

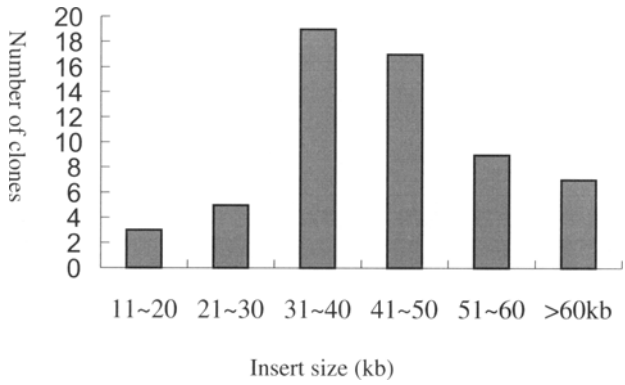

Figure 2. Distribution of insert sizes of clones in the transformation-competent artificial chromosome (TAC) library. Sixty TAC clones randomly selected from the library were checked for insert sizes.

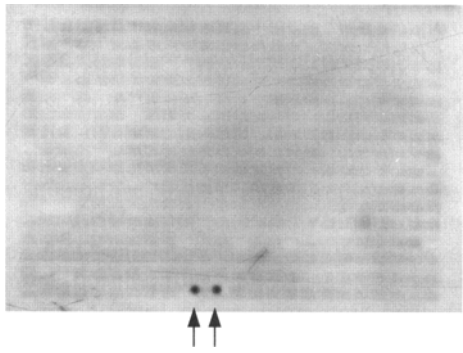

Figure 3. Hybridization analysis of the contamination of ctDNA and mtDNA in peach transformation-competent artificial chromosome (TAC) clones. Dots with arrows were positive controls of cpDNA and mtDNA, respectively.

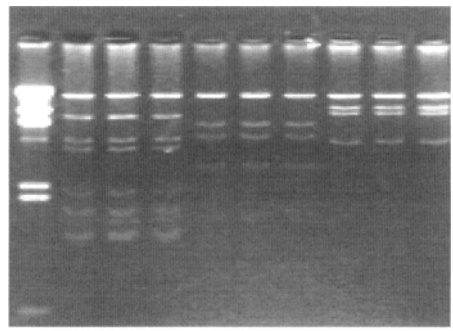

Figure 4. Comparison of the *Hind* III digestion patterns of 3 randomly selected transformation-competent artificial chromosome (TAC) clones in generations 0, 50, and 100, respectively. M, molecular weight marker λ DNA / *Hind* III; A, generation 0; B, generation 50; C, generation 100.

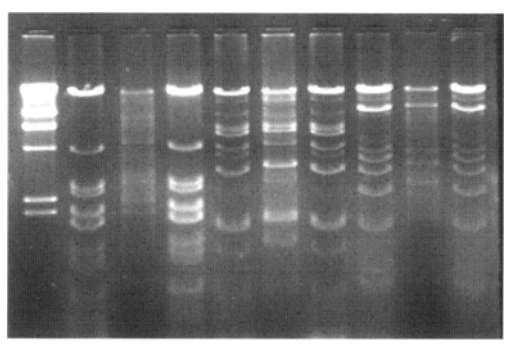

Figure 5. Stability test of transformation-competent artificial chromosome (TAC) clones in *Escherichia coli* and *Agrobacterium tumefaciens*. M, molecular weight marker λ DNA / *Hind* III. A, digestion pattern of 3 random TAC plasmid DNA from *E. coli*. B, digestion pattern of the same TAC plasmid DNA from *A. tumefaciens*. C, digestion pattern of the same TAC plasmid DNA from *E. coli*.

clones. Plasmid DNA was electroporated into *A. tumefaciens* strain EHA105, re-isolated from the *Agrobacterium* transformants, and transferred back into *E. coli*. Restriction analysis of these plasmids indicates that clones remained intact (Figure 5).

