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Construction and characterization of a bacterial artificial chromosome library of apple

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Abstract A bacterial artificial chromosome (BAC) library has been constructed from apple (*Malus* × *domestica* Borkh.) using the variety "Florina", which is resistant to scab (*Venturia inaequalis*) by virtue of the *Vf* gene. Since apple leaves are rich in polyphenols, high-molecular-weight DNA was extracted from leaf nuclei with a protocol adapted to apple. The nuclei were then embedded in agarose microbeads and partially digested by varying ratios of *Eco*RI to *Eco*RI methylase. The resulting DNA fragments were size-selected by pulsed-field gel electrophoresis, ligated to the BAC cloning vector pECBAC1 and transformed into *Escherichia coli* cells by electroporation. A total of 36 864 recombinant clones (BACs) were obtained. The library has an average insert size of 120 kb and represents approximately 5 × apple haploid-genome equivalents. It was screened with six cDNA probes using the chemiluminescent DIG system. An average of 4.4 clones was detected for each locus. The apple BAC library will be used to isolate the *Vf* scab resistance gene through map-based cloning. In this connection the library was screened with a marker closely linked to the *Vf* gene and six positive clones have been isolated. This library should thus be well suited for map-based gene cloning, in particular for the isolation of the *Vf* gene and for the construction of a physical map of the apple genome.

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Introduction

Molecular linkage maps of apple, *Malus* × *domestica* Borkh., have been developed (Gardiner et al. 1994; Hemmat et al. 1994; King 1994; Maliepaard et al. 1998) and many loci for genes determining agronomically important traits have been located on these maps. We are particularly interested in the *Vf* scab resistance gene, which was introgressed into some cultivated apple varieties from a small-fruited selection of the Asiatic *Malus* species *M. floribunda* 821 (Hough 1944). Closely linked DNA markers have been identified on both sides of this gene (Gianfranceschi et al. 1996; Tartarini 1996) and have provided a powerful means for the manipulation of scab resistance by marker-assisted selection. These markers may also be useful in the isolation of the *Vf* gene through map-based cloning. Isolating the *Vf* gene through map-based cloning requires a large-insert apple genomic-DNA library, which as yet has not been available. Two cloning systems have been used in plants to construct large-insert genomic DNA libraries: the yeast artificial chromosome (YAC) (Burke et al. 1987) and bacterial artificial chromosome (BAC) (Shizuya et al. 1992).

Plant BAC libraries with insert sizes ranging from 110 to 160 kb have been constructed (Woo et al. 1994; Wang et al. 1995; Zhang et al. 1996; Frijters et al. 1997). Although they have smaller average insert sizes compared to those of the most recently developed plant YAC libraries, i.e. average insert sizes of 245 kb (Bonnema et al. 1996) and 420 kb (Creusot et al. 1995), it has been demonstrated that they are well-suited to map-based cloning and genome physical mapping. The BAC system also has several advantages over the YAC system. BAC constructs are transformed into

Escherichia coli by electroporation and the YAC constructs are transformed into *Saccharomyces cerevisiae* through spheroplasts. The transformation efficiency of BACs is thus much higher than that of YACs. BACs are also more stable in the host cells than YACs. No chimeric clones have been detected in plant BAC libraries to-date, while up to 50% of the clones have been found to be chimeric in YAC libraries (Anderson 1993). And while BAC DNA can be extracted as easily as conventional plasmid DNA, YAC DNA has to be extracted as high-molecular-weight (HMW) DNA and is difficult to purify from the yeast host chromosomal DNA. We have therefore chosen the BAC system for the construction of a large-insert genomic-DNA library of apple.

To construct a large-insert genomic-DNA library, HMW DNA of high quality and yield is required. It can be extracted either from plant protoplasts or plant nuclei. We used the nuclei method, developed by Zhang et al. (1995), because of its high suitability for the isolation of HMW DNA from different species, its simplicity, economy and the low chloroplast DNA contamination of the genomic DNA. Because of the high polyphenol content of apple leaves, the extraction buffer used by Zhang et al. (1995) was modified so as to reduce polyphenol contamination of the extracted nuclei since these polyphenols could interact with DNA and make it inaccessible to restriction enzymes.

