IBN AL- HAITHAM J. FOR PURE & APPL. SCI. VOL.19(4) 2006

Molecular Cloning of large DNA Fragments of The Salt Tolerant wild Tetraploid bermudagrass *Cynodon Dactylon L.* Using A Bacteriophage Cloning Vector. 2. Ligation and *in vitro* packaging of The Recombinant Phage DNA Molecules

S. A. Ibraheem
IPA Agricultural Research Center. Baghdad

Abstract

This paper represent the second step in a molecular cloning program aiming to clone large DNA fragments of the salt tolerant bermudagrass (*Cynodon dactylon* L.) DNA using the bacteriophage (EMBL3) as a vector.

In this work, a yield of about 100 µg bacteriophage DNA per one liter culture was obtained with a purity ranging between (1.7-1.8). The vector DNA was completely double digested with the restriction enzymes BamH1 and EcoR1, followed by purification of vector arms. Bacteriophage arms were also efficiently ligated with the partially digested bermudagrass DNA (prepared earlier). The optimum ligation ratio of arms: inserts (EMBL3: bermudagrass) was found to be 4:1 respectively. The recombinant DNA was successfully *in vitro* packaged and plated on a P2 lysogen *E. coli* (strain NM539). An efficiency of about $1.3x10^6$ pfu/ µg recombinant phage DNA was determined in these experiments.

Introduction

Salinity and other environmental stresses constitute the major limit on many if not all crop plants production (1). Nevertheless, variations to such stresses exist among cultivated crops which form a prerequisite of any plant breeding programs. Also, since most of these crops have been

bred on favorable conditions and out side of their natural habitat, their gene pools have become limited (2) .Since recent advances in the application of recombinant DNA technology have made possible the introduction and expression of foreign genes from wild into cultivated plants, they have enriched their gene pools (3), and has opened new perspectives to plant breeders (4, 5). Presently, the molecular and genetic basis of salinity stress is not very well clarified. Nevertheless, a number of molecular investigations have been reported involving studies on mRNA transcription under salinity stress in barley (6). Gene induction and repression by saline stress in different tissues of the salt tolerant wheat, tolerant amphiploid, and the salt sensitive chinese spring were reported by (7).

In Iraq several attempts have been made (e.s.Amash et al. personal communication) to study and characterize halophilic bacteria, to identify and clone the genes responsible for salinity tolerance as an alternative source for foreign genes (8, 9).

Since gene cloning is the only practical way for any molecular biology study to be carried out on individual genes (10), and in order to provide a possible source of salinity genes from wild plants. The objective of the present study, (which forms a continuation of a project initiated by (11)) was therefore, the preparation of an efficient genomic library for the wild salt tolerant tetraploid bermudagrass (*Cynodon dactylon* L. pers) (12). This was performed by cloning large DNA fragments of target DNA prepared earlier (11) in the bacteriophag lambda EMBL3 replacement cloning vector Fig. (1) (13). The cloning was achieved by ligation of bacteriophage arms and bermudagrass DNA, and by *in vitro* packaging of recombinant DNA molecules in the packaging extracts (promega, USA). The EMBL3 replacement phage vector is particularly useful vector for eukaryotes. Partial Sau3A digests of genomic DNA are cloned at BamH1 sites of the vector. The restriction enzyme sites on the polylinker sequences of the vector are arranged to give EMBL3 phage with sal1-BamH1- EcorR1 sites in opposite orientations at both ends of the stuffer (middle) fragment. The polylinker sequences allow inactivation of the stuffer fragment by digestion of the vector DNA with both BamH1 and Ecor1.

Recombinant phage can be selected by plating the packaged library onto a P2 lysogenic host. The insert (cloned) DNA can be cleanly removed from the recombinant phage DNA as one or more fragments by cleavage with sal l restriction enzyme.

Material And Methods

- **Isolation of EMBL3 bacteriophage DNA:** To isolate DNA from the cloning vector, a large scale method based on a method developed by (14) and modified by (15) was emploid. This involved, the following main steps; one liter of *E. coli* strain NM 538 culture grown on LB medium to an OD600 of 0.3, was inoculated with 5 ml of phage stock (1×10^{10} particle/ml) prepared earlier. The contents were divided into two flasks and shaken at 180 rpm at 37°c until lysis was complete, then bacteria debris was pelleted by centrifugation at 2000xg. Bacterial nucleic acids were denatured by the addition of solid DNase and RNase to final concentration of 10 µg/ ml. After this 2% Nacl and 8% polyethylene glycol 6000 were added.

