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TECHNICAL ADVANCE

An improved method of partially digesting plant megabase DNA suitable for YAC cloning: application to the construction of a 5.5 genome equivalent YAC library of tomato

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Summary

An improved method for preparing partially digested tomato DNA has been developed, that is suitable for YAC cloning. It involves (i) isolation of high molecular-weight DNA from agarose-embedded leaf protoplasts, (ii) controlled partial digestion *in situ* using *Eco*RI endonuclease in the presence of *Eco*RI methylase (M. *Eco*RI), and (iii) fractionation of the partial digest on a Clamped Homogeneous Electric Fields (CHEF) gel. Unlike methods commonly used for generating partial digests, the present method allows one to produce digests in which the bulk of restriction fragments are of the desired size. Use of these partial digests in constructing YAC libraries of the tomato lines Moneymaker-*Cf4* and VFNT Cherry resulted in libraries (total 21 060 clones, 5.5 genome equivalents) in which 80% of the YACs have inserts between 200 and 600 kb. Both libraries have been screened with selected RFLP markers linked to the *Cladosporium fulvum Cf4* locus on chromosome 1, using a three-dimensional PCR-based screening technique. To this end, the RFLP markers have been sequenced to allow for the synthesis of specific primers. Thus, for each marker tested several YAC clones have been isolated, including a family of clones that carry leucine-rich repeat sequences located around the *Cf4/Cf9* locus.

Introduction

Isolation and manipulation of DNA that is at least several megabases in size is an essential element in physical mapping and YAC cloning experiments. Usually, high molecular-weight plant DNA is prepared by *in situ* lysis of agarose-embedded leaf protoplasts using proteinase K and an ionic detergent (Cheung and Gale, 1990; van Daelen and Zabel, 1994; van Daelen *et al.*, 1989; Devos and Verkruysse-Dewitte, 1989; Ganal and Tanksley, 1989; Sobral *et al.*, 1990). The agarose matrix protects DNA from shearing and allows entry of enzymes and reaction buffers by diffusion.

For the construction of complete YAC libraries consisting of a set of overlapping clones, the high molecular-weight DNA needs to be partially digested in a controlled manner. Commonly, this is accomplished by incubating the agarose-embedded DNA with limiting amounts of *Eco*RI endonuclease and limiting digestion times (Edwards *et al.*, 1992; Guzman and Ecker, 1988; Imai and Olson, 1990; Klein-Lankhorst *et al.*, 1994; Kleine *et al.*, 1993; Lee *et al.*, 1992; Martin *et al.*, 1992; McCormick *et al.*, 1989; Qin *et al.*, 1993; Riley *et al.*, 1992; Wing *et al.*, 1993). However, because of the relatively low diffusion rate of the enzyme through the mass of DNA within the agarose matrix, the outer DNA molecules become digested almost completely, whereas the interior DNA molecules remain virtually excluded from digestion. As a result, only a small proportion of the DNA molecules is digested to the desired size range.

A more uniform digestion of the target DNA throughout the agarose matrix has been aimed at by using limiting concentrations of the co-factor Mg^{2+} (Albertsen *et al.*, 1989), or by increasing the surface area of the agarose matrix that is in contact with the enzyme incubation mix (Wang and Schwartz, 1993; Wing *et al.*, 1993). The former method indeed resulted in mammalian YAC libraries with high average insert sizes (Albertson *et al.*, 1990; Libert *et al.*, 1993). Even though the latter method resulted in a tomato YAC library with 35% of the clones containing inserts larger than 350 kb, still over 50% of the clones carried inserts smaller than 100 kb (Wing *et al.*, 1993).

An alternative procedure of producing partially digested DNA involves the combined use of *Eco*RI endonuclease and its cognate methylase (M.*Eco*RI), which both act on identical recognition sites (Larin *et al.*, 1991). Competition for the substrate between both enzymes results in partial digestion of the DNA, with the size range depending on the ratio between the endonuclease and the methylase. As

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the reactions run until complete modification of all sites, the size of the restriction fragments is independent of digestion time (Hanish and McClelland, 1990; Hanish *et al.*, 1991; Hoheisel *et al.*, 1989). Unlike the aforementioned methods, controlled digestion by combined incubation with *Eco*RI endonuclease and M.*Eco*RI does generate digests in which a high percentage of the restriction fragments is of the desired size. Use of these digests in YAC cloning resulted in mammalian YAC libraries with very high average insert sizes (620 and 700 kb) and uniform size distributions (Larin *et al.*, 1991). Surprisingly, this effective procedure has not been used as yet in the construction of plant YAC libraries.

In this paper we demonstrate that controlled partial digestion of high molecular-weight tomato DNA embedded in agarose plugs using *Eco*RI endonuclease and its competitor M.*Eco*RI allows the production of digests in which the bulk of the restriction fragments are between 200 and 800 kb. Use of these partial digests for YAC library construction resulted in libraries in which 80% of the clones carried inserts ranging from 200 to 600 kb.

Results

Partial digestion of high molecular-weight tomato DNA

Prior to digestion, the quality of the isolated DNA was checked by Clamped Homogeneous Electric Fields (CHEF) gel electrophoresis using a portion of an agarose plug. When most of the DNA remained in the slot and only a very small amount migrated into the compression zone at approximately 2 Mb, the preparation was considered to be suitable for YAC library construction (Figure 1, lane 3). As noted previously (van Daelen *et al.*, 1989; van Daelen and Zabel, 1994), the quality of the DNA was strictly correlated to the quality of the protoplasts used as source material. Good protoplasts were only isolated from young tomato plants, which had been transplanted twice at most after sowing.

