Construction of genomic library of rice using a bacteriophage lambda vector

KUNISUKE TANAKA and KENJI MATSUI

Abstract : The method for construction of genomic library using a bacteriophage lambda vector EMBL 3 was modified so as to increase its efficiency for plant DNA. Genomic DNA was prepared from rice embryos, but to be a substrate of a restriction enzyme it had to be purified by equilibrium centrifugation in a cesium chloride density gradient. The isolated DNA was partially digested with *Mbo* I, then ligated with bacteriophage lambda vector EMBL 3 arms substantially according to Frischauf *et al.* (J. Mol. Biol. *170*, 827(1983)). The efficiency for ligation between the bacteriophage DNA and the rice embryo DNA was improved by addition of polyethylen glycol(PEG) 6000. Moreover the use of PEG 6000 raised the efficiency of *in vitro* packaging. This improved procedure for the ligation and packaging of the recombinant DNA will be generally applicable to the construction of plant genomic libraries.

Key words : bacteriophage lambda vector(EMBL 3), genomic library, *in vitro* packaging, ligation, polyethylene glycol 6000, rice(*Oryza sativa* L.)

Abbreviations : CH buffer, 40 mM Tris-HCl(pH 8.0), 10 mM $MgCl_2$, 1 mM spermidine, 1 mM putrescine, 0.1% 2-mercaptoethanol and 7% dimethyl sulfoxide ; PEG, polyethylene glycol ; TE, 10 mM Tris-HCl(pH 8.0)and 1 mM EDTA ; SDS, sodium dodecyl sulfate ; pfu, plaque forming unit(s); kbp, kilo base pair(s)

Introduction

cDNA encoding the rice glutelin, a major storage protein, has been cloned by Tanaka *el al.* (unpublished). Glutelin is synthesized vigorously in several days from 5 days after flowering, and constitutes at least 80% of the total protein in the mature rice grain[1]. This high rate of glutelin synthesis must depend on the presence of a strong promoter lying upstream of the open reading frame. This powerful promoter would regulate the site-and time-specificities of the glutelin synthesis. In order to analyze the controlling system in the gene expression of rice glutelin, genomic library of rice is necessary.

The development of efficient *in vitro* packaging system made it available to construct genomic library of eukaryotic DNA. Bacteriophage lambda substitution vector which can accept inserts up

* Laboratory of Biochemistry, Faculty of Agriculture, Kyoto Prefectural University, Shimogamo, Kyoto 606, Japan
** Laboratoty of Food Production, The Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan
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to 20 kbp in length is often used for this purpose. The method to construct genomic libraries involves partial digestion of genomic DNA to make insert DNA, ligation of the insert DNA and the lambda vector, and *in vitro* packaging of the recombinant DNA. Many methods have been developed for such genetic engineering techniques, but most of them are useful only for prokaryotes. Althogh much effort has been made for the development of the methods for mammalian cells, little has been done for those plant cells. In constructing genomic DNA library of higher plants, the genomic DNA must be highly purified so as to be digestible with restriction enzymes because contaminants in the DNA preparartion reduce the restriction enzyme activities. Moreover, some plants have much larger genomes, therefore, large numbers of independent recombinants are needed to cover all the sequences.

Here, we describe an application of the bacteriophage lambda clonig system for the construction of genomic library of rice, which includes a rapid method for isolating high-molecularweight DNA from rice embryos and practical application of the effect of PEG 6000 on ligation between DNA molecules. We discovered that PEG 6000 also increased the rates of *in vitro* packaging of bacteriophage lambda DNA. Using PEG 6000 made it possible to construct available genomic library of rice.

Materials and Methods

Materials - Embryos of rice (*Oryza sativa* L. Japonica cv Nipponbare) were isolated from the rice bran. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo Co., and *Mbo* I from Pharmacia Co. DNA gel electrophoresis was carried out with a mini-gel electrophoresis system "Mupid" (Cosmo Bio Co.) provided with 80 mM Tris-phosphate (pH 8.0) and 2 mM EDTA at 50 V for 1-1.5 hr.

