Amplification of Long Targets of Approximately 50 kb from Cloned Cosmid Inserts of Arabidopsis thaliana

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(Received 26 March 1996)

Since the robust amplification of targets greater than approximately 5 kb has been reported,1 the long polymerase chain reaction (PCR) method has become a labor-saving alternative cloning method in the analysis of larger genomic segments.2~4 This method has been used to amplify up to 22 kb of human genomic DNA,2 42 kb from the genome of λ bacteriophage,2 and 16.3 kb of the 16.6-kb human mitochondrial genome.3 No reports on the amplification of long targets from cloned cosmid inserts as templates have been published, however. In the present study, we report the amplification of long targets up to approximately 50 kb from cloned cosmid inserts of Arabidopsis thaliana.

Five cosmid clones that constitute a contig near the abt1 locus on chromosome 4 of A. thaliana,5,6 were used as templates for the amplification of inserted DNA fragments. Escherichia coli cells containing cosmid clones were grown for 16 h at 37°C while being shaken at 200 rpm in super broth (SB) medium supplemented with 50 μg/ml ampicillin. Cosmid DNA templates were then prepared using the Automatic Plasmid Isolation System (Model PI-100, KURABO, Osaka, Japan) and purified using the Qiagen Plasmid Mini Kit (Qiagen Inc., CA, USA) according to the manufacturer’s instructions. The amplification of inserts was performed using the Takara LA PCR Kit Ver. 2 (Takara Shuzo Co., Kyoto, Japan) in a THERMAL SEQUENCER TSR-300 (IWAKI GLASS Co., Ltd., Chiba, Japan). The reaction mixture (50 μl) consisted of 20 ng of cosmid DNA, 1 x LA PCR buffer II (Mg2+ plus), 400 mM dNTP, 0.2 μM of each primer, and 1.5 units of Takara LA Taq. Primers were chosen from the β-lactamase gene and the 5' flanking region of the 70 K heat-shock gene; regions flanking the cloning sites of the cosPneo cosmid vector.7 Primer sequences were 5’-CGGGGCGAAAACTCTCAAGGATCTTACCGC-3’ (cosPneoF4) and 5’-CAGAGTGAGAGAGCATTAGTG-CAGAGGGG-3’ (cosPneoR1). Thermal cycling profiles consisted of 30 cycles of denaturation at 99°C for 45 s and annealing and extension at 68°C for 30 min. All the runs included an initial denaturation at 99°C for 60 s and a final hold at 72°C for 10 min. Amplified PCR products were separated on 1% agarose gels (NA, Pharmacia, Uppsala, Sweden) by clamped homogeneous electric-field (CHEF) gel electrophoresis with CHEF DRII (Bio-Rad, Tokyo, Japan) at 200 V for 12 h with switching times ramped from 1 to 5 s. The long targets up to approximately 50 kb were amplified from five cosmid DNA templates (cosE4-6, cosE4-4, cosH7-47, cosH7-26 and cosH7-48; Fig. 1A).

To verify that the amplified fragments were derived from the cosmid templates, amplified PCR products and cosmid DNA treated with A-Terminase were analysed by agarose gel electrophoresis. In all the cosmid templates analysed, PCR products were about 9 kb smaller than the cosmid DNA, the size of which corresponds to that of the cosPneo cosmid vector7 (Fig. 1A). Furthermore, the digestion pattern of the fragment amplified from the cosH7-47 cosmid DNA with EcoBI and SalI was compared with that of the cosH7-47 cosmid DNA (Fig. 1B). The digestion patterns were identical, with the exception of a band (black arrowhead in Fig. 2) derived from the cosPneo cosmid vector and a band (white arrowhead in Fig. 1B) corresponding to the region between the primer and the recognition site of the restriction enzyme in the amplified fragment. Similar results were obtained with another cosmid DNA (data not shown). These results indicate that long PCR faithfully amplified the target sequences from the cosmid DNA templates of A. thaliana.

We found that freshly purified cosmid template was more effective for the amplification of inserts than the cosmid templates stored at 4°C for 1 month or longer, and that 1.5 units of Takara LA Taq was more effective than 2.0 or 2.5 units. Furthermore, we found that 20 ng of cosmid template gave better results than 10 ng or less. These results indicate that long PCR faithfully amplified the target sequences from the cosmid DNA templates of A. thaliana.

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It has been reported that target DNA of up to 35 kb can be amplified routinely.4 Successful amplification of targets of up to 50 kb may have been possible because of the PCR conditions used, including primers, amount

Communicated by Mituru Takanami
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**Figure 1.** A. Amplification of long targets from the cosmid templates. Five cosmid templates (cosE4-6, cosE4-4, cosH7-47, cosH7-26 and cosH7-48) were used for amplification of target DNA. High Molecular Weight DNA Marker from GIBCO-BRL (Gaithersburg, MD) (lane 1); long target fragments amplified from cosmid DNA (L; lanes 2, 4, 6, 8 and 10); cosmid DNA treated with λ-Terminase (C; lanes 3, 5, 7, 9 and 11). Samples were electrophoresed on 1% agarose gel in 0.5 X Tris-borate (TBE) buffer by CHEF gel electrophoresis. B. Pattern of restriction enzyme digestion of cosmid DNA (cosH7-47) and the fragment amplified from the cosmid DNA. Cosmid DNA (lane C) and the fragment amplified from the cosmid DNA (lane L) were digested with EcoRI and SalI and electrophoresed on a 0.7% agarose gel in TBE buffer. The black arrowhead indicates a DNA fragment derived from the cosPneo cosmid vector. The white arrowhead indicates a DNA fragment derived from a region between the position of the primer and the recognition site of SalI in the amplified fragment. Lane M represents λ DNA digested with HindIII.

of template and enzyme, and the ratio of template to enzyme. Furthermore, *A. thaliana* DNA may be a good template for long PCR.

In vitro amplification of long targets by the long PCR technique is useful because it avoids the DNA rearrangement and gene toxicity problems of in vivo cloning, and will probably be useful in the management of long genomic DNAs that are cloned in cosmid vectors.

**Acknowledgements:** We thank Drs. Jérôme Giraudat and Jeffrey Leung for providing the cosmid subclones. This work was supported by a grant for Genome Research from RIKEN. M.S. was supported by a fellowship from the Science and Technology Agency of Japan.

**References**