Incubation of the agarose-embedded high molecular-weight DNA with one unit *Eco*RI resulted in complete digestion (Figure 1, lane 1), indicating that all the DNA was accessible to the restriction enzyme and no local impurities were inhibiting the digestion. The amount of M.*Eco*RI needed to partially inhibit the digestion so as to obtain DNA fragments in the range of 200–800 kb was tested by digesting a series of miniplugs with 1 unit of *Eco*RI in the presence of increasing amounts of M.*Eco*RI (60–160 units) and fractionating the digests by CHEF gel electrophoresis. As expected, restriction fragments increased in size with increasing amounts of methylase (Figure 1, lanes 1, 4–8). One of the problems associated with preparing partial digests in the 'traditional' way using limiting amounts of *Eco*RI and limiting digestion times, is that only a small

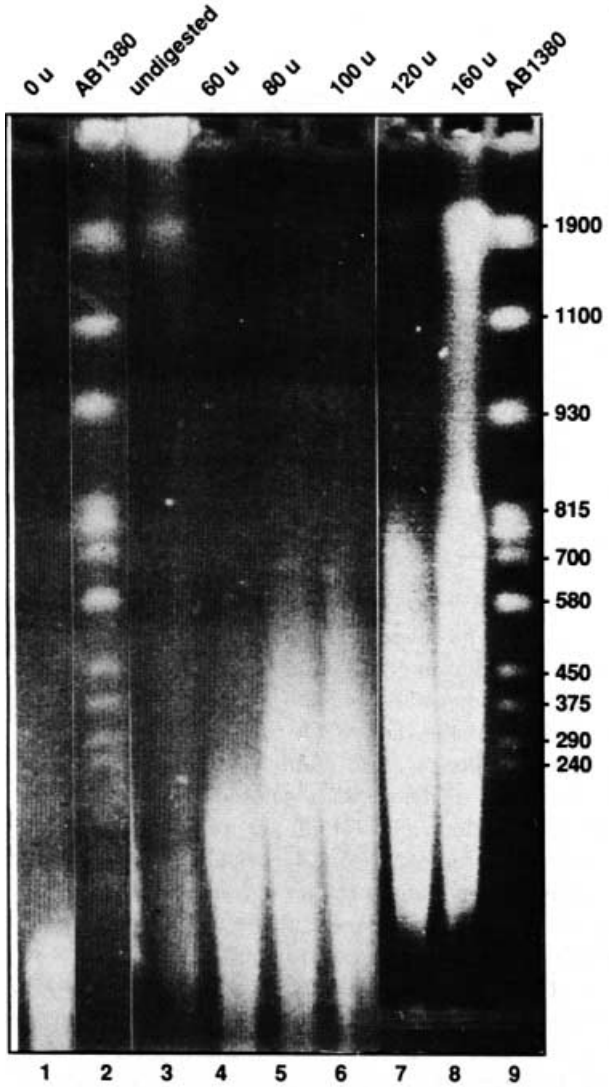

Figure 1. CHEF gel analysis of complete and partial *Eco*RI-digested tomato MM-*Cf4* DNA.
The megabase-sized genomic DNA, embedded in agarose plugs, was digested with 1 unit *Eco*RI in combination with the indicated number of units M.*Eco*RI. Lanes 2 and 9 contain AB1380 yeast chromosomes as size markers, lane 3 contains undigested MM-*Cf4* DNA. A 1% agarose CHEF gel in 0.5×TBE buffer was run at 200 V, 14°C, with a switch time of 60 sec for 15 h, followed by 4 h with a switch time of 90 sec. Sizes are in kb.

portion of the restriction fragments generated falls in the size range desired for YAC cloning (data not shown). In contrast, combined incubation with *Eco*RI and its cognate methylase was highly effective in narrowing the size range of partially digested DNA fragments to the distribution desired for cloning (Figure 1). The combination of 1 unit *Eco*RI and 160 units of M.*Eco*RI yielded most DNA fragments in the range of 200–800 kb and was chosen for constructing the YAC libraries. The size distribution of restriction fragments at the various *Eco*RI/M.*Eco*RI ratios was found to be highly consistent between DNA samples from different isolations (data not shown).

Table 1. Characteristics of the MM-*Cf4* and VFNT Cherry YAC libraries

Characteristic	MM-*Cf4*	VFNT Cherry	Total
Number of clones	8640	12420	21060
Average YAC size[a]	252 kb	240 kb	245 kb
Genome equivalents	2.3	3.2	5.5
% YACs with cpDNA[c]	5	n.t.[b]	–

[a]Average YAC size is based on 109 randomly selected clones for the MM-*Cf4* library and on 45 randomly selected clones for the VFNT library.
[b]Not tested.
[c]YACs hybridizing to the Rubisco Large Subunit chloroplast (cp) clone.

Construction and characterization of the YAC libraries

The partial *Eco*RI digest was size-fractionated on a CHEF gel and restriction fragments larger than 250 kb were collected from the compression zone, ligated to pYAC4 vector DNA and transformed into AB1380 yeast spheroplasts. No additional size fractionation was applied after ligation, as the presence of non-ligated, dephosphorylated vector DNA did not interfere with YAC transformation and contamination of the compression zone with smaller fragments appeared minimal (see below).