Preparation of genomic DNA from rice embryos - Rice embryos were washed with distilled water and frozen in liquid nitrogen. The frozen embryos were powdered by a Bio-mixer (Nihon Seiki Co.) in the presence of liquid nitrogen. After liquid nitrogen had evaporated, the powdered embryo was incubated in 0.5 M EDTA (pH 8.0) containing 0.5% (w/v) Sarcosyl and 100 μg/ml of proteinase K (Sigma Co.) at 50℃ for 3 hr. After removing of the debris by filtration through cheese cloth and centrifugation at 10,000×g for 10 min, the DNA in the supernatant was purified by the phenol extraction method. The DNA in the aqueous layer was dialyzed against 50 mM Tris-HCl (pH8.0) containing 10 mM EDTA and 10 mM NaCl. This DNA preparation was contaminated with RNA, but the RNA was able to remove at the later step, therefore, the digestion with RNase was omitted. The dialyzed DNA solution was further purified by equilibrium centrifugation in a cesium chloride-ethidium bromide density gradient ($\rho = 1.70$).

Ethidium bromide was added to give 600 μg/ml and centrifugation was carried out at 36,000 rpm for 36 hr at 25℃ in an RP65T rotor (Hitachi Koki Co.). This centrifugation resulted in only one band of DNA, which was collected through a hypodermic needle inserted into the side of the tube. Ethidium bromide was removed by repeated partitioning against CsCl saturated isopropanol, then CsCl was removed with extensive dialysis against TE.

The purified DNA was partially digested with *Mbo* I (3.0×10^{-3} units/μg DNA) at 37℃ for 30 min in 10 mM Tris-HCl (pH 7.5) containing 75 mM NaCl, 10mM $MgCl_2$, 1 mM 2-mercaptoethanol and 1 mg/ml bovine serum albumin. Dephosphorylation of 5'-terminal of the DNA was performed with 0.44 unit μg DNA^{-1} of calf intestine alkaline phosphatase (Boehringer Mannheim) at 37℃ for 30 min in 50 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA. The reaction was terminated by inactivation of the phosphatase at 68℃ for 15 min in the presence of 0.5% (w/v) SDS[2], but the step of gel filtration was omitted.

In vitro packaging - Lambda cloning vector EMBL 3 purchased from Promega Biotec. was amplified by *in vitro* packaging. Before use, the vector DNA was extracted with 0.5% (w/v) SDS and

50μg/ml proteinase K from purified bacteriophage particles. The arms were prepared from the isolated bacteriophage lambda EMBL 3 DNA substantially according to Frischauf *et al.*[3]. For removal of short linkers, the EMBL 3 DNA was subjected to sucrose density gradient centrifugation (10-40% w/v in 1 M NaCl containing 20 mM Tris-HCl (pH8.0) and 5 mM EDTA) in an RPS 25 rotor (Hitachi Koki Co.) at 22,000 rpm for 24 hr at 20℃, because partial precipitation with isopropanol was unsuccessful. *In vitro* packaging extract was prepared from *E. coli* lysogens BHB2690 and BHB2688 mainly according to Hohn[4], but UV-irradiation was omitted because particles which packaged exogenous DNA can be easily distinguished from those packaged endogenous DNA in bacteriophage lambda EMBL 3 cloning system[3]. Packaging reaction performed according to the modified Hohn's method as described by Maniatis *et al.*[5].

Results and Discussion

Preparation of rice genomic DNA - To clone a foreign DNA into the lambda vector, the DNA requires enzymatic manipulations to generate 15-20 kbp fragments which have cohesive ends compatible with those of the vector arms. Thus, DNA should be prepared in a form of more than 50 kbp in length. Rice genomic DNA more than 50 kbp in length was prepared by the freeze-thaw treatment following incubation in Sarkosyl and proteinase K. However, the prepared DNA could not be digested with restriction enzymes such as *Eco* RI and *Mbo* I. In this regard the purification of the DNA both by repeated ethanol precipitation and by extensive dialysis against TE showed no effect. This may be attributed either to contaminated polysaccharides which reduce restriction enzyme activity, or to the chromatin packing which cannot be attacked by the enzymes. Therefore, the purification of the DNA by centrifugation in a cesium chloride-ethidium bromide gradient was performed. After the centrifugation, no other bands consisting from genomic DNA can be seen under UV light, which is probably due to that the source of the DNA is the rice embryo where chloroplasts or mitochondria have not sufficiently developed.