Bacteriophage particles were pelleted by centrifugation at 10000xg and then resuspended in lambda diluent (100mM tris– Hcl pH 8,1mM EDTA, 10mM Mgso4). A CsCl density gradient was prepared by adding 0.71 gm CsCl/ml and centrifugated at 45,000 xg for 24 hr. The white band which represents bacteriophage particles was carefully recovered and dialysed against lambda diluent at 4c°. To isolate bacteriophage DNA, several phenol – chloroform –isoamyl extractions were performed according to (16). The DNA was recovered by ethanol precipitation and resuspend in TE buffer. A sample of this DNA was run on 0.7% gel electrophoresis along with lambda Hind 111 DNA molecular weight marker using TBE running buffer (16).

- **Preparation of EMBL3 arms:** The vector arms were prepared according to the method described by (13). 20 µg DNA was digested with Bam H1 restriction enzyme (2 units/µg DNA) using a medium salt buffer according to the supplier recommendations. After the digestion was completed, the DNA was heated at 68°c, then recovered by phenol/chloroform , ethanol precipitation and resuspended in 20 µl TE buffer. To remove Bam H1 site from the middle (stuffer) fragments, the

phage DNA was cleaved with EcoR1 restriction enzyme (2 units / µg DNA) in a high salt buffer as above. The phage arms were purified by phenol/ chloroform extraction and then recovered by isopropanol precipitation (14). DNA pellets were resuspended in TE buffer as above. A sample of the double digested vector DNA was taken for run on 0.7% gel electrophoresis.

- **Ligation:** Ligation experiments were carrieed out in attempt to join vector arms prepared here with the target (bermudagrass) DNA fragments (insert) prepared earlier (11) using T4 DNA ligase enzyme (NTL, UK). In order to determine the optimum conditions for ligation, a serial trial of ligation testes were setup generally according to (16) including: (i) different arms: insert ratios (2:1, 3:1 and 4:1 respectively). (ii) Two ligation reaction buffers were used for all of these ratios. The first reaction buffer was that recommended by the suppliers (1x: 67 mM Tris-Hcl pH=7.8, 607 mM MgCl, 10 mM DTT, 66mM ATP), the second reaction buffer was somewhat similar to the first one, but contained 1mM spermidine. (iii) Different incubation times and temperatures were also tested, first by incubation at 4°c for 18-24 hours according to (17). The second incubation time was that at 12.5°c for 12 hours (18). Samples of ligation tests were run on 0.7% gel electrophoresis as above.

- ***In vitro* packaging and plating the genomic library:** The recombinant DNA molecules obtained from ligation experiments (4:1 ratio), were *in vitro* packaged using promega lambda DNA packaging system (promega, USA). Two different reactions were setup by the addition of 5µl ligated mixture containing 0.8 µg DNA to the first, and to the second mixture 3µl of ligated DNA (0.48 µg) was added. After one hour of incubation at room temperature (according to suppliers recommendation), 0.5 ml of lambda diluent was added, then 25 µl of chloroform. A serial dilutions (10^{-2}, 10^{-4}, 10^{-6}) of the packaged recombinant phages were plated on top agar LB plates to determine the titer of library on the P2 lysogen *E. coli* strain NM539 (19).

Results and Discussion

- **Lambda DNA isolation and λ arms purification:** EMBL3 DNA obtained in these experiments was found to be intact and with high purity ranging between 1.7-1.8 (based on absorbance measurements at 260/280 nm). Highly purified phage DNA is necessary for successful cloning experiments. DNA yields obtained from these experiments were over 100 μg per liter O/N *E. coli* culture (strain NM 538) inoculated with 5 ml of phage stock suspension 1×10^{10} particle /ml (prepared previously). The EMBL3 DNA (42.2 kb) appeared as a single intact band on 0.7% agarose gel Fig.(2, A and B).