Transformation efficiencies ranged from 50 to 1000 clones per plate (50 ng of ligated tomato DNA transformed to 9×10^7 spheroplasts), depending on the quality of the spheroplasts. In control experiments, only 10–100 colonies were obtained after transforming AB1380 spheroplasts with 1 μg dephosphorylated *Eco*RI/*Bam*HI-digested vector pYAC4, while the transformation efficiency of non-digested vector DNA ranged from 10^3 to 10^5 colonies per μg DNA.

At present the two tomato YAC libraries thus prepared together contain 21 060 clones (Table 1). CHEF gel electrophoresis of 109 clones randomly selected from the MM-*Cf4* library showed that 67% carried inserts of 200–350 kb and 13% inserts of 350–600 kb. Only in 20% of the clones were the inserts smaller than 200 kb (Figures 2 and 3). With an average YAC size of 245 kb and a genome size of 9×10^5 kb (Arumuganathan and Earle, 1991), the two libraries thus represent 5.5 tomato genome equivalents. Colony filter hybridization using a Rubisco Large Subunit clone as probe (van Grinsven *et al.*, 1986), showed that only 5% of the YACs contained chloroplast DNA (data not shown).

As for the major applications of the YAC libraries, our interest mainly lies in the construction of an integrated physical and genetic linkage map of chromosome 6 (Weide *et al.*, 1994; van Wordragen *et al.*, 1994) and the isolation of clones corresponding to markers that are tightly linked to the loci conferring resistance to *Cladosporium fulvum* (*Cf4* on chromosome 1; Balint-Kurti *et al.*, 1994) and root-knot nematodes (*Mi* gene on chromosome 6; Ho *et al.*,

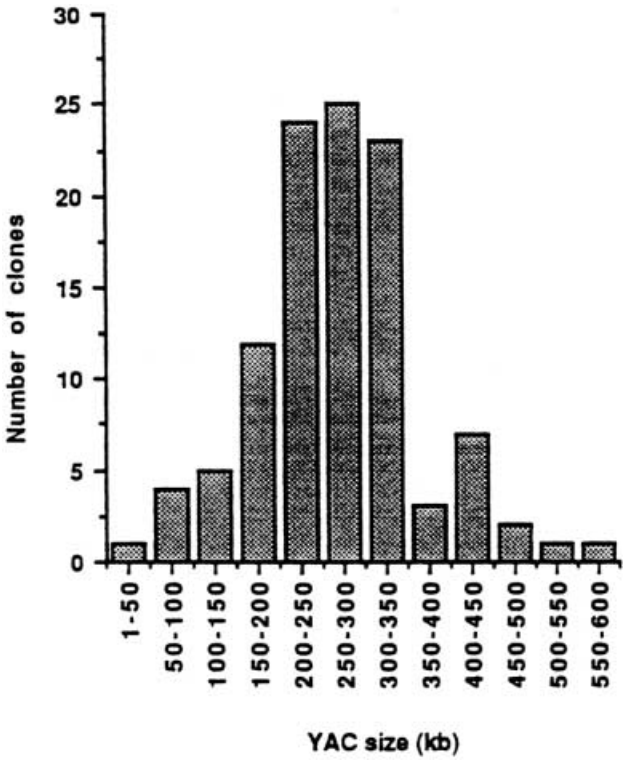

Figure 2. Size distribution of 109 randomly chosen YAC clones from the MM-*Cf4* YAC library.
The calculated mean YAC size is 252 kb, the median is 250 kb.

1992), respectively. We have, therefore, screened both libraries with selected RFLP markers from the respective chromosomal regions, using a three-dimensional PCR screening technique. With that object the markers were sequenced at both ends to allow for the synthesis of specific primer pairs (Table 2). Screening of both libraries with primers corresponding to RFLP markers linked to the *Cf4* resistance gene resulted for each marker in the identification of several YAC clones with insert sizes ranging from 90 to 650 kb (Table 2).

To further evaluate the quality of the libraries, two other features were tested, clone stability and chimerism. As for the former, chromosomal DNA was isolated from five single colonies grown from each YAC clone mentioned in Table 1, separated by CHEF gel electrophoresis, transferred to Genescreen and hybridized to the labelled RFLP probe. In all cases, the five YACs had identical sizes, demonstrating that clone instability is a feature that should be of minor concern in employing the present libraries (data not shown). Besides clone instability, chimerism may seriously affect the efficacy of YAC libraries in chromosome walking. Many of the well-established YAC libraries contain up to 30–50% chimeric YACs. Preliminary evidence obtained from the analysis of 10 individual YAC clones strongly suggests, that the percentage of chimeric YAC clones in the present libraries is rather low. For example, genetic and physical