The rice genomic DNA prepared was more than 50 kbp in lenght (Fig. 1A). Faint bands in low molecular weight region may be contamination of RNA, but it did not disturb the later processes.

After removing ethidium bromide and cesium chloride, the purified DNA could be digested with a restriction enzyme *Mbo* I (Fig. 1B). For construction of genomic DNA library by using lambda vector EMBL 3, the prepared DNA must be partially digested to 15-20 kbp fragments. To establish the conditions for the partial digestion, the prepared DNA was digested with various amouts of *Mbo* I. The results of this experiment (Fig. 1B) indicated that 3.0×10^{-3} units of *Mbo* I μg DNA^{-1} was suitable for the partial digestion.

Construction of recombinant DNA - Bacteriophage lambda cloning vector EMBL 3 was used for construction of a rice genomic DNA library. Extracted EMBL 3 DNA was digested with *Eco* RI and *BamH* I, and size-fractionated by centrifugation through sucrose density gradients (10-40% w/v). The left (9.24 kbp) and right (19.3 kbp) arms, and the stuffer fragments (13.7 kbp) were collected through the bottom of tube. The short linkers (110 bp and 17 bp) in the upper region of the tube were discarded. 5'-Termini of the *Mbo* I-digested 15-20 kbp DNA fragments were dephosphorylated with calf intestine alkaline phosphatase. The resultant DNA is referred as the insert DNA.

In constructing a genomic library which has capacity to screen any genes, the ligation reaction of the insert DNA and the vector arms is preferred to be highly efficient. Ligation would occur not only between the vector arms and the insert DNA, but also between the single-stranded complementary *cos*-sites of the vector arms for the DNA to be assembled into lambda particles.

Macromolecules such as polyethylene glycol or serum albumin at high concentrations stimulate the rates of ligation of short cohesive ends[6]. PEG 6000 was used to enhance the efficiency of the ligation

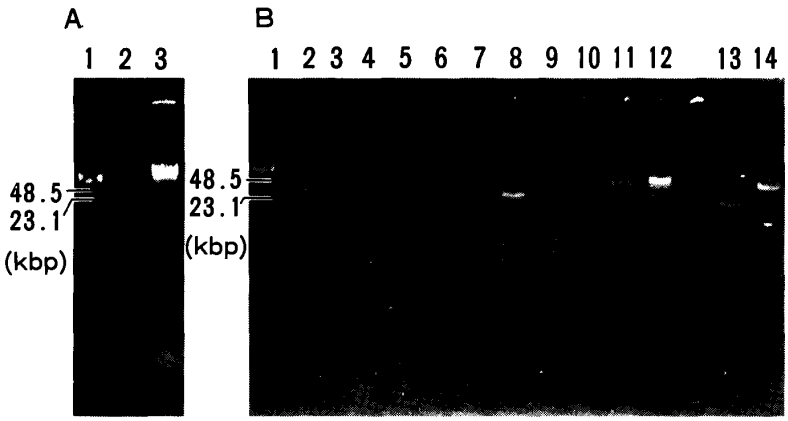

Fig. 1 K. Tanaka K. Matsul

Fig. 1. A; Electrophoretic analysis of genomic DNA isolated from rice embryos. - Samples were analyzed on a 0.4% agarose gel at 50 V for 1.5 hr. Lane 1, undigested lambda DNA ; Lane 2, lambda DNA digested with *Hind* III ; Lane 3, 1 μg of DNA purified from rice embryos after the equilibrium centrifugation in CsCl.