EMBL3 DNA was found to be completely digested and efficiently with restriction enzyme BamH1. This can be noticed by clear bands shown on 0.7% agarose gel Fig.(2, C) representing, the left and right arms joined at cos sites, followed by left arm (19.3 kb), middle fragments (13.7 kb) and right arms (9.2 kb).

- **Ligation:** The results of ligation experiments between EMBL3 and partially digested bermudagrass DNA are presented in Fig. (2, A, D and C). The lack of both vector DNA bands and bermudagrass DNA smear in this figure demonstrate that the vector has been reconstructed. The optimum ligation ratio of arm: inserts tested was found to be 4:1 respectively. Better results were found using ligation

buffer containing 1mM spermidine and incubation at 4°c for 20 hrs. This may be expected since spermidine is know to be one of nucleic acids stablizers.

The successful ligation was also verified still further at the *in vitro* packaging step shown bellow.

- ***In vitro* packaging and plating the library:** Successful results were also obtained from the *in vitro* packaging reaction using (4:1) arms: inserts ratio. These results were determined by plating serial dilutions of the packaged reaction on the p2 lysogen *E. coli* (strain NM 539). A number of 1.3x 10^6 pfu/μg recombinant phage DNA was obtained Fig. (3). The reason for plating on this strain (NM 539) is based on genetic selection that it is allows only recombinant phages to lyse and form plaques (19). Normal phages are inhibited by P2 prophage, which is integrated in this strain (spi^+). Insertion of target DNA fragments

(bermudagrass DNA) into the cloning site causes a change from spi^+ to Spi^- (no longer inhibited by P2 lysogen). Therefore, no control tests are necessary in such experiments.

Cloning vectors based on lambda bacteriophage have been widely used for constructing genomic libraries (16, 20). For instance, (21) reported an efficiency of about 1×10^6 pfu/µg DNA in constructing a library of rabit tumor cells using lambda Charon 4A as cloning vector.

EMBL3 bacteriophage was used as a cloning vector first by (13) in constructing genomic libraries. They report an efficiency of 3×10^7 pfu/µg rabit DNA.

Although the genomic size of bermudagrass plant is not known, however, the efficiency of recombinant obtained may well suggest that the most of the genome is covered in this library, since most wild plants usually have small genomic sizes (3).

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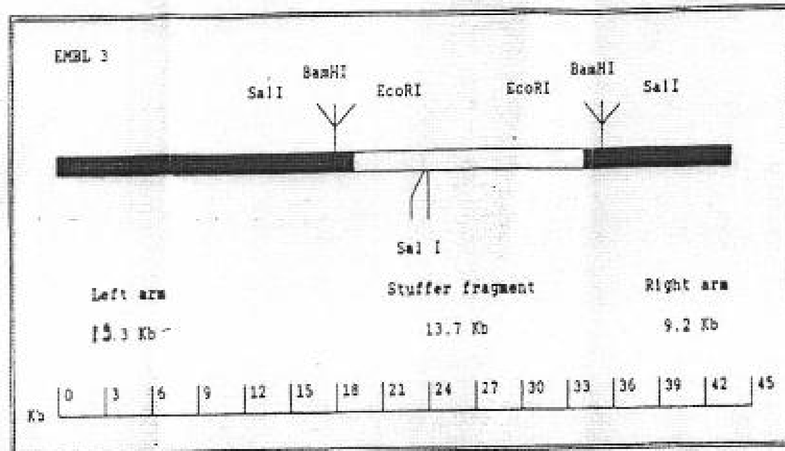

Fig.(1) A digram of the main features of the restriction map of EMBL3 bacteriophage DNA. The polylinker sequences are arranged to give EMBL3 phage with Sali- BamH1- EcoR1 sites in opposite orientations at both ends of the stuffer fragment.

