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通过微切－扩增建立植物(蚕豆 *Vicia faba*)染色体区段特异性基因文库研究初探

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摘要 以蚕豆(*Vicia faba*, 2n=12)根尖为材料，采用改良方法制备染色体标本，在光镜下切割分离一段大M染色体核仁组织区(NOR)特定区段(约含0.9 pg DNA)，通过单一引物—聚合酶链式反应法(Single Unique Primer-PCR)随机扩增微切DNA后，获得近60 μg DNA。经琼脂糖电泳分析测定扩增产物分子片段大小介于200－900 bp。以地高新(Digoxigenin)标记蚕豆总体DNA，作为探针与扩增产物进行Southern杂交，证实扩增得到的DNA与蚕豆DNA同源，来自微切染色体。部分扩增产物经EcoRI酶切后，连入经同酶切割后的pUC18载体质粒，转化大肠杆菌(*E coli* JM109)。琼脂糖电泳分析得到的部分克隆，得知插入子长度介于0.25－0.9 kb。本文将用于动物材料的单一引物—聚合酶链式反应法应用于植物染色体的微切微扩增，并作了一定程度简化，初步建立起一套包括微切、扩增、检测和克隆的便捷、经济的实验室制备植物染色体区域特异性基因文库的方法。

关键词 蚕豆大M染色体核仁组织区；微切；单一引物—聚合酶链式反应；Southern杂交；克隆；染色体区域特异性基因文库

A PRELIMINARY STUDY ON A SIMPLE PROCEDURE TO CONSTRUCT MICRODISSECTION LIBRARIES OF DEFINED REGION FROM AMPLIFIED CHROMOSOME SEGMENT IN PLANT CELL (*VICIA FABA*)

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Abstract A simple method to create chromosome region specific library in plant materials, including microdissection, amplification, characterization and cloning, is described. A single NOR region of chromosome I from *Vicia faba* was microdissected and directly amplified by SUP-PCR in a magnified system. The sizes of the PCR products ranged from 200 to 900 bp. Southern hybridization with total genomic DNA demonstrated the reliability of the method. Part of the products were cloned into plasmid pUC 18. The inserts ranged between 0.25 and 0.9 kb.

Key words Chromosome region specific library; *Vicia faba;* Microdissection; NOR region; Chromosome I; Southern hybridization; Clone

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1 Introduction

Positional cloning by microdissection and microcloning has been proved to be an extreemly useful technique. Chromosome or subchromosome libraries can provide both high-density RFLP markers for genetic linkage map construction and specific probes for genomic library screening.

The microdissection of metaphase chromosomes with glass needles[1] or laser microbeams[2, 3] and subsequent amplification and cloning is a technique which was just developed in the last five years.

Since 1981, after the first successful example of microdissection and direct cloning by manipulation of polytene chromosome in *Drosophila*[1], the cloning of subpicogram quantities of DNA had been hampered by the difficulties in getting sufficient chromosome materials, in micromanipulation and the relatively low cloning efficiency. Usually at least 100 chromosomes or segments were needed to be collected per cloning cycle[4].

The introduction of Polymerase Chain Reaction (PCR) into the microcloning protocol had greatly improved the efficiency of this technique[1,5]. To date, several ways of sequence-independent amplification of microdissected DNA are available, which could be sorted into two types: 1) Methods to use specific primers to amplify microdissected DNA, which has been priorly ligated into vector or adaptors providing primer-binding sequence. The linkers including pUC/M13 sequencing plasmid[1,6], Mbo I linker-adaptor[7－10] and Sau3A primer-linker[11,12]. 2) Random amplification with partially random sequence as a primer[13,14]. However, both of the approaches have been almost exclusively employed in human and animal chromosomes.