Partial digestion by restriction enzymes is generally used to obtain overlapping DNA fragments in the size range from 100 to 300 kb. This can be accomplished by varying the ratios of methylase to endonuclease, both enzymes recognizing the same target sequence (Larin et al. 1991). When the ratio of methylase to endonuclease is increased, fewer sites are accessible to the endonuclease and larger fragments are generated. The methylation method was successfully used in plants for the construction of a tomato YAC library by Bonnema et al. (1996) who employed *Eco*RI and *Eco*RI methylase. Until recently, this method could not be used for the construction of BAC libraries because no BAC vector with a unique *Eco*RI cloning site was available. Frijters et al. (1997) modified the BAC cloning vector pBeloBAC 11 (Kim et al. 1996) into pECBAC1 by destroying the second *Eco*RI site in the chloramphenicol resistance gene of pBeloBAC 11. The vector pECBAC1 therefore contains a unique *Eco*RI cloning site in the *LacZ* gene for recombinant selection. We utilized pECBAC1, along with partial digestion by *Eco*RI and *Eco*RI methylase, for the construction of the apple BAC library.

The apple BAC library was constructed from the commercial variety "Florina", which is resistant to apple scab because of the presence of the *Vf* gene. Our objective is to utilize this library to clone the *Vf* gene by map-based cloning employing the markers identified by Gianfranceschi et al. (1996) and Tartarini (1996) as a starting point for chromosome walking. The present study reports the construction and characterization of an apple BAC library and discusses its utility for map-based cloning of apple genes and the *Vf* gene in particular.

Materials and methods

Plant material and chemicals

Leaves were collected from juvenile shoots of adult trees of the variety "Florina" from a field in Auer (South Tyrol, Italy), frozen in liquid nitrogen, and stored at −80°C before use. ElectroMAX DH10B *E. coli* cells were purchased from Gibco BRL (USA). *Eco*RI methylase was purchased from New England Biolabs (USA). GELase was purchased from Epicentre Technologies (USA). T4 DNA Ligase was purchased from US Biochemicals (USA). Restriction enzymes were purchased from Promega (USA) and Gibco BRL (USA). All chemiluminescent labelling and detection reagents were purchased from Boehringer Mannheim (Germany). Trizma base, sucrose and other chemicals were purchased from Sigma (USA).

BAC vector preparation

pECBAC1 (Frijters et al. 1997), used as the cloning vector for construction of the apple BAC library, was kindly provided by Dr. R. W. Michelmore, University of California, Davis. The vector DNA was isolated from a 1.5-l overnight culture by the alkaline-lysis method of Sambrook et al. (1989), followed by several purification steps with PEG and ammonium acetate and an RNase treatment. The pECBAC1 DNA was digested and de-phosphorylated after Zhang et al. (1996).

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

Nuclei were prepared from apple leaves after Zhang et al. (1995) with modifications. The HB (1×) nuclei extraction buffer (10 mM trizma base, 80 mM KCl, 10 mM EDTA, 1 mM spermidine, 1 mM spermine, 0.5 M sucrose, 0.15% β-mercaptoethanol, pH 9.4–9.5) was modified by adding PVP-40 at a ratio of 2% (w/v), the same ratio used by Zhang et al. (1994) in the cotton nuclei extraction buffer. After incubating the ground apple leaves in the extraction buffer for 10 min, the suspension was filtered through two layers of cheesecloth and one layer of miracloth and centrifuged at 1900 g for 20 min. The pellet was re-suspended in wash buffer (Zhang et al. 1995) using a paintbrush and filtered through two layers of miracloth. The nuclei were then washed twice, each wash step consisting of centrifugation at 1200 g for 20 min and re-suspension in wash buffer. Pelleting at 1200 g made it possible to re-suspend the nuclei after each wash without using a paintbrush. After the second wash, low centrifugation at 57 g for 2 min was performed. The supernatant was transferred carefully to a new tube with a plastic transfer pipette and pelleted again at 1200 g for 20 min. The nuclei were then re-suspended, embedded in low-melting-point agarose microbeads and incubated in the lysis buffer for 24 h after Zhang et al. (1995).

