Plant Molecular Biology Reporter
14 (1) 1996

pages 58–67

Protocol

Rapid and Reliable Screening of a Tomato YAC Library Exclusively Based on PCR

Klaus Pillen, Kevin B. Alpert, James J. Giovannoni, Martin W. Ganal, and Steven D. Tanksley

sdt4@cornell.edu
(KP, KBA, STD) Dept. of Plant Breeding & Biometry, Cornell University, 255 Emerson Hall, Ithaca, NY, 14853, USA; (JJG) Dept. of Horticultural Sciences, Texas A&M University, College Station, TX, 77843, USA; (MWG) Institute of Plant Genetics and Crop Plant Research, Corrensstr. 3, D-06466 Gatersleben, Germany; (KP permanent address: Institute of Agronomy & Plant Breeding, University of Bonn, Karzenburgweg 5, 53115 Bonn, Germany)

Key Words: Tomato, YAC library, PCR screen

Abstract: An improved procedure is presented to select clones from a tomato yeast artificial chromosome (YAC) library. The procedure is based exlcusively on the polymerase chain reaction (PCR). We combined DNA from approximately 36,000 YAC clones in pools containing 96-single YAC clones from one master plate and further in super pools representing 10 master plates. This pooling strategy allows the selection of single YAC clones homologous to a target sequence after three rounds of PCR using super pools, single pools, and single YAC clones as a template. Single YAC clones were spheroplasted prior to the third PCR round in order to omit the conventional radioactive colony hybridization step. To date, we applied this PCR-based selection strategy to isolate clones homologous to ten different sequence-tagged sites (STS) that are linked to genes targeted for map-based cloning. The selection of YAC clones can be readily accomplished within three days. The PCR-based screening strategy is easy to set up and contributes to a further acceleration of the construction of YAC contigs.

The number of YAC libraries has increased steadily since Burke et al. (1987) first demonstrated how to construct a library based on yeast artificial chromosomes. Currently, YAC libraries are available for a variety of plant species, including *Arabidopsis thaliana* (Grill and Sommerville, 1991; Ward and Jen, 1990; Ecker, 1990), tomato (Martin et al., 1992; Nakata et al., 1993), maize (Edwards et al., 1992), barley (Kleine

et al., 1993) and sugar beet (Eyers et al., 1992; Kleine et al., 1995). These libraries provide a powerful means to construct long-range YAC contigs in plant genomes (Putterill et al., 1993; Giovannoni et al., 1995; Schmidt et al., 1995) and are the basis of map-based gene cloning (Martin et al., 1993).

Soon after YAC libraries became available, strategies were developed to screen systematically the large number of YAC clones present in such libraries. Three major approaches have been reported that utilize either colony hybridization (Coulson et al., 1988; Schmidt and Dean, 1993), PCR (Libert et al., 1993) or a combination of both techniques (Green and Olson, 1990) to accomplish the selection of YAC clones.

Small libraries are easily replicated on nylon membranes, and YAC clones can be selected within several days by colony hybridization. If, however, a library includes tens of thousands of clones, as would be needed for large genome species, colony hybridization becomes laborious, expensive, and time-consuming due to the increased effort to hybridize filters and to survey autoradiograms. Furthermore, a low signal-to-noise ratio can result in a high frequency of selected false positives (Green and Olson, 1990).

To screen large YAC libraries, Green and Olson (1990) introduced an improved screening approach that is based partly on PCR. The PCR screen generally utilizes primers derived from STS (Olson et al., 1989) and pooled YAC DNA as a template. Due to its ability to amplify small amounts of template DNA, the PCR-based screening approach allows to assay thousands of YAC clones in one reaction tube and, thus, to screen a complete YAC library in one round of PCR. Since, however, a direct PCR of intact yeast cells seemed to be impractical, the final detection of single, positive YAC clones is still achieved by running a colony hybridization step of YAC clones present in a candidate master plate pool (Green and Olson, 1990).

Our goal was to further facilitate the PCR-based YAC screening procedure by increasing the degree of automation and omitting the final hybridization step. For this, we pooled the YAC clones in two steps and replaced the final hybridization step with a direct PCR screen using spheroplasted yeast cells as DNA template.

Procedures

Tomato YAC library

The tomato YAC library employed was constructed by Martin et al. (1992). To date, this library includes approximately 36,000 clones with an

average insert size of 220 kb (unpublished results). The YAC inserts originate from three different tomato sources (VFNT cherry, Rio Grande PtoR, and tomato accession TA208).