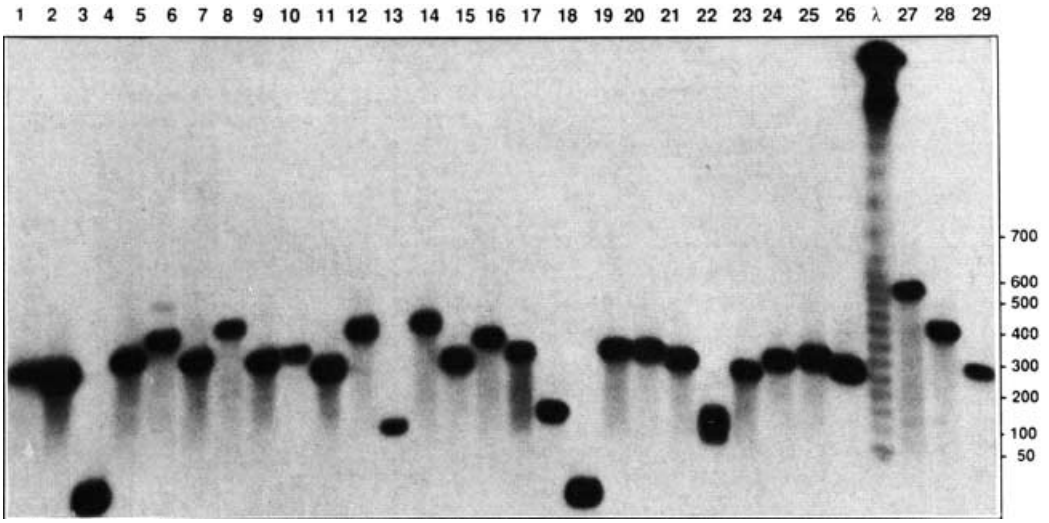

Figure 3. Autoradiogram of a CHEF gel with 29 random YAC clones, hybridized with pBR322 DNA.
Lanes 3 and 18 contain DNA of yeast clones, which do not continue to grow on double selective medium. λ, λ-ladder; sizes are in kb. One per cent CHEF gel in 0.5× TBE buffer was run at 200 V, 10°C, with a switch time of 50 sec for 22 h.

Table 2. Isolation of YAC clones corresponding to RFLP markers linked to the *Cladosporium fulvum* resistance gene *Cf4* on chromosome 1 (the primer sequences used for PCR screening of the YAC libraries, the size of the PCR amplification product and the coordinates of the positive YACs are listed)

RFLP marker	Primer sequence	Length PCR product (bp)	Number of YAC clones screened	Positive clones combined MM*Cf4* (C) and VFNT (V) YAC libraries[a,b]
TG24	F:ATGGAGTATATGAATGGAAAACGG R:GGACTATGGATTATTGGTTTGTTAGTTG	218	17 760	C43e3 (250), V116f2 (200), V116g2 (200)
TG51	F:GGCTAT GAAATCGGTGAGTCAG R:TGAAGGCTAATGTAAGACATGAAG	1500	4320	C40c5 (170)
TG67	F:GAACTTGATGAGCTGTGAGCC R:GTTTATGTTCATGTTTCGCTCTC	1000	17 760	C47f3 (250), V84e11 (250)
TG125	F:TGACTGAGACAGGGGAGCTCCATG R:GAAGGGTGTCCTCCTTTTCTGTAG	2800	6 240	C47a6 (130)
TG184	F: TCTTCATGTGGTAAGTTGCTCTTTG R: CCACTATTCATCTCTTCCAAAGGTC	1155	17 760	V116f2 (200), V116g2 (200)
CT87	F:CAAATTACAGAACCATTCAGTAGGG R:GACTTGGATATGCTTGTGGAGAC	127	17 760	C55b8 (190), C57a8 (250), V45b2 (650), V80g9 (250
CT116	F: AATATCTTCGAGGCCGATTGA R: TAAAAGCCCATGAATGTTGAGG	1700	17 760	C47f3 (250), V84e11 (260)
CT268	F:ATGAAAATGCTCAAATGTTGTTG R:CTTGGATCTTCTGGATTCTACTACC	267	17 760	V32e12 (240), V60h8 (270), V61b4 (90), V77f9 (270)
FT33	F:AGAAGGATAAAGCTCAACTACGG R:TTATTTACGATTTTACAGAAGCTGC	201	20 160	C3b6 (230), VF57c7 (250)
LRR[c]	F: ATCCAATTCCCTAACTGG R: ATAGATCGTGGGACTTTCC	540	20 160	C31c3 (280), C52e12 (260), C59a4 (240), V39d7 (200), V43b9 (240), V49d12 (240), V52e8 (230), V58f10 (240), V100c12 (240)

[a]Clone designation refers to YAC library from which clone was isolated (C, MM-*Cf4* and V, VFNT Cherry), number of the microtitre plate and well number.
[b]YAC sizes are indicated in kb between parentheses.
[c]The LRR primers have been designed on basis of the sequence of the *Cf9* gene (Jones *et al.*, 1994).

mapping of end probes recovered from eight YAC clones selected with PCR primers amplifying a leucine-rich repeat (LRR) sequence derived from the *Cf9* gene (Jones *et al.*, 1994), allowed the construction of three contigs, representing three loci containing LRR sequences. All but one end fragment of five YACs, constituting two contigs, were