BAC library construction

The HMW apple DNA was partially digested by varying ratios of *Eco*RI methylase to *Eco*RI after Bonnema et al. (1996) with modifications. The microbeads were incubated for 1 h on ice in the *Eco*RI/*Eco*RI methylase buffer (100 mM Tris-HCl, pH 8.0, 100 mM

NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 2.6 mM spermidine trichloride, 0.5 mg ml^{-1} bovine serum albumin, and 80 mM S-adenosylmethionine). The enzymes *Eco*RI and *Eco*RI methylase were then added to the buffer at the pre-determined desirable ratio followed by another hour of incubation on ice. The reaction was subsequently incubated at 37°C for 3 h and stopped by adding 1/10 vol of 0.5 M EDTA, pH 8.0. The microbeads were then loaded on a 1% LMP agarose gel in 0.5 × TBE (Sambrook et al. 1989). CHEF gel-electrophoresis was performed with a CHEF DRIII or a CHEF Mapper (BioRad, USA) at 6 V/cm, 11°C and a switch time of 90 s for 20 h. The region of the gel corresponding to the *S. cerevisiae* chromosomes and the lambda ladder between 150 and 600 kb was selected and cut into six slices perpendicular to the direction of migration. When two size-selections had been performed, the slices from the first size-selection were melted at 68°C for 5 min and loaded onto a new 1% LMP agarose gel in 0.5 × TBE. The conditions of the second size-selection with the CHEF system were 4 V/cm, 11°C and a switch time of 5 s for 11 h. The bands of DNA were then excised from the gel and treated with GELase as specified by the manufacturer (Epicentre, USA). Ten microliters of the DNA solution derived from each gel slice were loaded on a 1% agarose gel and the DNA concentration was estimated by comparison with lambda DNA used as a standard.

Ligations were performed by adding the linearized and de-phosporylated pECBAC1 DNA to the size-selected apple DNA at a molar ratio of 5 vector DNA: 1 apple DNA. The mixture was incubated at 60°C for 10 min and then cooled to room temperature. Following this, 10 × ligation buffer and T4 DNA ligase (USB, USA), at a rate of 2 units per 100-µl ligation reaction, were added. The ligation was incubated at 16°C overnight and used immediately for a test transformation. One microliter of ligation mix was mixed with 20 µl of *E. coli* ElectroMAX DH10B cells (BRL Gibco, USA). Transformation was performed using a Cell Porator and Voltage Booster system (BRL Gibco, USA) after Zhang et al. (1996), but at 350 V as suggested by Frijters et al. (1997) instead of 400 V as suggested by the manufacturer. The transformed cells were grown at 37°C in SOC medium (Sambrook et al. 1989) for 50 min for cell recovery, then plated on LB agar containing 12.5 µg ml^{-1} of chloramphenicol, X-gal and IPTG, and grown at 37°C for at least 36 h to allow blue and white color to develop completely.

The average insert size of each ligation mix was determined by analyzing 10–20 individual BAC clones as described by Zhang et al. (1996). The ligation mix chosen for the construction of the library was further characterized by analyzing an additional 59 clones. The recombinant white clones were then individually picked up, put into 384-well microtiter plates and stored at −80°C.

BAC library screening

Six random apple cDNA clones, MC7, MC13, MC16, MC38, MC108 and MC111 (kindly provided by Dr. A.W. Van Heusden, CPRO-DLO, the Netherlands), were used as probes to screen the apple BAC library. Plasmid DNA was extracted by the alkaline-lysis method (Sambrook et al. 1989) and the cDNA inserts were labelled with DIG-dUTP by PCR using the universal primers Sp6 and T7 and the PCR DIG-labeling Kit (Boehringer Mannheim, Germany). The probe AL07 was also labelled with the same kit using sequence-specific primers and the plasmid DNA of the cloned AL07 RAPD band as a template (Tartarini et al., personal communication).