Extraction and pooling of YAC DNA

YAC clones were transferred from master plates onto solidified YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose) using a 96-pin replicator and individually grown at 30 °C (Martin et al., 1992). After two days, single YAC colonies from each master plate were harvested and pooled. Subsequently, yeast cells were spheroplasted in SE+zymolyase (0.9 M sorbitol, 0.1 M EDTA pH 8.0, 0.1 mg / mL zymolyase 100T; ICN-Biomedicals) for 60 min. at 37 °C. Genomic yeast DNA was extracted with 1% SDS in TE, and proteins were precipitated with 0.5 vol. 4 M sodium acetate. After centrifugation (10,000 rpm, 10 min.) the supernatant was transfered into a new tube. The DNA was then precipitated with 1 vol. isopropanol and sedimented (10,000 rpm, 10 min.). After washing with 70% w / w ethanol, the yeast DNA pool was resuspended in 10 mM Tris / HCl pH 8.0 to a final concentration of 5 ng / µL. Sets of ten single pools were further combined to one YAC super pool. Hence, each super pool contains DNA from 960 single YAC clones.

Selection of primers

To identify YAC clones homologous to a target RFLP marker, we first transformed the corresponding single-copy probe into a sequence-tagged site (Olson et al., 1989). For this, we sequenced the ends of the DNA probe and designed suitable PCR primer pairs ranging from 20 to 25 nucleotides in length.

PCR screen to identify marker-specific single YAC clones

A successful screen was accomplished after three PCR rounds (Fig. 1). The first PCR screen included template DNA from 38 super pools representing the complete tomato YAC library. The second PCR screen used template DNA from each single pool present in a positive super pool. The final third screen identified homologous YAC clones after direct PCR of all 96 single YAC clones that are covered in one positive YAC pool. A PCR reaction contained 50 µL of the following PCR mix: 10 mM Tris / HCl pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.02% gelatin; 200 µM each dNTP, 0.1 µM each primer, 1 unit *Taq* DNA polymerase. One µL of super pool or single pool DNA (5 ng) was added to the first and second PCR rounds, respectively. The third PCR round included yeast cells instead of purified yeast DNA. For direct PCR, yeast cells were transfered

