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Microamplification of specific chromosome sequences; an improved method for genome analysis

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ABSTRACT

An improved method was developed for microdissection and cloning of metaphase as well as pachytene chromosomes. The protocol incorporates efficient ligation of chromosomal DNA with linker adaptors, abolishment of microcloning steps and the reduction of micromanipulation. The threshold for amplifying genomic DNA template was in the range of 2–20 femtogram. The amplification products had a size distribution between 200 and 1300 bp (average 500 bp). Using pachytene chromosomes of maize the selectivity for segment-specific libraries can be increased between 10- and 20-fold. The approach described here is being applied to the fine mapping and isolation of genes conveying resistance against plant pathogens.

Generating molecular probes is one of the crucial starting points in genome research. Defining chromosome regions is a prerequisite for the analysis of genetic linkage and physical mapping, and in the search for genes coding for important phenotypes. Consequently, a chromosome microcloning technology was developed in 1981 (1). Subsequently, it has evolved into an efficient tool for generating chromosome segment-specific DNA libraries of many species (2–4). Modifications including the use of laser light (5,6) and the introduction of different PCR-based techniques (7–10) simplify the strategy. Although the approaches were straightforward, the need for micromanipulation as well as handling very small volumes have hampered the development of routine techniques. Here we introduce a simple and efficient adaptor–PCR-mediated method for obtaining analytical amounts of DNA from specific chromosomal regions, especially from higher plants.

Chromosome spreads were manipulated under microscopical control (11). The microdissection was performed on the short arm of chromosome 6 of maize. We used glass needles and also employed laser light to cut the chromosomes giving the same results (data not shown). Segments were scraped off and transferred to a collection drop (100 nl) of GP-buffer (4 vol 87% glycerol, 1 vol 0.05 M sodium/potassium phosphate, pH 6.8) on a coverslip. After completing the transfer, the volume of the drop was adjusted to 5 µl with 2 mg/ml proteinase K (Boehringer), 0.25% SDS, 10 mM Tris–HCl pH 7.5, 1 mM EDTA. The coverslip was incubated in a moist chamber at 37°C for 1.5 h. After proteinase K treatment, the reaction mixture was transferred to a 0.5 ml test tube and extracted three times with 5 µl phenol–chloroform (1:1) saturated with TE (10 mM Tris–HCl pH 7.5, 1 mM EDTA). The solution was microdialysed (0.025 µm membrane pore size) according to the manufacturer's protocol (Millipore) against 10 ml TE for 45 min. Then DNA was digested with the restriction enzyme *Mbo*I (10 U) in a volume of 10 µl of reaction buffer (50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) at 37°C for 1 h. The reaction was stopped by heat-inactivating at 65°C for 20 min and ligation to the adaptor molecules was carried out in 15 µl 0.5× buffer (1× buffer: 50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% polyethylene glycol-8000) with 1 Weiss unit T4-ligase (Life Technology) and 0.45 µM Mbo-adaptor (12) at 15°C for 12–16 h. After ligation, fragments were amplified in a volume of 100 µl, containing 15 µl ligation product, reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 9.0), 250 µM of each dNTP and 1 µM primer (Mbo 20) (12). After denaturing (95°C, 10 min) 2.5 U of *Taq* polymerase (Pharmacia) were added. Amplification conditions for 40 cycles were 94°C for 1 min, 45°C for 1.5 min, 72°C for 2 min and at 72°C for 5 min. The reaction was performed under mineral oil in a thermal cycler (Gene ATAQ Controller, Pharmacia). Tests for sensitivity of the PCR reaction and controls were performed according to the same protocol (Fig. 1).

We have modified previously described protocols for 'micro-amplification' (8,12) to simplify the generation of chromosome segment-specific DNA probes. The new method works without oil chamber and mircopipetting. All purifications and biochemical reactions were performed in microliter volumes. The compensation of the very small quantities of dissected chromosomal DNA in enlarged volumes was driven to completion by adding a large excess of synthetic linker–adaptors. Other primer systems, like plasmid vectors in comparison to short adaptor molecules seem to be less efficient in the ligation reaction. Libraries generated from adaptor PCR products yield a 10–100 times higher number of recombinants compared to using vector PCR (7,12). A convenient feature of this adaptor PCR method is the variable composition of the adaptor–primer sequence. There is no limitation to a target region in the genome. Coding or non-coding regions may be selected by choosing appropriate sites for restriction endonucleases. We optimized conditions for amplification of chromosomal DNA to detect and amplify quantities between 2 and 3 femtogram (fg) of DNA.

The advantages and disadvantages of direct or PCR mediated methods to generate chromosome segment-specific DNA probes were discussed previously (13,14). Generally, the requirements