Hybond N+ filters of 12 cm × 8 cm were placed on lids of 96-well microtiter plates containing LB agar and 12.5 µg ml^{-1} of chloramphenicol. A Beckman 2000 Automated Workstation was used to spot the clones of the BAC library onto the filters, each containing a duplication of each colony of four 384-well microtiter plates. The entire library was inoculated onto 24 filters. The filters were incubated at 37°C overnight, and when the colonies reached a size of 2–3 mm in diameter, the filters were processed after Zhang et al. (1996) and baked at 80°C for 2 h. The 24 filters of the library were pre-hybridized at 68°C for 4 h in a plastic box containing 150 ml of hybridization solution [5 × SSC, 1% blocking reagent (Boehringer Mannheim, Germany), 0.1% sodium-lauroylsarcosine, 0.02% SDS, 50 µg ml^{-1} yeast RNA]. Hybridization was then carried out in 150 ml of fresh hybridization solution, containing two cDNA probes at a time, at 68°C overnight. Post-hybridization washes were carried out four times, the first two in 2 × SSC, 0.1% SDS at room temperature for 5 min each and the other two in 0.1 × SSC, 0.1% SDS at 68°C for 15 min each. The detection of hybridization signals was according to the manufacturer (Boehringer Mannheim, Germany). The hybridized filters were autoradiographed for 20 h using Lumi-Film Chemiluminescent Detection Film (Boehringer Mannheim, Germany). Between one screening and the other we stripped the filters, incubating them for 20 min in NaOH 0.2 N and SDS 0.1% at 37°C by shaking. The filters were then washed in 2 × SSC for 10 min and stored in the fridge in fresh 2 × SSC in a box until the next screening.

RFLP analysis of positive clones

Ten microliters of each BAC DNA, prepared from a 5-ml overnight culture after Zhang et al. (1996) and re-suspended in 50 µl of TE, were digested with six units of *Eco*RI in a volume of 20 µl for 4 h. Electrophoresis was carried out at 2 V/cm on a 0.8% agarose gel in 1 × TAE buffer (Sambrook et al. 1989). The DNA was blotted onto positively charged nylon membranes (Boehringer Mannheim, Germany) by capillary blotting using 10 × SSC as a transfer buffer (Sambrook et al. 1989 with modifications). The DNA was fixed on the filters by baking at 120°C for 30 min. Hybridization and detection were performed as described for the colony filters.

Results

Extraction of high-molecular-weight DNA from apple leaf nuclei

The method for extracting HMW DNA from plant nuclei was first described by Zhang et al. (1995). Zhang et al. (1994) adapted this protocol to the extraction of HMW DNA from cotton, a species rich in polyphenols, using a different extraction buffer containing PVP-40 to reduce co-precipitation of the polyphenols with the nuclei. Since apple leaves are also rich in polyphenols, we added PVP-40 to the extraction buffer developed by Zhang et al. (1995) to reduce the content of polyphenolic substances in the apple-leaf homogenate. Centrifugation speeds during washing were decreased compared to those employed in the original protocol to allow re-suspension of the nuclei without using a paintbrush to reduce mechanical shearing. To further purify the nuclei, low-speed centrifugation was performed after the washing steps to precipitate cell debris and intact cells. These precautions yielded a white pellet of nuclei.

After the nuclei were embedded in agarose microbeads and lysed in the lysis buffer, the color of the microbeads changed to light brown, probably due to the oxidation of the remaining polyphenols; this did not seem to have any effect on the digestibility of the DNA. Pulsed-field gel electrophoresis (PFGE) of the

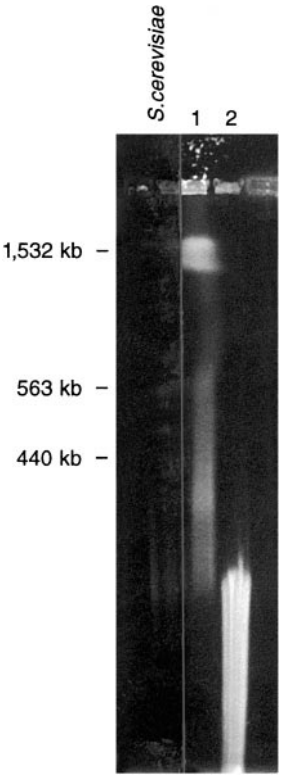

Fig. 1 PFGE analysis of HMW apple (*Malus* × *domestica*) DNA embedded in agarose microbeads. *Lane 1*: *S. cerevisiae*, *lane 2*: apple *DNA*, *lane 3*: *Eco*RI-digested apple DNA. PFGE was performed with a CHEF DRIII (Biorad) under the following conditions: 1% agarose gel in 0.5 × TBE, T = 11°C, V = 6 V/cm, pulse time = 90 s, and run time = 18 h