Screening of TAC library with PGIP gene

We used specific primers synthesized according to the conservative region of the PGIP gene in GenBank to amplify peach genomic DNA with PCR. A fragment of approximately 1.2 kb was obtained and cloned. The DNA sequence had an open reading frame (ORF), and the nucleotide and deduced amino acid sequences of the DNA fragment showed high similarity to PGIP genes in *Prunus armeniaca*, *Prunus mahaleb*, and other plants. To evaluate the potential for identifying genomic regions associated with the PGIP gene, the TAC library was screened with pooled PCR by using primers of the PGIP gene. After PCR and confirmation by spot-hybridization, 21 positive clones were obtained, and the amplified

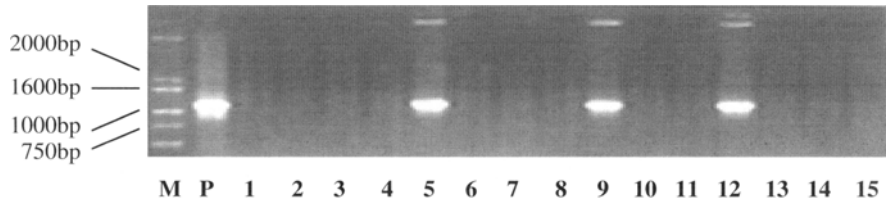

Figure 6. Screening of the transformation-competent artificial chromosome (TAC) library with polygalacturonase-inhibiting protein (PGIP) gene by using the pooled PCR method. M, DNA size marker DGL2000; P, PCR result of peach genomic DNA; 1-15, PCR results of partial TAC clones for screening; 5, 9, and 12 are positive clones.

fragment was approximately 1.2 kb (Figure 6). Because the TAC library covers 6 haploid genome equivalents of peach, the number of positive clones indicates that the PGIP gene is multicopy in peach. Results indicate that the TAC library represents the peach genome well.

Discussion

Because of a rigid cell wall, the most common methods for plant megabase-size DNA isolation include embedding isolated protoplasts or nuclei in an agarose matrix, cell lysis, and protein degradation (Woo et al., 1995). Large-scale protoplast isolation is time-consuming, costly, and tedious. Furthermore, HMW DNA prepared from plant protoplasts can contain significant amounts of cpDNA and mtDNA, which can mislead chromosome walking studies in gene map-based cloning (Cheung and Gale, 1990; Liu and Whittier, 1994).

In contrast, isolating nuclei from leaf tissue is simple and has been used successfully in grasses (e.g., wheat, sorghum, maize), legumes (e.g., soybean, bean), vegetables (e.g., cauliflower, squash, eggplant, watermelon, pepper), and trees (e.g., peach, walnut, willow) (Zhang et al., 1995). We modified the protocol for isolating HMW DNA from nuclei to make it suitable for peach. Most isolated peach DNA was greater than 2 Mb, contained no significant cpDNA or mtDNA contamination, and was suitable for constructing the TAC library.

Vector pYLTAC7, which is 22,530 bp, was used to construct the TAC library. Studies indicate that the cloning efficiency of this relatively large vector is lower than that of a commonly used BAC vector (~7 kb) (Shizuya et al. 1992). To obtain higher transformation efficiency, various ligation conditions were tested. Conventional methods for ligation include overnight incubation at 4°C or 16°C. Considering that ligase activity is low, that the ligation substrate is relatively stable at low temperatures, and that the situation is reversed at high temperatures, we used temperature cycling for a short time (~5 h) to increase ligation efficiency. In constructing the TAC library, ligation with temperature cycling was carried out according to Lund et al. (1996) with some modifications, resulting in higher transformation efficiency than with other methods.

PGIPs are ubiquitous plant cell wall proteins that are directed against fungal polygalacturonases (PGs), which are important pathogenicity factors (De Lorenzo and Ferrari, 2002). Cloning the peach PGIP gene is important for disease resistance breeding and genome research. Large-insert genomic DNA libraries can be

screened with 2 basic methods: hybridization and PCR. Using pooled PCR to screen for the target gene saves space and minimizes effort (Liu et al., 2000). In our system, the high-density filers used in screening BAC or YAC libraries are not needed. We identified positive clones containing the PGIP gene that can be used for future PGIP gene cloning and functional analysis.

Constructing a large-insert genomic DNA library such as YAC, BAC, or TAC is essential for gene map-based cloning. Such libraries have been used to clone many genes (Giraudat et al., 1992; Arondel et al., 1992; Song et al., 1995; Dixon et al., 1998), but functional complementation by candidate clone transformation was difficult in these studies. Peach is a model plant for molecular genetic research of fruit trees. Okubo is an elite peach variety and an important parent in Asian peach breeding that displays economically valuable agronomic traits. The TAC library of Okubo will be useful for candidate gene transformation and for isolating the whole gene corresponding to the cDNA fragment. With its high stability and good genomic coverage, the TAC library provides an efficient platform for gene cloning and functional complementation of target genes in peach.

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