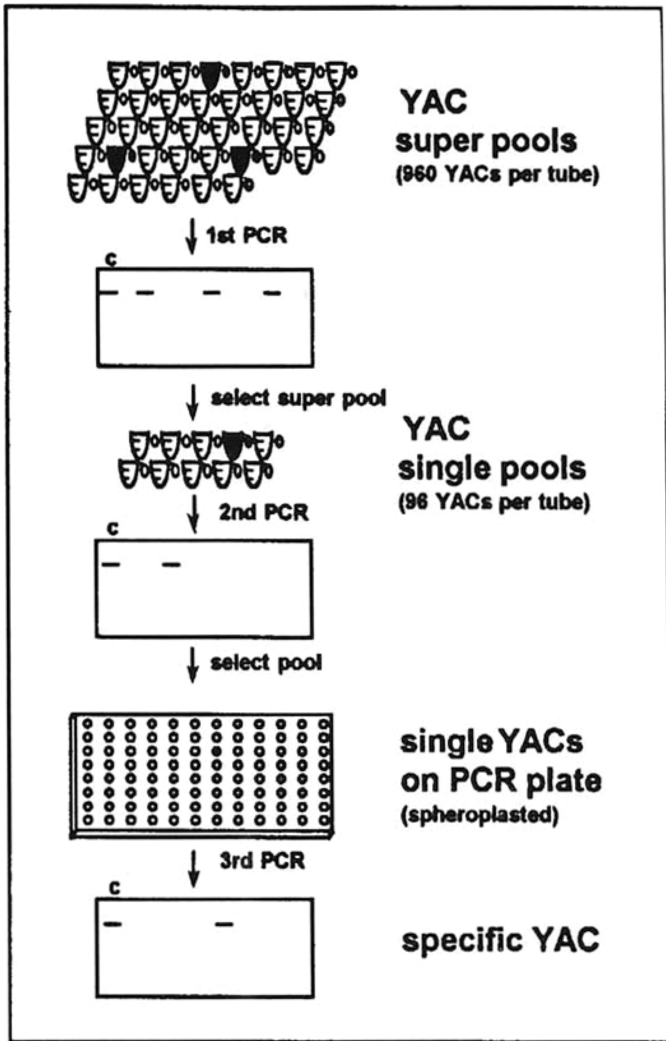

Fig. 1. Strategy of the PCR-based screening of the tomato YAC library. During the first round of PCR, STS primers are applied to template DNA from YAC super pools representing 960 YAC clones. Comparison of tomato control amplification (C) and PCR aliquots from super pools allows the selection of positive super pools after electrophoresis. Then, 10 single pools included in one positive super pool are used for a second round of PCR to identify the corresponding positive single pool. Finally, 96 YACs included in one positive single pool are spheroplasted and, subsequently, applied to a third round of PCR. Electrophoresis of the PCR aliquots detects a single YAC clone specific for the STS primer pair used from each positive single pool.

from a 96-well master plate into a 96-well PCR plate (Costar) containing 25 µL of zymolyase 100T in dH_2O (0.1 mg/mL). After one hour incubation at 37 °C, cell lysis was terminated by placing the PCR plate on ice. Thereafter, 25 µL of 2x concentrated PCR mix (see above) was added. PCR reactions were run in a 96-well thermal cycler (MJ Research) under the following conditions: 4 min. 94 °C; 35 cycles of 1 min. 94 °C, 1 min. 50 or 55 °C, and 2 min. 72 °C and, finally, 5 min. 72 °C. Subsequently, 20 µL of each PCR reaction were electrophoresed in 1.5 % agarose to identify DNA amplifications from either super pool, single pool or single YAC DNA.

Hybridizations

Southern hybridization was used to verify the homology of each selected YAC clone with its specific STS. Purified YAC DNA (Ausubel et al., 1995) and genomic tomato DNA (0.1 and 5.0 µg, respectively) were digested with *Eco*RI, electrophoresed, blotted onto Hybond N^+ membrane (Amersham) and hybridized with STS probes (Bernatzky and Tanksley, 1986). YAC clones were considered specific when the STS probe revealed the same restriction fragment for YAC and tomato control DNA.

Results and Discussion

PCR-based identification of YAC clones homologous to RFLP marker GP125

We applied the PCR-based screening approach to identify YAC clones homologous to various STS. Here, we illustrate the identification procedure by selecting YAC clones specific for the RFLP marker GP125.

After sequencing the ends of the plasmid clone GP125, we designed two oligomers (GP125F: 5' GGCATTAACGAGCACATGATG and GP125R: 5' TTATTCATTTGTGGAGTGCCC), which were then utilized as primers in PCR experiments. PCR with the GP125 primers and either the original plasmid GP125 or one of the three tomato lines included in the YAC library gave rise to the same 1.3-kb PCR fragment. The initial PCR screen with primers GP125F and GP125R included template DNA from 38 YAC super pools. Besides the positive controls, super pools #23 and #32 (lane 1 and 5 in Fig. 2A) gave rise to a 1.3-kb PCR fragment. Subsequently, each subset of 10 YAC pools represented in super pools #23 and #32 were used as templates during the second round of PCR. A GP125 fragment could be amplified in YAC pools #312 (lane 7 in Fig. 2B) and #613. Prior to the final PCR round, yeast replicates of master plates #312 and #613 were spheroplasted with zymolyase. The final PCR