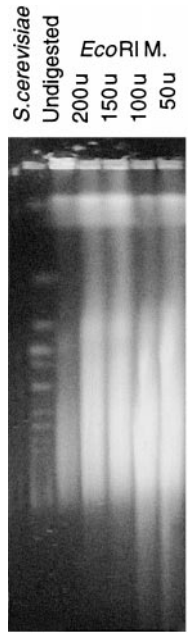

Fig. 2 CHEF-gel analysis of partially digested apple HMW DNA. *Lane 1*: *S. cerevisiae*, *lane 2*: undigested apple DNA, *lanes 3–6*: DNA digested with 4 units of *Eco*RI in the presence of the indicated number of units of *Eco*RI methylase. PFGE was performed with a CHEF DRIII (BioRad, USA) under the following conditions: 1% agarose gel in 0.5 × TBE, T = 11°C, V = 6 V/cm, pulse time = 90 s, and run time = 18 h

undigested and digested DNA showed that most of the extracted DNA is of megabase size and is readily accessible by restriction enzymes (Fig. 1). The apple DNA so extracted is thus suitable for the construction of a BAC library.

Partial digestion of apple HMW DNA

The HMW DNA embedded in the microbeads was partially digested using different ratios of *Eco*RI to *Eco*RI methylase. Partial digestions were performed by fixing *Eco*RI to an amount that completely digested the DNA within 2–3 h and varying the amount of *Eco*RI methylase. A clear shift towards fragments of higher molecular weight was found when increasing the ratio of *Eco*RI methylase to *Eco*RI. By using 4 units of *Eco*RI in the presence of 50–100 units of *Eco*RI methylase most fragments were in the desirable size range from 100 to 400 kb, as verified by PFGE (Fig. 2).

Construction of the apple BAC library

The partially digested apple HMW DNA was subjected to PFGE, and the region of the gel containing fragments in the size range from 150 to 600 kb was excised and cut into six parallel slices, perpendicular to the direction of migration. Half of each slice was used for ligation and the other half was subjected to a second size-selection. After the first size-selection, the smaller fragments which were still trapped in the larger fragments, reduced the average insert size of the clones obtained after ligation to the pECBAC1 cloning vector and transformation into *E. coli*. After the second size-selection, the smaller fragments were significantly removed, and thus the average insert size of the clones was increased (Table 1). However, when a slice was cut from a region that was too low in molecular weight, such as slices 1 and 2, the average insert size of the resulting clones was too small even after the second size-selection. If on the other hand the size-selected

Table 1 DNA concentration, transformation efficiency and average insert size of the clones obtained after ligation of the DNA fragments from different gel regions after first and second size-selection

Slice	Gel region (kb)	DNA ($ng\,\mu l^{-1}$) in the gel	Transformation efficiency ($cfu\,\mu g^{-1}$)[a]	Average insert size (kb)
After first size-selection:				
1	150–280	3	3×10^7	70
2	280–350	3	1×10^7	80
3	350–440	3	6×10^6	95
4	440–500	2	3×10^6	70
5	500–550	2	2×10^6	n.a.[b]
6	550–600	2	1×10^6	n.a.[b]
After second size-selection:				
2	280–350	3	3×10^6	105
3	350–440	5	6×10^5	120
4	440–500	2	n.a.[b]	60
5	500–550	2	n.a.[b]	45

[a] Only recombinant transformants counted
[b] n.a. = not available

fragments were too large, such as in slice 4 and 5, their ligates with the vector could not be efficiently transformed into bacterial cells by electroporation; only the small fragment ligates were transformed into the cells. These results show that only a very restricted gel region enables the construction of a library with acceptable insert sizes. The location of this region is unpredictable because even slight changes in DNA concentration seem to influence its mobility during electrophoresis, so the DNA size marker does not provide exact indication of fragment size. Yet the region can be identified by conducting individual ligations of different gel slices, thereby producing the highest average insert size of BACs. In our case slice 3, which yielded the highest average insert size of BACs, was used for the construction of the apple BAC library.

For transformation we used the electroporation conditions suggested by Frijters et al. (1997) and for the DNA fragment/vector ligates of gel slice 3 achieved an efficiency of 6×10^5 cfu μg^{-1} (approximately 400 recombinant colonies per µl of ligation mix). This high efficiency, along with the high DNA concentration (approximately 5 ng μl^{-1}) made it possible to produce the whole BAC library, which consists of 36 864 clones, from this slice.

