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RAPD-based screening of genomic libraries for positional cloning

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ABSTRACT

RAPD markers are frequently used for positional cloning. However, RAPD markers often contain repeated sequences which prevent genomic library screening by hybridisation. We have developed a simple RAPD analysis of genomic libraries based on the identification of cosmid pools and clones amplifying the RAPD marker of interest. Our method does not require the cloning or characterisation of the RAPD marker as it relies on the analysis of cosmid pools or clones using a simple RAPD protocol. We applied this strategy using four RAPD markers composed of single copy or repeated sequences linked to avirulence genes of the rice blast fungus *Magnaporthe grisea*. Cosmids containing these RAPD markers were easily and rapidly identified allowing the construction of physical contigs at these loci.

RAPD markers (Random Amplified Polymorphic DNA; 1) are widely used as starting molecular markers for positional cloning of a gene of interest (2). However RAPD markers frequently contain repeated sequences (3,6) that prevent the screening of genomic libraries by hybridisation. To identify single copy sequences within such RAPD markers, a large number of subclones must be analysed individually by Southern hybridisation using genomic DNA. Such a strategy might be time consuming or unsuccessful. RAPD markers can also be cloned and sequenced. Oligonucleotides (20–24 bp) complementary to ends of the RAPD marker are designed to specifically amplify a fragment of this locus (SCAR; 4). Pools of cosmids, BACs or YACs from genomic libraries are screened by PCR using SCAR oligonucleotides (5). Although this strategy can be successful, the molecular characterisation of RAPD markers is time consuming.

We present here a rapid and efficient strategy to screen a genomic library for clones bearing a RAPD marker. First, we performed a RAPD analysis of cosmid pools from a genomic library. Second, the RAPD marker of interest was used as a probe on colony filters of cosmid pools amplifying the RAPD marker. Third, each cosmid clone hybridising to the RAPD marker was analysed for amplification of the marker using a RAPD protocol. The efficiency of this strategy was evaluated with RAPD markers composed of single copy or repeated sequences.

Four RAPD markers linked to avirulence genes of the rice blast fungus *Magnaporthe grisea* were identified by bulk segregant analysis (6). OPE-Y13 (1.3 kb) was linked to avirulence gene *Avr*Irat7-1. OPE-D16 (0.3 kb) and OPE-M18 (1.2 kb) were linked to avirulence gene *Avr*Mednoï-1. OPE-S9 (0.9 kb) was linked to avirulence gene *Avr*Ku86-1. OPE-S9 and OPE-M18 were shown to contain repeated sequences, while OPE-D16 and OPE-Y13 contained only single copy sequences. We constructed two *M.grisea* genomic libraries, one with 4100 clones (four times the genome size) using isolate 96/0/76 and cosmid vector pMOcosX and the other with 2880 clones using isolate Guy11 and vector pHC79. The 96 cosmids from each microtitration plate were pooled for growth and their DNA extracted (7). RAPD analysis of cosmid pools and clones were performed with a PTC100 MJ-Research PCR apparatus with standard conditions: one cycle of 2 min 30 s at 95°C and 45 cycles of 1 min at 95°C, 1 min 30 s at 37°C, 1 min 30 s at 72°C followed by one cycle of 15 min at 72°C. Four Operon primers were used: S9 (CCTGGTCCCC), M18 (CACCATCCGT), D16 (AGGGCGTAAG) and Y13 (GGGTCTCGGT) at 1 µM with 50 ng of DNA, 2.5 µl of 10× buffer (Appligene: $MgCl_2$ at 1.5 mM final), 0.2 mM dNTP (Eurobio), 1 U Taq polymerase (Appligene) and autoclaved water up to 25 µl. Amplification products were separated by gel electrophoresis on 1% agarose gel in TAE at 5 V/cm for 3 h. For hybridisation, RAPD markers were separated by gel electrophoresis, extracted from the gel by glassmilk purification (Jetsorb, Bioprobe) and labelled with α-^{32}P (Amersham) by random priming (Pharmacia). Hybridisation was performed at 65°C overnight in buffer 1 (6× SSC, 0.5% SDS, 5× Denhardt's) with two washes at 65°C with buffer 2 (0.1× SSC, 0.2% SDS) for 1 h.

RAPD analysis of cosmid pools from 96/0/76 genomic library with Operon primer Y13 showed that two pools (D31 and D42, Table 1) amplified a marker similar in size to OPE-Y13 (1.3 kb). Colony filters corresponding to these positive pools were probed with OPE-Y13. We did not detect hybridisation signals in pool D42, while a strong signal was detected in pool D31 (cosmid D31C12, Table 1). A marker similar in size to OPE-Y13 was amplified from cosmid D31C12. The screening of the whole 96/0/76 cosmid library by hybridisation to the single copy marker OPE-Y13 led to the detection of the same cosmid. RAPD analysis of cosmid pools from 96/0/76 library with Operon primer D16 revealed six pools (D11, D12, D27, D28, D35 and D37; Table 1) amplifying a marker similar in size to OPE-D16 (0.3 kb). Colony filters corresponding to these positive pools were probed with OPE-D16. One cosmid from each pool strongly hybridised to OPE-D16 (Table 1). A RAPD marker