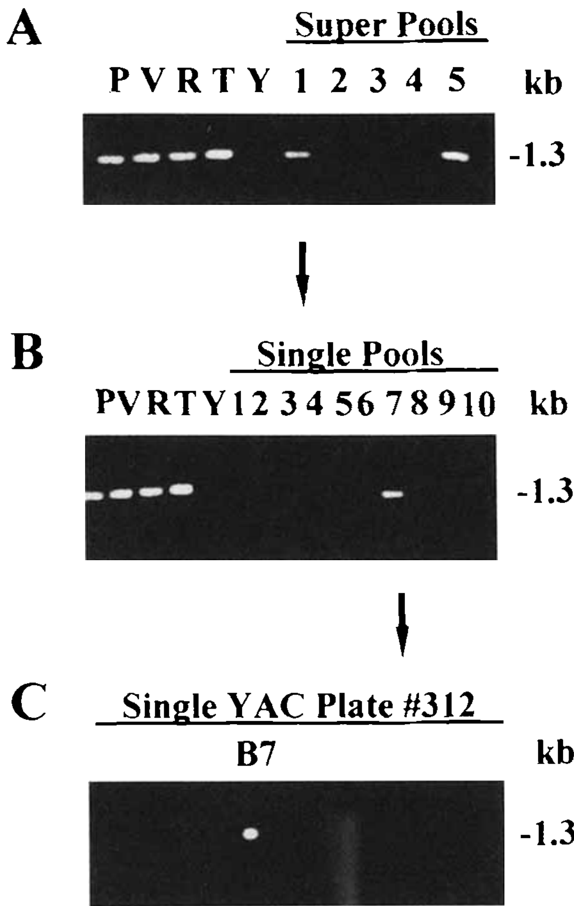

Fig. 2. PCR-based identification of YAC clones homologous to RFLP marker GP125. Panel A, B, and C show PCR amplification of a 1.3-kb fragment using GP125 primers and template DNA from super pools, single pools, and single yeast spheroplasts, respectively. Template DNA from plasmid GP125 (P), VFNT cherry (V), Rio Grande PtoR (R), accession TA209 (T), and yeast strain AB1380 (Y) served as positive and negative controls, respectively. The first PCR screen identified two specific super pools (lane 1 and 5 in panel A). Single pool #312 (lane 7 in panel B) was then recognized as specific among 10 single pools included in super pool 1. Finally, YAC 312.B7 was classified as one specific clone for GP125 after direct PCR of spheroplasted yeast cells from pool #312 (panel C).

identified the homologous YAC clones y312.B7 and y613.D8 (results for plate #312 are shown in Fig. 2C).

To confirm the specificity of the selected YAC clones, *BstNI*-digested DNA from YACs y312.B7 and y613.D8 were hybridized with probe GP125. As expected, both YAC clones revealed the same *BstNI* fragment as the control tomato lines, whereas non-specific YAC clones remained without a signal (Fig. 3). This result proved that the GP125 primers allowed the isolation of two YAC clones that are homologous to the original probe GP125.

The PCR-based screening technique reported here replaces the final colony hybridization step of the Green and Olson (1990) protocol with a direct PCR step of spheroplasted yeast cells. The spheroplasting step prior to PCR proved to be sufficient for reliable detection of a positive yeast clone, whereas PCR of yeast cells frequently resulted in false positives. So far, direct PCR from spheroplasted yeast cells never failed to identify the positive single YAC clone that is present within a positive YAC pool.

We used a 96-well thermal cycler for PCR to increase the number of assays per PCR run. In addition, this thermal cycler allows the direct assay of YACs from one master plate during the third PCR round. With our approach, YAC clones specific for an STS can be obtained readily within three to six days, depending on the number of hits found. The selection of three positive YAC clones, for instance, requires 356 PCR reactions. A 96-well thermal cycler makes it possible to finish this screen after five PCR runs (one super pool, one single pool and three yeast plate runs). If necessary, the pace of PCR-based detection can be increased by simultaneous PCR amplifications of two or more STS. In this case, however, non-interference of each primer pair should be confirmed in test PCR runs with genomic template DNA.

The selection of suitable STS primers for PCR with YAC DNA depends on several criteria. Each oligomer primer should ideally consist of 20 to

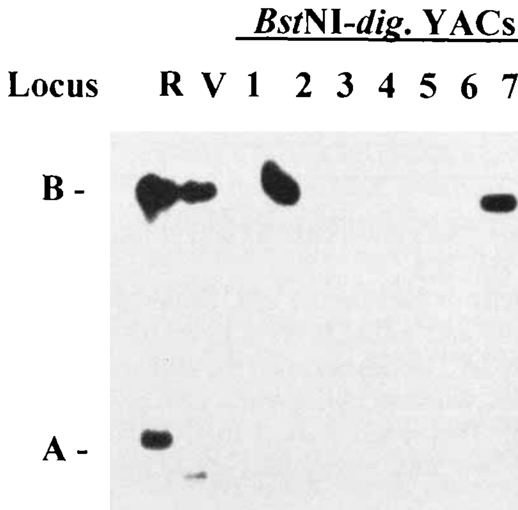

Fig. 3. Autoradiogram of *Bst*NI-digested DNA from selected YAC clones and tomato lines Rio Grande PtoR (R) and VFNT cherry (V) after Southern hybridization with probe GP125. A 12-kb *Bst*NI fragment (B) which is specific for locus GP125B is present in YACs 312.B7 and 615.B3 (lane 2 and 7) but missing in control YACs (lane 1 and 3 to 6). Both specific YACs were selected by PCR-based screening.