B; Effect of *Mbo* I concentration on the partial digestion of isolated DNA. - Purified rice DNA (1 μg) was digested with various amounts of *Mbo* I in 10 μl 10mM Tris-HCl (pH 7.5) containing 75mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 100 μg/ml bovine serum albumin at 37℃ for 30 min. Digestion was terminated by addition of 2 μl of 0.1M EDTA. Lanes 1 and 14, undigested lambda DNA ; Lanes 2, 8 and 13, lambda DNA digested with *Hind* III. The amounts of *Mbo* I (units/μg DNA) were ; 2.0×10^{-1} (lane 3), 1.0×10^{-1} (lane 4), 5.0×10^{-2} (lane 5), 2.5×10^{-2} (lane 6), 1.3×10^{-2} (lane 7), 6.3×10^{-3} (lane 9), 3.1×10^{-3} (lane 10), 1.6×10^{-3} (lane 11), 7.9×10^{-4} (lane 12).

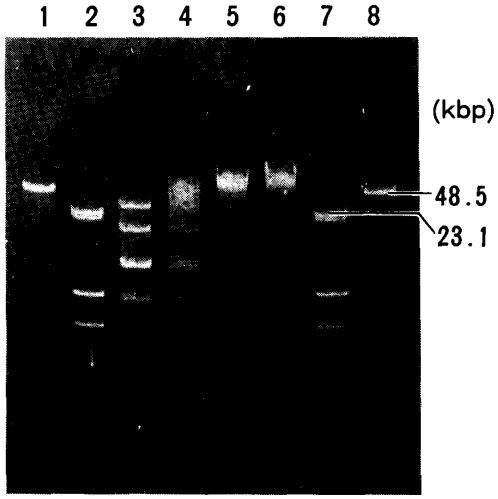

Fig. 2 K. Tanaka K. Matsul

Fig. 2. Effect of PEG 6000 on ligation reaction between vector and insert. - The vector arms (1.42 μg) and the insert DNA (0.47 μg) were ligated in the reaction mixture consisting of 66 mM Tris-HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP for 1 hr at 20℃. Lanes 1 and 8, undigested lambda DNA ; Lanes 2 and 7, lambda DNA digested with *Hind* III. In lanes 5 and 6 PEG 6000 (15% w/v) were added. T4 DNA ligase was added to 1.25 units/μg DNA (lanes 3 and 5) or 50 units/μg DNA (lanes 4 and 6).

reaction between the insert DNA and the vector arms (Fig. 2). In the absence of PEG 6000, ligated fragments of the left and right arms (18.5 kbp) can be seen, but no band could be detected in high molecular weight regions, which indicates that no recombinant was constructed (lane 3). When PEG 6000 was added to 15% in the reaction mixture, the left and right arms disappeared, and a broad band was detected in a higher molecular weight region than lambda DNA marker (lane 5), irrespective of the amount of T4 DNA ligase added. The broad band was expected to contain recombinants, and also concatenated DNA which was a consequence of the ligation of protruding complement cohesive ends originating from wild type lambda DNA (*cos*-sites). In the presence of 15% PEG 6000, 1.25 units μg DNA^{-1} T4 DNA ligase was sufficient.

Minton has theoretically suggested that excluded volume effects of macromolecular species such as PEG 6000 affect both rates and equilibria of biochemical reactions[7]. These suggestions can be introduced to the ligation reaction of the insert DNA and the vector arms. Before covalently binding, each DNA molecule must associate. PEG 6000 increases the equilibrium constant and reduces the free energy barrier of association reaction, thus the rates of association of DNA molecules will be increased. Furthermore, PEG 6000 also enhances the rates of the enzyme-catalyzed reaction[7]. Therefore, the rates of formation of a phosphodiester bond between adjacent 3'-OH and 5'-P termini in DNA are also expected to be enhanced.