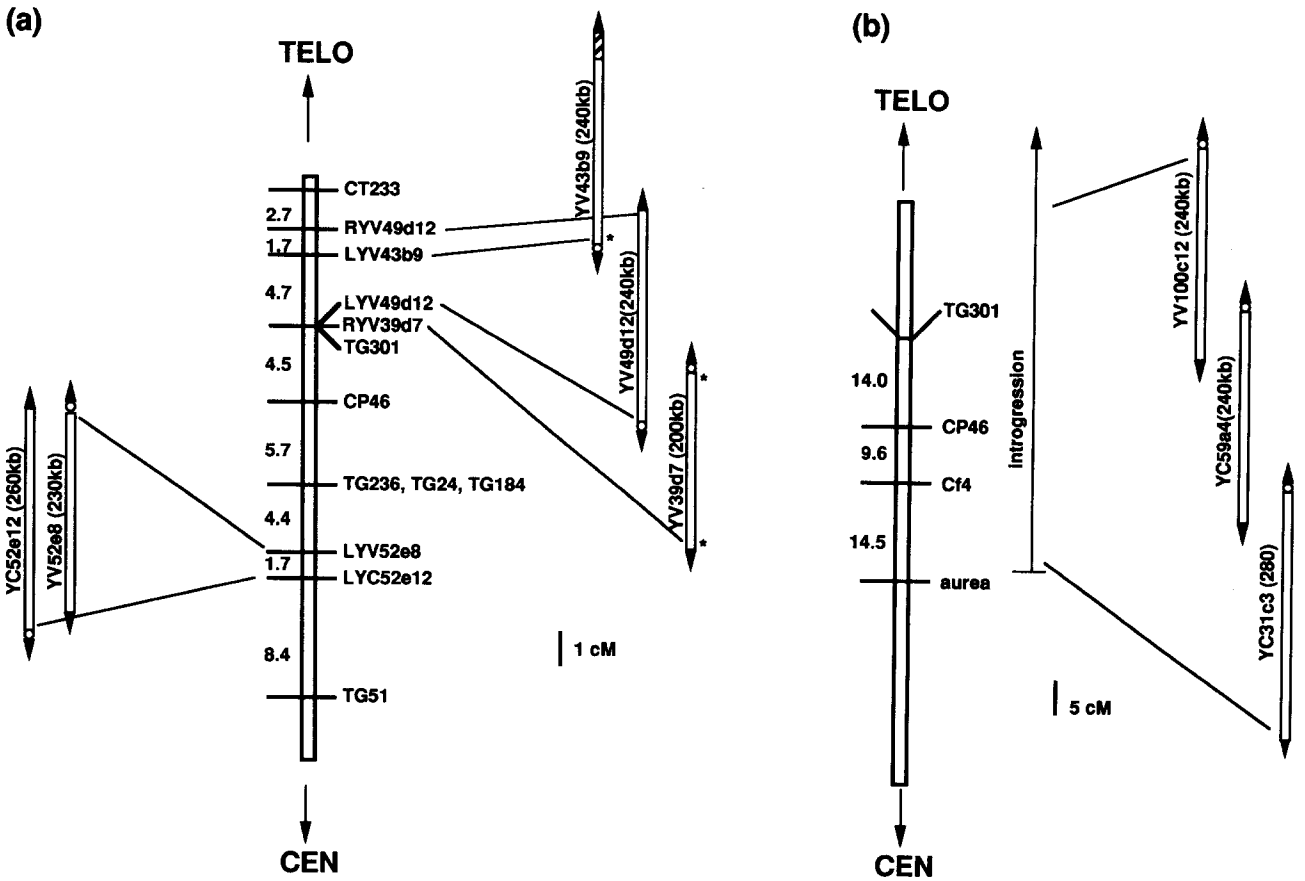

Figure 4. Genetic maps of the top of chromosome 1 showing the map position of a number of YAC end fragments.
In (a), recombination distances are based upon the analysis of 84 *L. esculentum* × *L. pennellii* LA716 F_2 plants (Odinot *et al.*, 1982). For the end fragments with an asterix, the map position was confirmed by analysis of 100 recombinants with cross overs between the marker CP46 and *aurea* selected from a MM-*Cf4*×MM-*aurea* F_2 population (n= 1471). The chimeric right end fragment of YAC V43b9 is shaded.
The map of (b) is based on the analysis of the MM-*Cf4*×MM-*aurea* F_2 population. The region of an *L. hirsutum* DNA introgression, carrying the *Cf4* gene, is indicated (Balint-Kurti *et al.*, 1994). Physical overlap between the YAC clones was determined by restriction mapping. The centomeric (left end) arm of the YACs is indicated with a circle.

located on the short arm of chromosome 1 (Figure 4a). The end fragments from three YACs from a third contig could not be mapped by linkage analysis, due to their repetitive nature. However, Southern analysis of a pair of near-isogenic lines (MM and MM-*Cf4*), that only differ in an introgressed region on chromosome 1 (Balint-Kurti *et al.*, 1994), showed a couple of polymorphic bands, indicative of a map position in this region (Figure 4b). As these three YACs all contain LRR sequences that are characteristic for chromosome 1 (Jones *et al.*, 1994), these data strongly support their non-chimeric nature. From two other YAC clones selected with a chromosome 3 marker linked to the *Alternaria alternata* resistance gene, one was found to be chimeric (Hille, personal communication). Thus, in total, two chimeric clones have been found among 10 clones analysed. Importantly, the observation that the average YAC size corresponds to the average size of the restriction fragments used for cloning, is also in support of the finding that the percentage chimerism is low.