Only till the last three years, had the application of microcloning in plants been reported. A direct cloning of microdissected B-chromosomes of rye obtained only 20 clones[15]. The vector-mediated PCR method was used to construct microdissection libraries of *Beta patellaris* chromosome conferring nematode resistance[16], and of the short arm from chromosome 1H of barley[17]. Two to five pieces of chromosome arms of wheat cells were amplified in a simplified adaptor-mediated PCR reaction[18].

Single unique primer-PCR (SUP-PCR) proved to be one of the most effective methods designed to amplify a single microdissected segment of human chromosome with primer BVE22cc[2,19]. The present paper applied it to clon a single plant chromosome specific region—the Nucleolar organizer region (NOR) of chromosome I from field bean (*Vicia faba*) with a simplified procedure. Not only the use of micromanipulation was eliminated, but the quantity of the PCR products of once-microdissected materials was increased as compared to that by the original method. It could facilitate the study of chromosome microdissection and microcloning in plant.

2 Materials and methods

2.1 Preparation of metaphase chromosome spreads

Seedlings of *V. faba* with 2-cm-long side roots were incubated successively in 1.25 μmol/L hydroxyurea, double distilled H_2O and 4 μmol/L APM (Amiprophosmethyl) at 25 ℃ as described by Schübert et al.[20] to get metaphase cells. After being fixed in 70% ethenol for above 24 hours at -20 ℃, the harvested root tips were squashed individually and quickly on cold clean wet cover slip in a drop of 3:1 methanol-acetic acid solution. Air dried, stained with 1:10 Giemsa solution (in phosphate buffer, pH6.8) for 5 min.

2.2 Microdissection

Siliconized rods with an external diameter of 1.0 mm were pulled on a pipet puller to form microneedle with a tip of 1−2 μm, and bended to an angle of 120°, UV treated for 20 min before use.

The cover slip carrying chromosomes was put upside down and fixed on a glass chamber. Microdissection was performed under a Nikon microscope of 40×magnification using microneedles controlled through a micro-manipulator. The tip of the needle sticking a single NOR segment was broken off into the bottom of a 0.5 ml microtube, and added 10 μl super pure water, stored at 4 ℃ overnight. Then DNA-free proteinase K was added to a final concentration of 1.0 μg μl^{-1}, incubated at 37 ℃ for 2 h to deproteinize completely. Inactivation of proteinase K was at 75 ℃ for 15 min.

2.3 SUP-PCR amplification of the microdissected DNA

All the steps, as well as the sequence of the primer, followed essentially the previous description by Hanado et al.[2], except for a magnified reaction system.

The first step of PCR: samples ready for amplification were added with the first PCR mixture[2] to a final volume of 20 μl, covered with paraffin oil. Six rounds of low stringency cycles were performed: 5 min at 90 ℃ and 1 h at 24 ℃, then the temperature was increased gradually to 50 ℃ and was kept for 20 min.

The second step: added the second PCR mixture to a 60 μl final volume, 92 ℃ for 2 min, 50 ℃ for 1.5 min, 72 ℃ for 4 min. Twelve cycles were performed.

The third step: 10 μl of the products were transferred to a new microtube containing 50 μl of the third PCR mixture, oil covered. Thirty-two cycles were carried out as followed: 1.5 min at 92 ℃, 55 ℃ for 1.5 min, 72 ℃, 3 min.

10 μl of the products were electrophoresed on 1.5% agarose gel. Another 2 μl from the products were taken out to detected DNA concentrations by their absorbing values at 254 nm UV light with the products of negative control as background.

2.4 Characterization of the amplified chromosome DNA by Southern hybridization

To confirm the bean origin of the amplified microdissected DNA, 1/6 of the final products in one tube, and of the negative control, were seperated on a 1.5% agarose gel then transferred to a nylon membrane (Hybrida) using SSC solution. The membrane was prehybridized for 1 h at 68 ℃ in a solution containing 5×SSC, 1% Blocking reagent (Boehringer Mannheim), 0.1% N-lauroylsarosine (Sigma), 0.2% SDS. Hybridization with total genomic DNA of *Vicia faba* was carried out at 68 ℃ overnight. The probes were labelled with digoxigenin by Random Priming Method. Signal detection followed the instructions in the specification enclosed in the "DIG labelling and detection Kit" (Boehringer Mannheim), with alkaline phosphatase as a detecting enzyme.