25 bp with a minimum GC content of 50 percent in order to ensure a successful PCR run under stringent annealing conditions. Lower GC contents can be compensated by an extension of the oligomer length. Since the 3′ region is the most critical part for primer elongation, and GC bonds are stronger than AT bonds, we generally select oligomers that end with at least one G- or C-nucleotide. Primer sequences should be checked for absence of self-annealing or primer dimer generation that would prevent the *Taq*-polymerase from fragment amplification. For efficient amplification and easy detection in agarose gels, PCR with a primer pair should result in fragments between 150 and 1500 bp in length. However, the selection of STS primers from short sequences—e.g., YAC end-sequences isolated by inverse PCR—can require one to choose a primer pair that is less than 100 bp apart. We had success in selecting specific YACs by electrophoresing an 80-bp PCR fragment in 2 percent agarose after PCR amplification (unpublished results). The selection of suitable primers can be accomplished manually or more readily from comercial software. Whenever possible, we applied the software program MacVector (Eastman/Kodak) for selection of STS primers using the outlined criteria.

Additional PCR screens

Besides the PCR screen described above, we selected YAC clones homologous to several additional STS (Table I). The STS primers are derived from sequence information of classical genomic probes, cDNA probes (Olson et al., 1989), RAPD fragments (Williams et al., 1990) or

Table I. Number of YAC clones selected from 10 independent PCR screens.

Locus	STS type	Chromosome	No. of YACs	Reference
R12	cDNA	9	2	Pillen et al. in prep.
GP125B	genomic	11	2	Pillen et al. unpublished
cos132	genomic	6	4	Pillen et al. unpublished
TG207	genomic	9	4	Pillen et al. in prep.
TG395	genomic	10	6	Giovannoni et al. 1995
OP1	RAPD	2	3	Alpert et al. unpublished
OP608	RAPD	2	3	Alpert et al. unpublished
i144R	inverse PCR	5	4	Giovannoni et al. 1995
i272.RA	inverse PCR	6	2	Pillen et al. unpublished
i617.RA	inverse PCR	9	2	Pillen et al. in prep.

YAC end-sequences (Ochman et al., 1988). The latter type of STS was used to extend existing YAC contigs. We did not observe any significant difference in screening efficiency that is related to the type of STS or the chromosomal region under study. Altogether, 32 YAC clones were selected for 10 STS located on six tomato chromosomes. Thus, the average PCR screen resulted in 3.2 positive YACs. The average YAC insert calculated from all selected YAC clones was 240 kb. This value is significantly higher than early estimates of the average insert length from the YAC library (140 kb). However, the size shift can be explained by improved cloning methods used during the construction of the second half of the library (Martin et al., 1992).

The PCR screening protocol reported here should further facilitate the selection of YAC clones from large libraries, which is a critical step in map-based cloning of target genes (Tanksley et al., 1995). Our PCR screen is particularly advantageous for systematic and repeated screening cycles, which are essential for the construction of long-range genomic YAC contigs by means of chromosome walking (Kere et al., 1992). Currently, we are constructing such YAC contigs for a variety of target genes that are of biological or agronomical relevance.

Acknowledgments: This work was supported in part by grants from the National Research Initiative Cooperative Grants Program, Plant Genome Program USDA (No. 58-1908-5-001), the Binational Agricultural Research and Development Fund (No. US-2427-94) and the U.S. Department of Agriculture grant 91-37300-6565 to SDT and by the International Human Frontier Science Program (HFSP) grant LT-628/93 to KP.

References

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. 1995. Short protocols in molecular biology. 3rd ed. John Wiley & Sons, Inc.

Bernatzky, R. and S.D. Tanksley. 1986. Toward a saturated linkage map in tomato based on isozymes and random complementary DNA sequences. Genetics 112:887-898.

Burke, D.T., G.F. Carle and M.V. Olson. 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236:806-812.

Coulson, A., R. Waterston, J. Kiff, J. Sulston and Y. Kohara. 1988. Genome linking with yeast artificial chromosomes. Nature 335:184-186.