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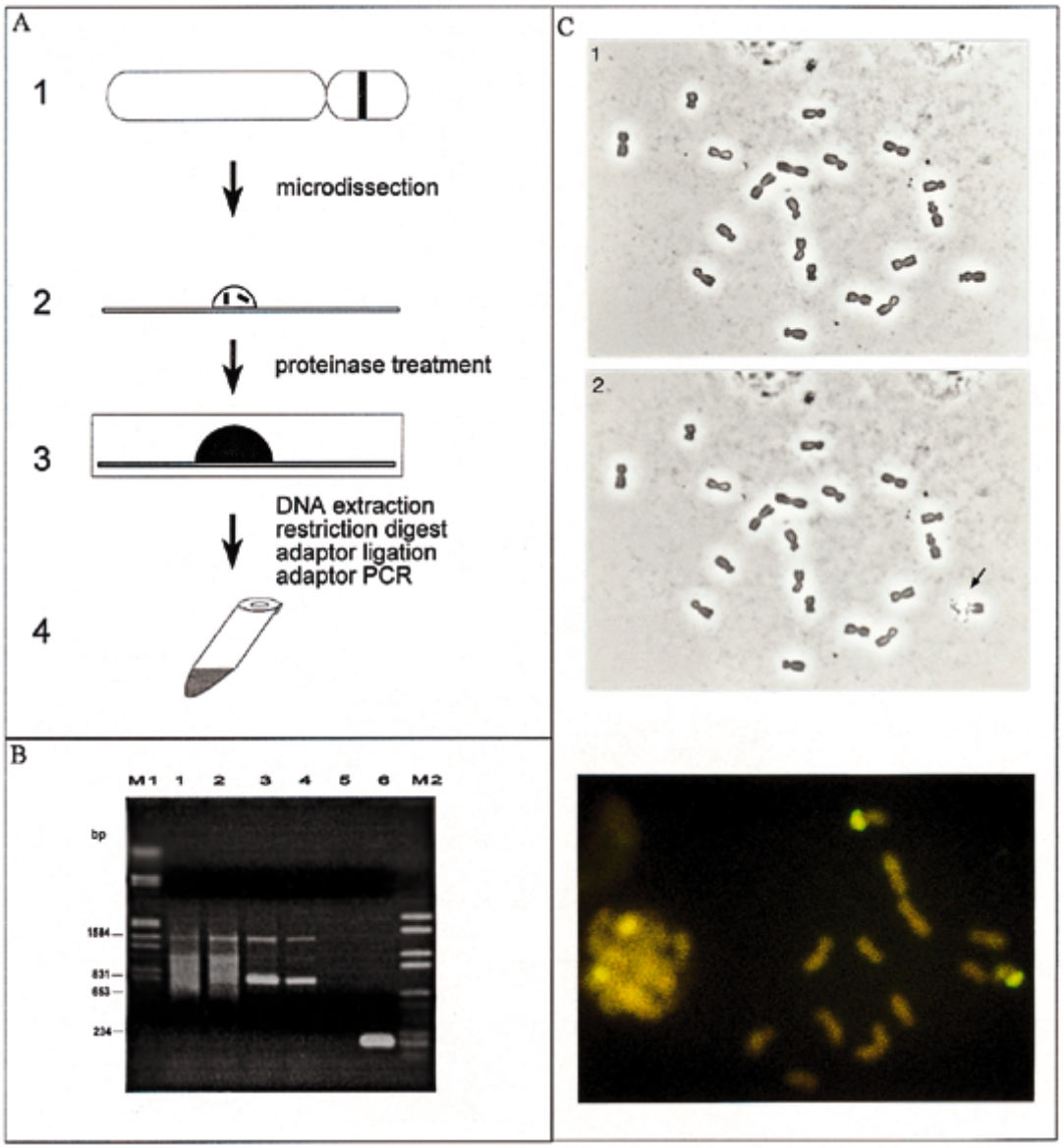

Figure 1. (**A**) Schematic representation of microdissection and microamplification of chromosomes. **1**, Microdissection of chromosomal material by a laser microbeam or a glass needle; **2**, transfer of dissected segments into a drop of glycerin-phosphate buffer (100 nl) on a coverslip; **3**, proteinase–SDS treatment on this coverslip after transfer to a moist chamber; 4. phenol extraction (5 µl), digestion with restriction endonucleases, ligation to synthetic adaptors and PCR amplification of the ligation products. (**B**) Sensitivity of microamplification of maize DNA. Gel electrophoresis of adaptor–PCR products (**1**, **2**) genomic maize DNA (PA405) and (**3**, **4**) specific Mbo fragment (700 bp/human). DNA of the following amounts (1: 21 fg, 2: 2.1 fg, 3: 30 fg and 4: 3 fg) was restricted, ligated with Mbo–adaptor, and amplified as described above. **5**, Negative control (no DNA); **6**, positive control, 10 ng plasmid DNA (Bluescript, Stratagene) amplified with sequencing and reverse sequencing primer. Molecular weight markers were applied to lanes **M1** and **M2**. (**C**) Microdissection and chromosome *in situ* suppression (*ciss*) hybridization of the amplification product. From a chromosome spread (mitotic metaphase/short arm of chromosome 6 of maize) (**1**) a segment was scraped off (**2**) (arrow) and transferred to the collection drop. After adaptor–PCR the complex amplification product (from six chromosome segments) was biotinylated and hybridized to spreads of metaphase chromosomes in the presence of competitor DNA according to the protocol of Lichter *et al.* (17) (**3**).

of sterility to prevent contaminations have to be the same in PCR as for tissue culture. A serious problem must be considered when minute amounts of DNA have to be amplified. The influence of potential contaminations of the PCR increases exponentially to the decrease of the quantity of template DNA. A specific problem is the contamination of enzyme preparations with DNA supplied by different companies (15). Approaches to solve this problem are the examination of the various enzyme preparations or to reduce the involved number of different enzyme reactions. Alternatively, the number of dissected chromosome segments for amplification has to be increased to change the ratio of contamination to template DNA.

With the method described here, chromosome segment-specific DNA probes can be generated rapidly for isolating molecular markers, establishing physical maps or employing in chromosome painting. Using this approach, a library was generated from the NOR region of maize to study resistance to *Maize Dwarf Mosaic Virus* (16).

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