Discussion

In this paper we have shown that partial digestion of high molecular-weight tomato DNA can be controlled effectively by combined incubation with *Eco*RI and its competitor M.*Eco*RI to consistently yield digests in which the bulk of the restriction fragments are within the desired size range. Other groups reporting similar results for agarose-embedded DNA from human and mammalian sources also recognized the reproducibility of the average size of the restriction fragments (Hanish and McClelland, 1990; Hanish *et al.*, 1991; Hoheisel *et al.*, 1989) and the suitability of the digests for producing YAC clones with average inserts as high as 620–700 kb (Larin *et al.*, 1991).

As for plant systems, YAC libraries prepared from DNA that has been partially digested with limiting amounts of a frequently cutting restriction endonuclease, have average insert sizes of approximately 150 kb (*Arabidopsis*: Grill and Somerville, 1991; beet: Klein-Lankhorst *et al.*, 1994; carrot:

Guzman and Ecker, 1988; maize: Edwards *et al.*, 1992; barley: Kleine *et al.*, 1993; tomato: Martin *et al.*, 1992; Wing *et al.*, 1993), except for the *Arabidopsis* YAC library constructed by Ecker (1990), which contains YACs with an average insert of 250 kb. Although some libraries have been reported to contain YAC clones with sizes over 400 kb, small YACs usually account for 50–75% (Kleine *et al.*, 1993; Martin *et al.*, 1992; Wing *et al.*, 1993).

In an attempt to overcome the problem of non-uniform digestion associated with DNA embedded in agarose plugs, Wing *et al.* (1993) recently reported an improved method for preparing partial digests by employing protoplasts encapsulated in agarose microbeads and thus increasing the surface area of the agarose matrix in contact with the restriction enzymes. Indeed, a relatively high proportion (35%) of YACs with inserts larger than 350 kb were obtained. However, 57% of the inserts were still smaller than 100 kbp. The generation of two size classes of YACs was ascribed to contamination of large fragments with smaller fragments in the compression zone during the size selection step by pulsed field gel electrophoresis (Wing *et al.*, 1993). Considering that the present library has been prepared, likewise, from fragments following a single size-fractionation step, but even so contains only 20% clones with inserts smaller than 200 kb, contamination does not seem to be a serious problem.

Apart from the size of the clones, the quality of a YAC library may be best assessed by its potential to provide specific clones corresponding to chromosomal regions of interest. In that respect, the present libraries are fulfilling our needs, as shown by the isolation of almost all clones searched for so far. Given the relatively large insert size and the ease by which clones of interest can be identified using the three-dimensional PCR screening strategy, our YAC libraries will be a valuable supplement to the other tomato YAC libraries currently in use. With a total genetic content of 11.5 genome equivalents, the combined 'Cornell' (Martin *et al.*, 1992; Wing *et al.*, 1993) and 'Wageningen' (this paper) YAC libraries should allow the isolation of virtually any tomato gene of interest. Requests for screening of the presented libraries can be sent to P.Z. and should be attended with a set of PCR primers.

Experimental procedures

Materials

YAC libraries were made in vector pYAC4 (Burke *et al.*, 1988) using the tomato (*Lycopersicon esculentum*) cultivar VFNT cherry and the line Moneymaker-*Cf4* (MM-*Cf4*). MM-*Cf4* is a near-isogenic line of the susceptible tomato cultivar Moneymaker (MM), developed by I. Boukema (Center for Plantbreeding and Reproductive Research (CPRO-DLO), Wageningen) from an F1 (Purdue 135×MM) that was backcrossed five times to the recurrent parent MM and thereafter selfed for three generations (Tichelaar, 1984).

Saccharomyces cerevisiae strain AB1380 (*Mat a, ura3, trp1, ade2-1, can1-100, lys2-1, his5*) was used for transformation. The enzymes *Eco*RI, M.*Eco*RI and T4 DNA ligase (high concentration) were purchased from Life Technologies.

Preparation of high molecular-weight DNA in agarose plugs

Leaf mesophyll protoplasts were isolated from plants grown *in vitro* on MS20 medium and DNA was prepared as described by van Daelen *et al.* (1989). Agarose blocks containing high molecular-weight DNA were stored at 5°C in 0.5 M EDTA, 1% sarkosyl.

Partial Eco*RI digestion of tomato DNA* in situ

The agarose plugs were washed three times for a few hours in a large volume of T10E10 (10 mM Tris, 10 mM EDTA), containing 40 μg ml^{-1} phenylmethylsulphonylfluoride (PMSF) at 5°C to inactivate the proteinase K, followed by another five washes in T10E10 to remove the PMSF. Hereafter, individual plugs (150 μl) were incubated twice in 10 ml 1× *Eco*RI equilibration buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$) at 5°C for 1 h while rotating. The plugs were sliced into five pieces of approximately 30 μl and each miniplug was incubated on ice for 1 h in 1 ml 1× *Eco*RI equilibration buffer. After removal of this buffer, 100 μl restriction digestion buffer was added, containing 100 mM Tris–HCl pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 2.6 mM spermidinetrichloride, 0.5 mg ml^{-1} bovine serum albumin, 80 μM S-adenosylmethionine, 1 unit *Eco*RI per 100 μl and 60–160 units of *Eco*RI methylase per 100 μl. The mini plugs were kept on ice for 1 h and then incubated at 37°C for 4 h. Reactions were terminated by placing the tubes on ice and adding EDTA to a final concentration of 20 mM.