Average insert size and insert size distribution of the apple BAC library

To estimate the average insert size and insert-size distribution of the apple BAC library, the DNA of 69 random BACs was isolated and digested with *Not*I, to release the inserts from the vector DNA, and subjected to PFGE (Fig. 3). The results showed that the average insert size of the clones was 120 kb. The insert sizes of these clones ranged from 75 to 190 kb, with approximately 50% of the clones having insert sizes between 100 and 125 kb (Fig. 4).

Representation of the BAC library for the apple genome

By assuming that the apple genome is 750 Mb/1 C (Arumanagathan and Earle 1991) in size and the average insert size of the 36 864 clones of the apple BAC library is 120 kb, the apple genome should be represented in the library by $5.9 \times$ haploid-genome equivalents; i.e. the probability to find any apple gene in the library should be higher than 99% (calculated after Clarke et al. 1976). To corroborate this estimation, the library was screened with six apple cDNA probes by colony hybridization. From 3 to 14 BAC clones were identified for each cDNA. These cDNAs hybridize to two loci in the apple genome (Maliepaard et al. 1998). Therefore, the number of positive clones found for each cDNA has to be divided by two in order to obtain the number of positive clones per locus. On average 8.8 clones were found for each cDNA (Table 2), i.e. 4.4 clones were found per locus.

The clones were confirmed by Southern-blot analysis of the *Eco*RI-digested BAC DNAs using the respective cDNAs as probes (Fig. 5). Two to four different classes of hybridization patterns were observed for each cDNA (Table 2). The number of patterns is probably due to the different grade of polymorphism at the two loci (four patterns, when both loci are polymorphic; three patterns, if only one locus is polymorphic; and two patterns if neither locus is polymorphic).

Screening the library with the cDNA probes thus showed that the apple genome is, in fact, well-represented in the BAC library and that the 99% probability of finding any apple gene in the library can be

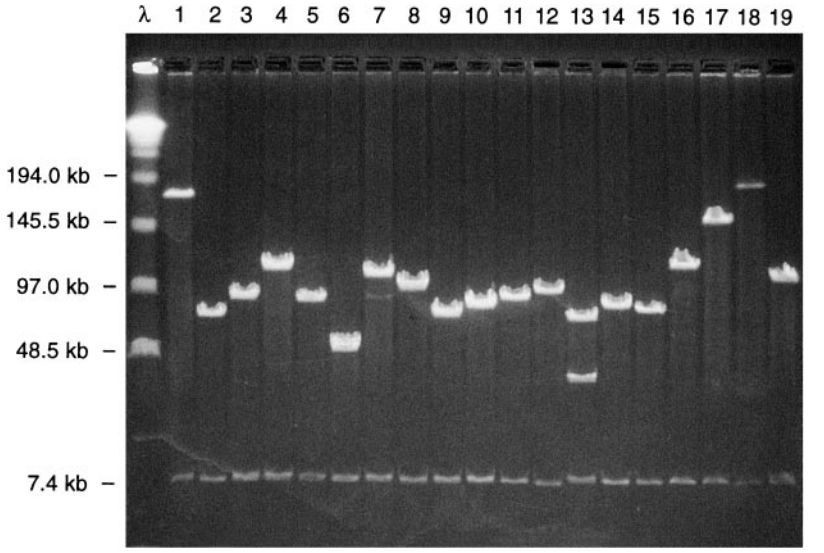

Fig. 3 Analysis of 19 randomly picked clones of the apple BAC library by PFGE. A lambda concatemer (New England Biolabs), as a molecular-weight marker, was loaded in *lane 1*. PFGE was performed with a CHEF DRIII (BioRad, USA) under the following conditions: 1% agarose gel in $0.5 \times$ TBE, T = 11°C, V = 4 V/cm, pulse time = 5–15 s with a linear ramping factor, and run time = 15 h