2.5 Cloning microamplified DNA into pUC18

One-twelfth of the microproduct was purified with phenol / chloroform extraction, and ethanol precipitation, followed by EcoR I digestion to create cloning sites. The DNA was ligated into pUC18 with T4 ligase. One-fifth of the ligation mixture was used to transform *E coli* JM109 competent cells prepared with $CaCl_2$ method. One-fifth of the transformed mixture was plated onto LB medium with ampicillin and IPTG-X-Gal.

The recombinant plasmids were isolated by alkaline lysis, subjected to electrophoresis (0.7% agarose gel).

3 Results

3.1 Microdissection

Root tips were used to provide sufficient metaphase chromosomes after treatment of hydroxyurea combining with APM. The highly effective synchronization method could create an MI of as high as 50% in *Vicia faba*[20]. In its karyotype (2n=12)[21] (Fig. 1) the chromosome I is easy to be distinguished by its reatively large size and NOR structure. A single segment was microdissected by siliconized glass needle (Fig. 2).

Fig. 1 Karyotypic cell of *Vicia faba* (2n=12) (550×Magnification, arrows indicate chromosome I)

Fig. 2 Microdissection of NOR region from chromosome I (650×Magnification, arrow indicates NOR region)
a. Chromosome I before microdissection;
b. Chromosome I after microdissection

3.2 Direct amplification of the microdissected DNA

A little amount of genomic DNA of *Vicia faba* was amplified as a positive control, and no DNA was added in the negative control. Fig. 2 shows the results. The product of genomic DNA has a larger mean size than that of microdissected DNA, reflecting some mechanical or biochemical damage of the chromosomal DNA during the spread preparation and the microdissection. UV absorbing value showed that approximately 60 μg DNA was obtained in every 3-step PCR tube, so totally about 360 μg of DNA could be the amplified amount of one microdissected segment. All the products appeared an additional smear in the gel, sizing below 110 bp, which had been decreasing greatly after a few days of storage at 4 ℃ (Fig. 3, Fig. 4 a). This problem was ignored as small primer polymers forming during amplification. Also the result of Southern hybridization was consistent with this possibility.

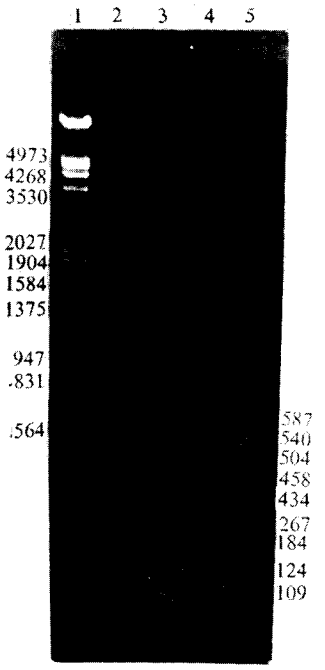

Fig. 3 SUP-PCR products (1.5% agarose gel)
Lane 1. λ DNA Hind Ⅲ /EcoR I markers
Lane 2. Negative control
Lane 3. Positive control (0.3 pg total genomic DNA as template)
Lane 4. Products of microdissected DNA
Lane 5. pBR 322 DNA Hae Ⅲ markers