In order to check the quality of the digest, the miniplugs were subjected to CHEF gel electrophoresis (Bio-Rad CHEF DRII). The ratio of *Eco*RI to M.*Eco*RI that resulted in restriction fragments of 200–800 kb was chosen for preparation digests for YAC library construction.

Size fractionation by PFGE

Miniplugs, containing partially digested DNA, were pooled and placed in a large well in a 1% low melting-point agarose gel (Seaplaque^{R}) in 0.5× Tris-borate-EDTA (TBE). DNA digests were subjected to CHEF electrophoresis at 160 V, 10°C, using a swich time of 10 sec for 16 h to compress DNA larger than 250 kb in the region of limiting mobility. Lanes containing molecular-weight markers and the outermost part of the preparative lanes were cut from the gel and stained with ethidium bromide to determine the position of the compression zone. The compression zone was cut out of the gel and stored in a large excess of T10E25 (10 mM Tris pH 8.0, 25 mM EDTA) or dialysed against ligation buffer for direct use. It was estimated that about 50% of the digested DNA was recovered in the compression zone.

Vector DNA preparation

One hundred micrograms of pYAC4 vector were digested to completion with *Bam*HI and *Eco*RI, and the ends were dephosphorylated using calf intestinal phosphatase (Boehringer) at 30°C for 30 min at an enzyme concentration of 0.1 unit μg^{-1} vector

DNA. The phosphatase was inactivated by adding EDTA to a final concentration of 20 mM, heating at 65°C for 10 min and phenol/chloroform extraction. The DNA was precipitated and dissolved in T10E1 (10 mM Tris, 1 mM EDTA) at a concentration of 1 μg μl^{-1}. The integrity of the cohesive ends of the vector was assessed in a ligation reaction in the presence and absence of T4 polynucleotide kinase. Reaction products were assayed by gel electrophoresis and by transformation to yeast spheroplasts. The preparation of vector DNA was only used for library construction when more than 90% of the DNA could be ligated in the presence of kinase and only a few colonies were formed upon transformation of yeast spheroplasts with 100 ng vector DNA, that had been ligated in the absense of kinase.

Ligation reaction

The agar slice containing size-selected tomato DNA was equilibrated on ice three times for at least 1 h in 50 volumes of ligation buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 125 mM NaCl). After removing the buffer and adding pYAC4 vector DNA, the agarose slice was melted for 15 min at 65°C in an eppendorf tube. A 1:1 vector to insert DNA weight ratio was used, which corresponded to a 25 molar excess of vector DNA at an average restriction fragment size of 250 kb. After a 10 min incubation at 37°C, the remaining components of the ligation reaction mixture were added to a final concentration of 1 mM ATP, 1 mM DTT, 0.5 μg BSA μl^{-1}, and five units T4 DNA ligase μg^{-1} insert DNA. The ligase was mixed by gently stirring with a pipette tip. The ligation mixture was kept at 37°C for 4 h, whereafter the agarose mix was pipetted into a precooled plexiglass mould to obtain slot-sized plugs. The solidified plugs were removed from the mould, collected in one volume ligation buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 125 mM NaCl, 1 mM ATP, 1 mM DTT, 0.5 μg BSA μl^{-1}, and 1.5 units T4 DNA ligase μg^{-1} insert DNA), and further incubated overnight at 15°C. The plugs were stored in a large excess of T10E25 (10 mM Tris, 25 mM EDTA), or immediately used for transformation.

Yeast transformation

Agarose plugs containing ligated tomato DNA and equilibrated in ligation buffer, were digested with β-agarase (1 unit per 75 mg plug). Plugs stored in T10E25 were first equilibrated with T10E1 buffer containing 100 mM NaCl, before agarase treatment. Preparation of spheroplasts from yeast strain AB1380 and transformation was essentially as described by Burgers and Percival (1987). For 50 ml cultures of AB1380 cells in YPD, between 400 and 750 units of lyticase were used to convert 80% of the yeast cells within 30 minutes into spheroplasts. For transformation generally 100 μl of spheroplasts (9×10^8 cells ml^{-1}) were mixed with 5 μl of ligation mix (containing about 50 ng of tomato DNA), plated in 7 ml of top agarose (minus uracil) on regeneration plates (minus uracil) and incubated at 30°C (Riley *et al.*, 1992). Colonies started to appear after a 3 day incubation, and were selected after 5 days.

Storage of the YAC library

Red colonies were picked from the top agarose with sterile toothpicks and transferred to plates containing solidified AHC medium lacking uracil and tryptophane (Brownstein *et al.*, 1989). Colonies that continued to grow after a 48 h incubation at 30°C were transferred to 100 μl liquid AHC medium in individual wells of microtitre plates. Two additional copies of the library were made in microtitre plates containing AHC medium. One copy was stored at 4°C as working stock. Colonies of the other two copies of the library were grown for another 24 h after addition of 1/3 volume of three times concentrated YPD medium and then stored at –50°C in 20% glycerol.