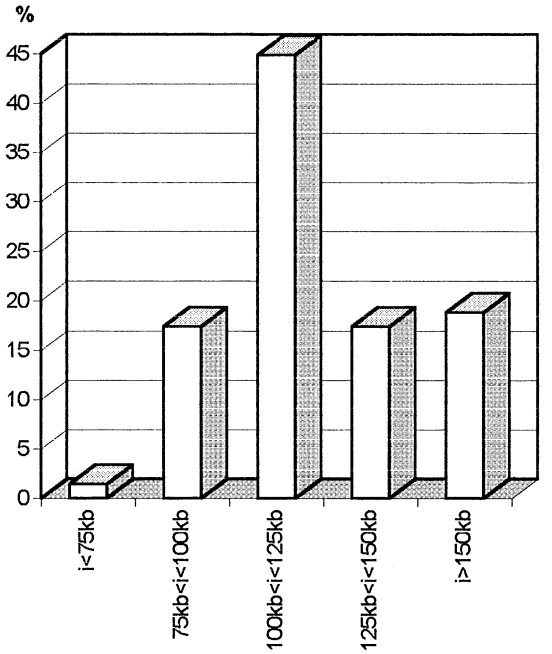

Fig. 4 BAC insert size distribution of the apple BAC library, based on 69 randomly picked clones. The average insert size of these clones is 120 kb

confirmed. Because of our intention to isolate the *Vf* scab resistance gene, the library was also screened with a probe derived from the closely linked AL07 RAPD marker (Tartarini 1996). Six positive clones were identified. PCR analysis of these clones with AL07 SCAR primers (Tartarini et al., personal communication) showed that three of them contain the allele of the marker associated with the resistant allele of the *Vf* gene and the other three contain the allele associated with the susceptible allele of the gene (Table 2). This finding also indicates that the chromosomal region containing the *Vf* gene is represented in the library as expected.

Discussion

Construction of the apple BAC library

The methods described in this report for the construction of the apple BAC library have been proven to be very efficient. Extracting DNA from apple-leaf nuclei was fast, economic, and made it possible to obtain HMW DNA, which was easily accessible to restriction enzymes. Embedding the DNA in agarose microbeads enabled us to handle the HMW DNA as if it were in liquid form and made its partial digestion by restriction enzymes more homogeneous than when embedded in agarose plugs since the ratio of surface to volume of the microbeads is larger than that of agarose plugs. Using varying ratios of *Eco*RI to *Eco*RI methylase for partial digestion made the attainment of DNA fragments of the desired size range between 100 kb and 300 kb easy and repeatable.

The DNA concentration in the microbeads was high compared to that used for other BAC libraries (Zhang et al. 1996; Frijters et al. 1997) and gave rise to a high concentration of DNA in the PFGE gel and in the ligation mix (4 ng/µl). A high DNA concentration during PFG electrophoresis should favor co-migration of DNA fragments of different size and reduce the efficiency of size selection in this way. We were, however, able to efficiently size-select the DNA fragments using two rounds of PFGE, obtaining almost exclusively clones with insert sizes of more than 75 kb in the apple BAC library.

During ligation a high DNA concentration could lead to chimeric clones, as discussed by Frijters et al. (1997). In the first attempts using the apple BAC library for chromosome walking no chimeric clones were found (Patocchi, personal communication). Therefore, we deduce that the pre-occupations over a high DNA concentration during ligation are not well founded.

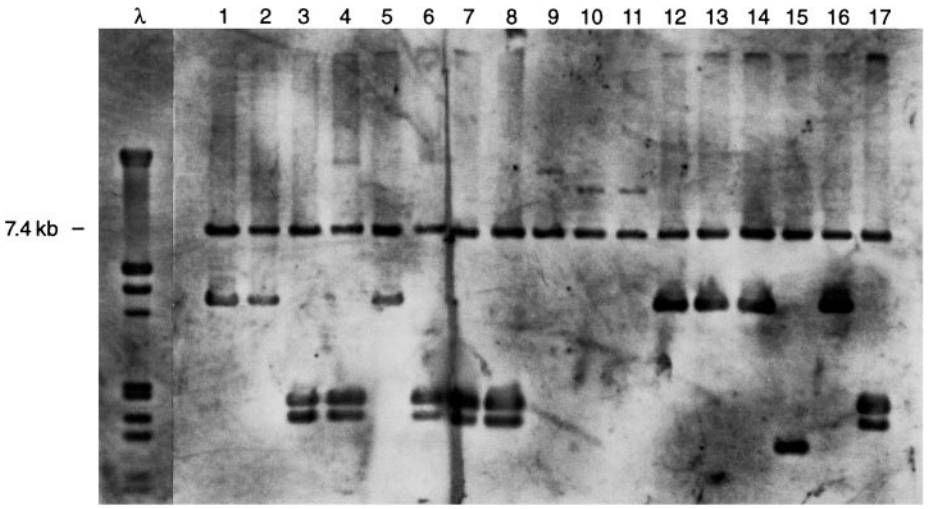

Fig. 5 Autoradiograph of blotted BACs positive for MC13 and MC16 after digestion with *Eco*RI and hybridization with a DIG-dUTP labelled MC16 probe. λ lambda *Eco*RI/*Hin*dIII ladder; *lanes 1–8* and *12–17*: BACs positive for MC16; *lanes 9–11*: BACs positive for MC13. The vector band running at approximately 7 kb can be seen in all clones. The different banding patterns are due to the polymorphism at the two loci of MC16