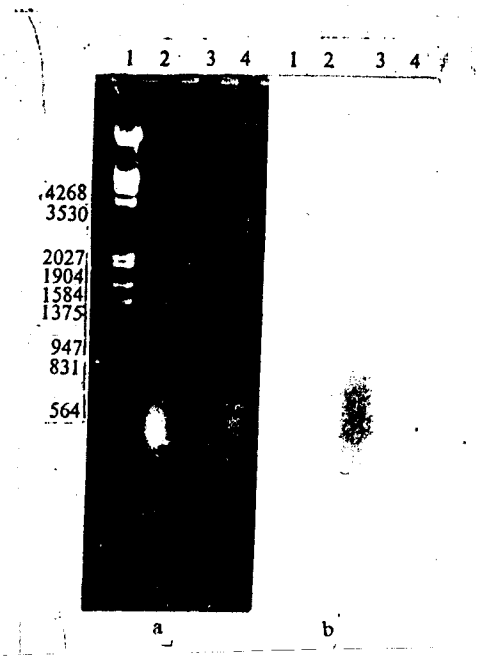

Fig. 4 Southern blot analysis of the amplified products of microdissected DNA
a. DNA seperated on 1.5% argarose gel
Lane 1. λ DNA Hind Ⅲ /EcoR I markers
Lane 2. Amplified product of microdissected chromosome
Lane 3. Negative control
Lane 4. pBR 322 DNA Hae Ⅲ markers
b. Southern blot analysis

3.3 Characterization of the PCR product by Southern genomic hybridization

The strong signal produced by hybridization between genomic probes and the

microproducts confirmed that the DNA obtained from the amplification did derive from the microdissected chromosome of *Vicia faba*. The small moleculars in products both of microdissected DNA and of the negative control got no signals detectable (Fig. 4).

3.4 Cloning of the amplified DNA from microdissection

One- a hundred and fiftieth of the transformed mixture was plated on LB-Amp-X-Gal plate, resulting in 43 white clones. The gel analysis showed that the recombinant plasmids were from about 2.94 to 3.6 kb, indicating that the sizes of the inserts were between 0.25 and 0.9 kb (Fig. 5). Detailed characterization and identification of the microclones were not carried out.

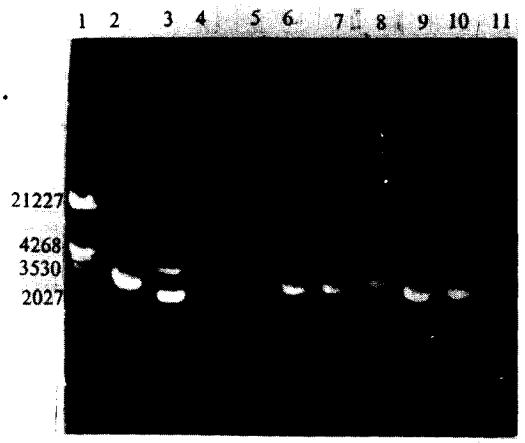

Fig. 5 The recombinant plasmids with the microamplified DNA inserts (0.7% agarose gel)
Lane 1. λ DNA Hind Ⅲ /EcoR I markers
Lane 2. Liner molecular of pUC18 (2.69 kb, EcoR I digested)
Lane 3－11. recombinant plasmids

4 Discussion

In order to establish a general way to construct chromosome specific DNA library, *Vicia faba* was selected as the materials for its easily obtained karyotype and well-recognized chromosomes. That made the microdissection easily performed.

To avoid the acid-induced depurination of chromosomal DNA, we employed the 70% ethenol as fix solution which has been used in chromosome preparation for microdissection in mamalian cells[4], as a substitution for 3:1 methanol: acedic acid. The root tips after fixiation were squashed directly on coverslip in a drop of 3:1 solution. So the time of the acid action was reduced to as short as several seconds.

SUP-PCR with BVE22cc primer was designed to amplify any unknown DNA sequences with a high sensitivity. Less than 10 fg of DNA template could be amplified effectively[2]. It has been used successfully in microdissection and microcloning of human chromosomes 4p 50－100%[2] and Xqter regions[19]. BVE22cc primer is a 22 mer sequence, with 3 restriction sites for EcoR I, Bgl Ⅱ and EcoR V respectively. It is different from the other random primers used in sequence-independant amplification by its defined sequence. The low stringent conditions in SUP-PCR cycles and the existence of polyethylene glycol (PEG) make it possible to prime DNA sequences nonspecifically. Usually a single piece of chromosome region is sufficient for the following amplification. A lot of work in microdissection was saved.