Determination of YAC insert sizes

Individual YAC clones were picked and grown in 2 ml AHC medium for 48 h. High molecular-weight DNA was isolated as described by van Daelen *et al.* (1989). The chromosomal DNA was separated by electrophoresis using a CHEF apparatus, transferred to Genescreen+ membranes and hybridized to radioactively labelled pBR322 DNA. To estimate the percentage of clones containing chloroplast DNA, colony filters containing the clones from one microtitre plate were prepared and hybridized to a clone encoding the large subunit of Ribulose-bisphosphate carboxylase (van Grinsven *et al.*, 1986). DNA was labelled with [α^{32}P]dATP using the random hexamer primer method (Feinberg and Vogelstein, 1983)

PCR screening of the libraries

A three-dimensional screening strategy was used (Barillot *et al.*, 1991; Green and Olson, 1990), in which all clones per plate and all rows and columns from 15 plates were pooled. The libraries were screened in two steps. First, PCR was carried out on DNA superpools consisting of the clones from five plates (5×96= 480 colonies). After identification of a superpool, positive for a molecular marker, the five individual plates and pools from rows and columns (20 reactions) were screened to identify the positive clone. Five single colonies of the positive clone were tested by PCR to check the purity of the positive clone and high molecular-weight DNA was isolated, separated on a CHEF gel, transferred to Genescreen+ membranes and hybridized to the molecular marker to determine the size and the stability of the clone.

To grow cells for DNA preparation, the libraries were replicated on to solid AHC medium and after a 48 h incubation at 30°C, transferred to microtitre plates containing 100 μl liquid AHC medium. After overnight incubation (30°C, 180 r.p.m.), 50 μl of three times concentrated YPD medium was added, followed by another overnight incubation (30°C, 180 r.p.m.). From each well 70 μl were used for preparing pools of plates, and 35 μl for pools of rows and pools of columns. Total yeast DNA, to be used for PCR screening, was isolated as described by Hoffmann and Winston (1987) with some minor modifications. After RNAse treatment of the samples, a second chloroform extraction was included to remove traces of phenol. As superpool DNA preparations (480 clones) thus prepared were often found to inhibit the PCR reaction, a further purification step using a Sephadex G50 spin column was included to remove the inhibitors. PCR was carried out in a total volume of 50 μl containing 300 ng superpool DNA or 30 ng DNA of pools of plates and pools of rows and columns, 100 ng of each primer in 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% Triton X-100, 0.2 mM dNTP and 0.5 units Taq DNA polymerase (Supertaq, SphearoQ).

Plasmid DNA of selected RFLP markers (Tanksley *et al.*, 1992) was isolated, purified using Qiaspin columns and the tomato DNA insert was sequenced at both ends using a PRISM Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit on an Applied Biosystems 373 DNA Sequencer (STRETCH). Forward and reverse primers were designed employing the program Primer (version 0.5, Whitehead Institute for Biomedical Research).

Isolation of YAC end fragments

Isolation of left end fragments by plasmid rescue and isolation of left and right end fragments by inverse PCR was essentially as described by Schmidt *et al.* (1992).

iPCR. Yeast DNA (250 ng) was digested with *Hin*cII and *Alu*I and ligated under dilute conditions (250 µl) to promote self-ligation. DNA was linearized by digestion with *Fsp*I for left end fragment isolation and with *Sma*I for right end fragment isolation and amplified by PCR in a total volume of 50 µl as described above, using 40 ng of template. The PCR program was as described by Schmidt *et al.* (1992). For right end fragment isolation the primers C70 and C72 were used, for left end fragment isolation the primers C78 and D72, together with the nested primer set D73 (5'-TCTTCAACAATTAAATACTCTCG-3') and C76 (5'-TTAACTGTGA-TAAACTACCGC-3') (Schmidt *et al.*, 1992). Southern analysis of genomic tomato DNA and YAC clone DNA was performed using iPCR probes from which all vector sequences were removed by digestion with *Eco*RI (cloning site) and *Alu*I or *Hin*cII.

Plasmid rescue for left end fragment. Yeast DNA (600 ng) was digested with *Xho*I or *Nde*I and self-ligated in a volume of 500 µl. The DNA was precipitated and dissolved in 10 µl H_2O. One microlitre of the DNA solution was transformed to electro-competent *Escherichia coli* SURE cells. Positive clones were characterized by digestion of miniprep DNA with *Eco*RI (cloning site) and *Nde*I or *Xho*I, electrophoresis, isolation of the tomato DNA insert from the gel and use of this fragment as a probe on Southern blots of digested tomato DNA or YAC clone DNA.

Genetic mapping of YAC end fragments

YAC end fragments were mapped by genetic linkage analysis using a *L. esculentum* cv. 'Allround' × *L. pennellii* LA716 F_2 population of 84 plants (Odinot *et al.*, 1992) and the mapping program JOINMAP (Stam, 1993). Map positions were further confirmed using 100 F_2 recombinant plants, selected from an F_2 population of 1471 plants of a cross between MM-*Cf4* and MM-*aurea* (a mutant line of MM), on the basis of having cross-overs between the molecular marker CP46 and *aurea* flanking the *Cf4* locus.

Parental DNA was digested with the restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, *Dra*I, *Taq*I, *Xba*I and *Bgl*II, fractionated on a 1% agarose gel, blotted on to Genescreen$^+$ membranes and hybridized with ^{32}P-labelled YAC end fragments.

YAC contig assembly

Undigested and *Mlu*I and *Bss*HII digested yeast DNA of YAC clones containing LRR sequences, homologous to the *Cf9* gene, was separated by CHEF gel electrophoresis, blotted and hybridized with gel-purified ^{32}P-labelled YAC end fragments and YAC vector arms.

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