Efficient method for *in vitro* packaging - *In vitro* packaging extracts were prepared from *E. coli* lysogens BHB 2690 and BHB 2688 mainly according to Hohn[4]. To determine the packaging efficiency, packaging with the wild type lambda DNA was performed. In packaging *in vitro*, wild type lambda DNA (1 μg) dissolved in 50 μl of CH buffer supplemented with 3.5 mM ATP was added to 50 μl of *in vitro* packaging extracts. Under these conditions, 1 μg of DNA produces no more than 10^3 pfu (Table 1). To cover every gene at the probability of 99%, more than 8.4×10^5 of independent recombinants which are carrying 17 kbp insert in average are needed because rice genomic DNA is 3.1×10^6 kbp in length[8].

In *in vitro* packaging, DNA must be catenated into linear DNA. Therefore, the cohesion of DNA duplex with *cos*-sites of lambda DNA must occur efficiently. To elevate the efficiency of *in vitro* packaging, we tried the addition of PEG 6000 at high concentrations (Table 1). PEG 6000 at 3.8% and 7.5% raised the efficiency of *in vitro* packaging of wild type lambda DNA about 10^3 fold. A genomic DNA library of rice which has more than 2×10^5 independent recombinants has been constructed in the presence of PEG 6000 at 7.5%.

Although the mechanism of this effect of PEG 6000 at high concentrations on packaging *in vitro* was not elucidated yet, it partly depends on sufficient cohesion of protruding cohesive ends of lambda

Table 1. Effect of PEG 6000 on *in vitro* packaging

PEG 6000 (%)	pfu/μg DNA
0	3.5×10^3
3.8	2.6×10^6
7.5	6.0×10^6
15	4.0×10^3

Wild type lambda DNA (1 μg) were packaged with 50 μl of *in vitro* packaging extracts. DNA was dissolved in 50 μl of CH buffer, and PEG 6000 was added at the indicated concentrations in the packaging reaction mixture. pfu were determined by counting the numbers of plaques formed on lawns of *E. coli* strains LE 392.

DNA 12 nucleotides in length(*cos*-sites). Zimmerman and Harrison[9] observed that macromolecules enhanced the rates of cohesion of the naturally cohesive ends of lambda DNA. The DNA substrate for packging *in vitro* is concatemeric lambda DNA, which is a tandem polymer of several DNA units, and linear monomeric DNA is not packaged[4]. Thus, the more lambda DNA is catenated over *cos*-sites, the more efficiently it will be packaged. Serwer *et al,* also discovered positive effects of macromolecules such as dextran 10 on *in vitro* packaging of bacteriophage T7[10]. They suggested that the macromolecules stabilize the proteins participating in packaging reaction and/or create the gradient of osmotic pressure which is thought to be necessary for DNA packaging across bacteriophage capsids(higher osmotic pressure outside). Additionally, as reported by Hamada *et al.*[11], in the defined *in vitro* system for packaging of bacteriophage T3 DNA, the higher molecular weight polyols enhanced the rates of the packaging efficiency.

Among the genomic DNA library of rice we constructed here, some clones hybridized with ^{32}P labeled probe obtained from plasmid pRH 3 which contains rice glutelin cNDA sequence. Now we are tending to sequence one of the clones.

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ラムダベクターを用いたイネ遺伝子ライブラリーの作製

田中國介*・松井健二**

要旨：バクテリオファージラムダベクターEMBL 3を用いた遺伝子ライブラリーの作製法を植物のDNAに対して効率が上がるように改変した。核DANはイネ胚芽より調製したが，制限酵素の基質とするためには，セシウムクロライド平衡密度勾配遠心で精製する必要があった。単離したDNAは*Mbo* Iで部分分解し，主にFrischaufら(J. Mol. Biol. *170* 827(1983))の方法により，バクテリオファージラムダベクターEMBL 3アームと結合した。バクテリオファージDNAとイネ胚芽DNAの結合効率は，ポリエチレングリコール(PEG) 6000の添加で上昇した。更に，PEG6000は*in vitro*パッケージングの効率も上げた。結合反応とパッケージングに対するこの改良された方法は，植物の遺伝子ライブラリー作製に一般的に適用することが可能であろう。