Here we applied SUP-PCR method from animal materials to amplify a defined region of plant chromosome. But the present protocol is not all the same as the original one. Instead of 1 μl collecting volume and 3 μl initial amplification system adopted in the previous description[2,19], we collected the chromosome DNA directly in microtube, and the initial volume of PCR was 20 μl. In the third-step cycles, 1/6 of the second PCR products were used in a final amplifying system of 60 μl. So all the 3-step products of one piece of NOR region from *Vicia faba* could amount to 360 μg in combination. In Hadano's report, the chromosome fragments were collected into a 3 μl PCR system in the first cycle, and 9 μl in the second. Both of them require micromanipulation, which is time-consuming and strenuous. In our work the micromanipulation was eliminated by adopting the magnified system. Gel analysis and Southern hybridization indicated that the simplified SUP-PCR method worked well in amplifying the microdissected chromosome of *Vicia faba*.

An important factor that would cause a great consequences in the experiment is extraneous DNA contamination. The following precautions must be taken: all the solutions should be prepared under sterile conditions in a laminar flow cabinet using reagents and equipments stringently autoclaved (123 ℃ , 90 min)[2]. New micropipette tips are consistently used (autoclaved). The microneedles and all the other equipments should be UV treated 30 min before use.

Genomic DNA from *Vicia faba* was used as probe to identify the microamplified DNA. The remarkable blotting signal showed that SUP-PCR did originate from the DNA of *Vicia faba*. Using microdissected DNA as probe is not feasible for 2 reasons below: 1) Usually the DNA amount of microdissected section from a chromosome is at fg (10^{-15} g) level, though chromosomes of *Vicia faba* is morphologically larger in this work, the greatest amount of the microdissected DNA cannot exceed 1 pg (10^{-12} g). But it's very hard to manage the probe labelling reaction when the template DNA is fewer than 1 ng (10^{-9} g). 2) The possible contaminated DNA in the microdissecting process could also produce hybriding signal, which cannot be the persuasive proof of the homogenesis. The best way of DNA characterization is *in situ* hybridization. But it's hard to control in plant cells and needs more work, which is not a good step in a simple and rapid laboratorial method comparing to the regularly used Southern blotting.

In the cloning of the microproducts, the smear of unsteady DNAs appeared in the gel analysis was neglected for several reasons: 1) they were decompositing automatically, 2) the possible primer polymers would be digested apart by EcoR I, 3) the sizes of the small DNA moleculars were not continuous with that of the microamplified DNA, so even if they were successfully cloned, it is still easy to identify.

The sizes of the liner moleculars of the recombinant plasmids ranging around 2.94—

3.59 kb (Fig. 5, the second bands from front end in all the recombinant plasmids) were roughly calculated according to the relationship between base-pair-number logarithm and DNA migrating distance on the gel under special conditions (concentration of the gel, temperature and time of electrophoresis, etc.). The size of pUC18 is 2.69 kb, so the inserts range from 0.25 to 0.9 kb, as consistent with the microamplified products. For the information about the NOR region of *Vicia faba* was not our focus study, detailed characterization and identification of the obtained clones and further completed cloning work of DNA from this region were not carried out.

In brief, the method reported here represents a simple and efficient protocol for the construction of subchromosome DNA libraries of specific region in plant, involving microdissection of specific chromosome region, direct amplification with single unique primer, and characterization by Southern hybridization as well as cloning into plasmid vector. This reproducible technique has also been used in microcloning the telemere of B-chromosome in rye and in amplification of every individual chromosome of rice as well.

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