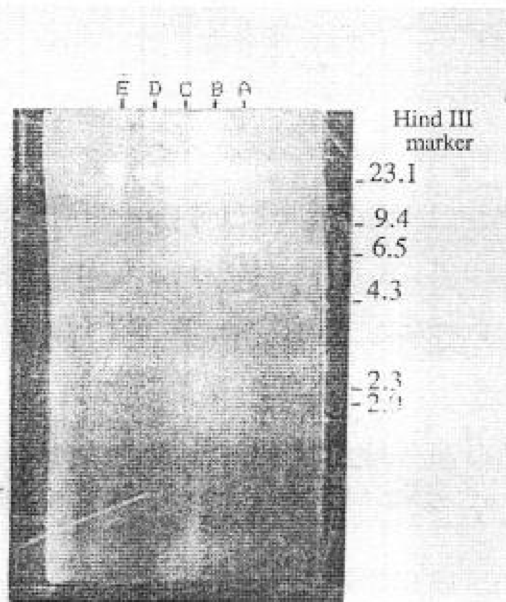

Fig. (2) Agarose gel (0.7%) electrophoresis of the ligation experiments.

A. Lambda DNA (Sam 7ts cl 857) digested with Hind III used as a molecular
weight marker.

B. Intact EMBL3 DNA.

C. EMBL3 DNA digested with BamH1, showing the left and right arms joined at cos. sites (nearest to the wells), followed by the left arm 19.3 kb , middle fragments 13.7 kb and right arms 9.2 kb.

D. Ligation using 3:1 (EMBL3 arms: bermudagrass DNA) ratio.

E. Ligation using 4:1 ratio.

The running buffer used is 1x TBE and with a constant voltage of 3 v/cm.

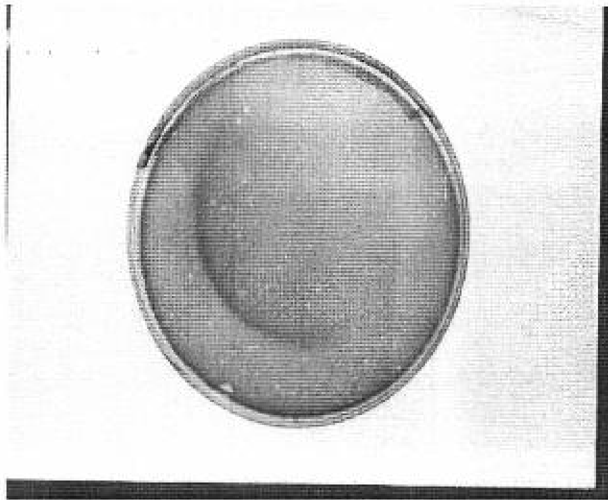

Fig. (3) A Petri dish of top agar LB plates showing the results of plating the *in vitro* packaged recombinant EMBL3 phage particles. The white spots represent plaques formed by the phage which had infected and lysed the indicator bacteria *E. coli* strain NM539.

الكلونة الجزيئية لقطع دنا كبيرة من نبات الثيل البري المقاوم للملوحة باستخدام العاثي كناقل كلونة

2. ربط وترزيم جزيئات الدنا المركبة.

سهيلة عائد إبراهيم
الشركة العامة للبستنة والغابات ، وزارة الزراعة، بغداد

الخلاصة

تمثل هذه الدراسة المرحلة الثانية من برنامج الكلونة الجزيئية والذي يهدف الى كلونة قطع كبيرة الحجم من دنا نبات الثيل البري .*Cynodon dactylon* L (رباعي المجموعة الكروموسومية) المتحمل للملوحة في بكتريا الاشريكيا القولونية *E.coli* باستخدام العاثي لامبدا EMBL3 كناقل كلونه. تم عزل وتنقية كميات وافيه من دنا العاثي EMBL3 تقدر بحوالي 100 مايكروغرام لكل لتر من الوسط الزرعي، وكانت نقاوته تتراوح بين 1.7 - 1.8 (اعتمادا على النسبه بين مقدار الضوء الممتص في جهاز الطيف الضوئي Spectrophotometer على طول موجي A 260/280). لتحضير اذرع من دنا العاثي فقد تم تقطيعه (هضمه) بانزيمي التقييد EcoR1, BamH1 .

تم الحصول على جزيئات دنا هجينه (مركبه) من خلال اجراء عملية التحام باستخدام نسب مختلفه من اذرع دنا العاثي مع قطع دنا نبات الثيل البري التي سبق وان تم تحضيرها عن طريق هضمه بانزيم التقييد Sau3A (الذي ينتج نهايات متوافقه مع الانزيم BamH1)، ولقد وجد ان النسبه 1:4 (دنا نبات الثيل: اذرع العاثي) قد اعطت افضل النتائج.