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Table 1. Cosmid pools and clones containing RAPD markers linked to *M.grisea* avirulence genes

RAPD	Type of sequence	Cosmid pools amplifying the RAPD	Cosmids hybridizing with RAPD[a]	Cosmids amplifying the RAPD[a]
OPE-Y13	Single copy	D31, D42	D31C12	D31C12
(1.3 kb)				
OPE-D16	Single copy	D11, D12, D27	D11E6, D12H4, D27C9	D11E6, D12H4, D27C9
(0.3 kb)		D28, D36, D37	D28E4, D36E5, D37B2	D28E4, D36E5, D37B2
OPE-S9	Repeated sequence	G14, G18, G21	G14F11, G18E6, G18F9	G27A2
(0.9 kb)		G27, G30	G21A1, G21A3, G21C6	
			G21D1, G27A2, G30B3	

[a]The first letter refers to the cosmid library: D for 96/0/76 and G for Guy 11. The first number refers to the microtitration plate number. The second letter and the second number refer to the row and column numbers of the microtitration plate.

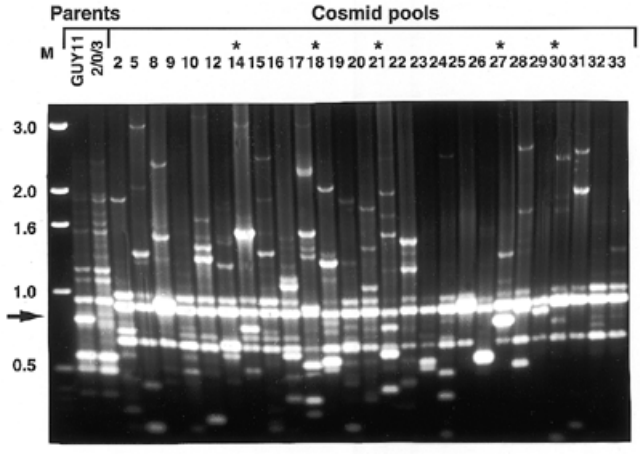

Figure 1. RAPD-based screening of cosmid pools from Guy11 genomic library with Operon primer S9. RAPD markers were amplified from DNA extracted from cosmid pools. Amplified products were separated by agarose gel electrophoresis. An asterisk indicates a cosmid pool amplifying a RAPD marker similar in size to OPE-S9 (0.9 kb) pointed out by an arrow. M, molecular weight markers.

similar in size to OPE-D16 was amplified from each of these cosmids. When the whole cosmid library was hybridised to the single copy RAPD marker OPE-D16, we only detected the six cosmids already identified by our RAPD-based screening.

This strategy was applied to RAPD markers containing repeated sequences. RAPD analysis of cosmid pools from Guy11 genomic library with Operon primer S9 showed that five pools (G14, G18, G21, G27 and G30; Table 1) amplified a fragment similar in size to OPE-S9 (0.9 kb; Fig. 1). Colony filters corresponding to the five positive pools of 96 cosmids were hybridised to OPE-S9 revealing nine strong hybridisation signals (2% of tested clones). Hybridisation of a repeated and dispersed sequence such as OPE-S9 to cosmid filters is expected to reveal clones containing homologous sequences from other loci. RAPD analysis of these nine positive cosmids showed that one clone from pool G27 (G27A2; Table 1) amplified a RAPD marker similar in size to OPE-S9. Identification of cosmids bearing OPE-M18 was unsuccessful. With Operon primer M18, we amplified a marker similar in size to OPE-M18 (1.2 kb) from all cosmid pools from 96/0/76 genomic library and from the cosmid vector alone. Hybridisation of OPE-M18 to RAPD amplification products obtained from cosmids pools did not reveal positive hybridisation signals. Independently, we detected cosmids containing OPE-M18 when analysing the contig constructed from OPE-D16. Such results could be explained by an inhibition of OPE-M18 amplification in the presence of the vector. The occurrence of false positive RAPD markers during the analysis of cosmid pools was also observed with other primers. Five cosmid pools amplified a fragment similar in size to OPE-S9 (0.9 kb), but only the RAPD amplified from pool G27 hybridised to OPE-S9 probe (Table 1). Cosmid pool D42 amplified a fragment similar in size to OPE-Y13 that did not hybridise to OPE-Y13 (Table 1). We assumed that some cosmid pools amplified RAPD markers similar in size to OPE-S9 or OPE-Y13 without any sequence homology to these markers. Such co-migrating RAPD products could be differentiated from the marker of interest by hybridisation of this marker to the set of RAPD products produced from the cosmid pools. This additional screening allowed the rapid selection of the right cosmid pools.

RAPD-based screening of genomic libraries is very efficient since we detected all the cosmids containing the RAPD marker of interest in our genomic library even when only one positive clone was present (cosmid D31C12; Table 1). RAPD marker amplification from cosmid pools and clones was highly reproducible in all the assays performed (at least three times). Cosmids containing a RAPD marker of interest were identified in less than a week and analysed by hybridisation to localise the marker on a restriction fragment (data not shown). The main advantage of this strategy is that it does not require the molecular characterisation of the marker and could be applied to RAPD markers containing either single copy or repeated sequences.

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