Table 2 Number and average insert sizes of BACs positive for several probes

Probe	Length in bp of the BAC *Eco*RI fragment to which the probe hybridizes (insert sizes of positive BACs in kb)	No. of positive BACs	Average instert size (kb)
MC 7	2250 (120); 2200 (120, 110, 105, 110); 2150 (95); 1500 (110)	7	110
MC 13	1600 (110); 1400 (130, 140)	3	123
MC 16	4000 (85, 130, 95, 125, 100, 110, 120); 1800 + 1600 (75, 120, 145, 130, 120, 120); 1300 (155)	14	117
MC 38	6200 (95, 95, 110, 95); 6000 (200, 110); 4800 (90, 120, 90, 100, 110)	11	111
MC 108	8000 (75); 7100 (110); 1400 (145, 100, 145, 120)	6	116
MC 111	20000 (140, 110); 18000 (115, 110); 15000 (15000 (150, 110, 110, 110); 10000 (125, 115, 150)	11	122
AL07	Resistant allele (140, 95, 110); susceptible allele (120, 105, 110)	6	113
	Total:	58	–
	Average:	8.8	116

Moreover, a high DNA concentration in the ligation mix allows a significant reduction of the cost of library construction, because of the relatively small number of transformation events necessary. We were able to obtain 36864 recombinant BAC clones, representing approximately $5\times$ apple haploid-genome equivalents, with 92 electroporations. Our conclusion is that the methods described in this report for the construction of the apple BAC library represent the best means of construction, considering the amount of cost and time spent and the attainment of average insert size and genome representation.

Screening of the apple BAC library using the non-radioactive DIG system

Probes labelled with ^{32}P are usually used to screen DNA libraries. We, however, have successfully utilized the non-radioactive chemiluminescent DIG system (Boehringer Mannheim, Germany). This system allowed us to screen the 24 filters containing all colonies of the library at the same time. The time necessary for one screening, from pre-hybrydization to the reading of the positive colonies on the X-ray films, was 2 days and made it possible to perform two screenings a week. We stripped the filters between each screening and were nevertheless able to obtain acceptable results from the same set of filters even after eight screenings. We obtained a good background signal of the grid of the non-positive colonies, which made it easy to identify the position of the positive colonies on the filters. We conclude that the DIG system is a valid alternative to the radioactive screening of BAC libraries.

The suitability of the apple BAC library for map-based cloning

A large-insert genomic library needs to meet the following criteria to be well suited for chromosome walking: a low ratio of chimeric clones, a low ratio of chloroplast DNA clones, a high average insert size and a good genome representation. It has been found that the ratio of chimeric clones in the existing BAC libraries is minimal (Woo et al. 1994; Kim et al. 1996) and also that the ratio of chloroplast DNA clones is very low in plant BAC libraries constructed from plant-leaf nuclei (Zhang et al. 1996). The apple BAC library with its average insert size of 120 kb and its genome representation by approximately $5\times$ haploid-genome equivalents should thus be well suited for chromosome walking.

The library was constructed from the cv "Florina", but its use is not restricted to the cloning of genes present in this variety; it can also be used for cloning genes of other apple varieties. For example, a DNA marker linked to a gene absent in "Florina" can be used as a probe to screen the BAC library and to perform a chromosome walk. The BAC clone containing the locus of the gene can then be employed as a probe to screen a cDNA library of a variety containing the gene allele of interest and then the gene can be isolated. The apple BAC library can therefore be applied to map-based cloning of genes from any apple variety.

Considering the representation of the apple genome in the BAC library, the library should also be useful for the construction of a physical map. The physical map could then be integrated with the existing molecular-linkage maps of apple, creating a powerful tool for future map-based gene cloning in this important perennial fruit species.

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