Ecker, J.R. 1990. PFGE and YAC analysis of the *Arabidopsis* genome. Methods 1:186-194.

Edwards, K.J., H. Thompson, D. Edwards, A. De Saizieu, C. Sparks, J.A. Thompson, A.J. Greenland, M. Eyers and W. Schuch. 1992. Construction and characterisation of a yeast artificial chromosome library containing three haploid maize genome equivalents. Plant Mol. Biol. 19:299-308.

Eyers, M., K. Edwards and W. Schuch. 1992 Construction and characterisation of a yeast artificial chromosome library containing two haploid *Beta-vulgaris* L. genome equivalents. Gene 121:195-201.

Giovannoni, J.J., E.N. Noensie, D.M. Ruezinsky, X. Lu, S.L. Tracy, M.W. Ganal, G.B. Martin, K. Pillen, K.B. Alpert and S.D. Tanksley. 1995. Molecular genetic analysis of the *ripening-inhibitor* and *non-ripening* loci of tomato: A first step in genetic map-based cloning of fruit ripening genes. Mol. Gen. Genet. 248:195-206.

Green, E.D. and M.V. Olson. 1990. Systematic screening of yeast artificial-chromosome libraries by use of polymerase chain reaction. Proc. Natl. Acad. Sci. USA 87:1213-1217.

Grill, E. and C. Somerville. 1991. Construction and characterization of a yeast artificial chromosome library of *Arabidopsis* which is suitable for chromosome walking. Mol. Gen. Genet. 226:484-490.

Kere, J., R. Nagaraja, S. Mumm, A. Ciccodicola, M. D'urso and D. Schlessinger. 1992. Mapping human chromosomes by walking with sequence-tagged sites from end fragments of yeast artificial chromosome inserts. Genomics 14:241-248.

Kleine, M., W. Michalek, A. Graner, R.G. Herrmann and C. Jung. 1993. Construction of a barley (*Hordeum vulgare* L.) YAC library and isolation of a *Hor* l-specific clone. Mol. Gen. Genet. 240:265-272.

Kleine, M., D. Cai, C. Eibl, R.G. Herrmann and C. Jung. 1995. Physical mapping and cloning of a translocation in sugar beet (*Beta vulgaris* L.) carrying a gene for nematode (*Heterodera schachtii*) resistance from *B. procumbens*. Theor. Appl. Genet. 90:399-406.

Libert, F., A. Lefort, R. Okimoto, J. Womack and M. Georges. 1993. Construction of a bovine genomic library of large yeast artificial chromosome clones. Genomics 18:270-276.

Martin, G.B., M.W. Ganal and S.D. Tanksley. 1992 Construction of a yeast artificial chromosome library of tomato and identification of cloned segments linked to two disease resistance loci. Mol. Gen. Genet. 233:25-32.

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

Nakata, K., H. Tanaka, T. Ito, N. Sasagawa, I.K. Chung, A. Ohta and M. Takagi. 1993. Construction and some characterization of a yeast artificial chromosome library from DNA of a tomato line having four disease resistance traits. Biosci. Biotech. Biochem. 57:1790-1792.

Ochman, H., A.S. Gerber and D.L. Hartl. 1988. Genetic applications of an inverse polymerase chain reaction. Genetics 120:621-624.

Olson, M., L. Hood, C. Cantor and D. Botstein. 1989. A common language for physical mapping of the human genome. Science 245:1434-1435.

Putterill, J., F. Robson, K. Lee and G. Coupland. 1993. Chromosome walking with YAC clones in *Arabidopsis*. Isolation of 1700 kb of contiguous DNA on chromosome 5 including a 300 kb region containing the flowering-time gene *co*. Mol. Gen. Genet. 239:145-157.

Schmidt, R. and C. Dean. 1993. Towards construction of an overlapping YAC library of the *Arabidopsis thaliana* genome. Bioessays 15:63-69.

Schmidt, R., J. West, K. Love, Z. Lenehan, C. Lister, H. Thompson, D. Bouchez and C. Dean. 1995. Physical map and organization of *Arabidopsis thaliana* chromosome 4. Science 270:480-483.

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

Ward, E.R. and G.C. Jen. 1990. Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly-sheared *Arabidopsis thaliana* DNA. Plant Mol. Biol. 14:561-568.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitray primers are useful as genetic markers. Nucl. Acids Res. 18:6531-6535.