JOURNAL OF PLANT PROTECTION RESEARCH
Vol. 39, No. 1 1999

CLONING OF THE DNA FRAGMENTS REFLECTING THE OPEN READING FRAME I AND II OF THE I-18 C GENE OF *CHIRONOMUS TENTANS* I. VIII. THE LIGATIONS OF THE INSERTS WITH THE pET-3a VECTOR AND THE IDENTIFICATION OF THE BACTERIAL COLONIES, CONTAINING THE RECOMBINANT PLASMIDS

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Abstract. The technology used for the cloning of the open reading frame (ORF) I and II reflecting DNA fragments in regard to the ligations of the inserts with the pET-3a vector and the identification of the bacterial colonies, containing the recombinant plasmids, is presented. The main steps include: calculation of the molar ratios of the vector to insert DNA, the ligation reactions, transformation of the competent *E. coli* cells of the XL-1 strain, isolation of the plasmids, digestion of the plasmid preparations with the Nde I and Bam HI to indicate the presence of the inserts cloned into the plasmids, using the agarose gel electrophoresis (2% gel, 1 x TBE buffer) of the plasmids samples, with the released inserts, after the digestion, transformation of the competent BL21 (DE3) cells of *E. coli* with the isolated recombinant plasmids, identification of the bacterial cells, carrying the pET-3a plasmids with the cloned inserts and the storage of these cells.

Key words: *Chironomus tentans*, The I-18 C gene, open reading frames (ORFs), Polymerase Chain Reaction (PCR), cloning, bluescript vector, pET-3a vector, expression, T7 RNA polymerase / promoter system

I. INTRODUCTION

The experiments were performed to clone the open reading frame (ORF) I and II reflecting DNA fragments of the I-18 C gene [1-8] of *Chironomus tentans* [10] into the bluescript vector [12], as the intermediate one and finally into the pET-3a, the translational vector, in order to express these sequences, at the level of polypeptides in the T7 RNA polymerase / promoter system in BL21(DE3) cells of *E. coli* [13,15].

Here, the technology applied for the ligations of the inserts with the pET-3a vector and for the identification of the bacterial colonies, containing the recombinant plasmids, is described.

II. THE TECHNOLOGY

These were the sticky ends ligations i.e. the ends of the vector – pET-3a and the inserts, were sticky [12]. The length of the pET-3a vector was 4603 bp [15]. About 40 ng of the pET-3a vector was used per one ligation reaction and the molar ratio of the vector to insert

DNA was 1:3. The molar ratio for both inserts i.e. the ORF I and II reflecting DNA fragments have been calculated from the adequate formula and these calculations are written below.

40 ng of the vector (i.e. pET-3a) x [420 bp (i.e. the length of the insert – the ORF I reflecting DNA fragment with sticky ends) -3 (bases of the last – stop-codon of the ORF I reflecting DNA fragments, not amplified by the PCR) + 6 (additional bases of the sticky ends of the ORF I reflecting DNA fragment due to creating the Nde I and Bam HI restriction sites)]

4603 bp (the length of the pET-3a vector)

$$= \frac{40 \times 423}{4603} = 3.676$$

3.676 x 3 = 11.028 ng [the amount of the insert i.e. the ORF I reflecting DNA fragment, at the 1:3 of the vector to the insert molar ratio]

40 ng of the vector (i.e. pET-3a) x [273 bp (i.e.the length of the insert – the ORF II reflecting DNA fragment with sticky ends) – 3 (bases of the last – stop-codon of the ORF II reflecting DNA fragment, not amplified by the PCR) + 6 (additional bases of the sticky ends of the ORF II reflecting DNA fragment due to creating the Nde I and Bam HI restriction sites)]

4603 bp (the length of the pET-3a vector)

$$= \frac{40 \times 276}{4603} = 2.398$$

2.398 x 3 = 7.194 ng (the amount of the insert DNA at the 1:3 of the vector to the insert molar ratio)

The ligation reaction [12,16] mixture was composed of the following ingredients: 1) vector i.e. the pET-3a, after the Nde I and Bam HI digestion in the amount of about 40 ng of the 3.5 µl volume, 2) insert i.e. the ORF I or the ORF II reflecting DNA fragments, with sticky ends i.e. after the Nde I and Bam HI digestion in the amount calculated above i.e. at the 1:3 of the vector:insert molar ratio in the volume of 1 µl, 3) H_2O – sterile, redistilled – 2 µl, 4) 10 x ligation buffer – 1 µl, 5) 0.1 M. Dithiothreitol – 1 µl, 6) 10 mM ATP – 0.5 µl, 7) the bacteriophage T4 DNA ligase – 1 unit i.e. 1 µl (from Boehringer Mannheim, Germany). The total one reaction volume was 10 µl. The ligation reaction was performed at room temperature for 2 hours. The composition of the 10 x ligation buffer was the following: 1) 200 mM Tris-HCl pH 7.6, 2) 100 mM $MgCl_2$, 3) 500 µg / ml [final concentration] bovine serum albumin (Fraction V from Sigma Chemical Company, St. Louis, Missouri, USA). After the ligation, the particular ligation reaction products were used to transform the competent *E. coli* cells of XL-1 strain. It was done by adding the particular ligation reaction contents to the aliquots of the bacterial cells. The competent *E. coli* XL-1 cells, $CaCl_2$

treated, were prepared as described earlier and were aliquoted and frozen at – 70°C in the freezer. Each aliquot was used only once and only once was defrozen by putting it on ice in the wet ice bucket. So, the particular ligation reaction contents were added to the aliquots of the bacterial cells placed on ice and the contents of the aliquots, in Eppendorf tubes were mixed a little bit. Such samples were left on ice for 30 minutes then were put for 1 minute to 42°C in water-bath and next were put back on ice for 2 minutes. Then 900 µl of the LB medium was added to each sample in Eppendorf tube. The samples were then incubated in water-bath at 37°C for 1 hour and were mixed a little bit from time to time during that period. The grown bacteria were transferred to the agar plates (with the ampicillin) [11]. The agar plates were prepared previously, as described and were kept at +4°C. Finally, after the incubation of the bacterial cells, the 150 µl of bacteria from each sample were transferred to the agar plate and the bacteria were uniformly distributed on the agar surface. The rest of each sample was centrifuged at 6 000 RPM for 4 minutes at the room temperature in the laboratory microcentrifuge. The supernatant was discarded into the bacterial waste container (then autoclaved) and the pellet was dissolved in a drop of supernatant and was equally distributed on the surface of the new agar plate. So, each sample of the transformed bacterial cells was transferred to the two agar plates (with the ampicillin) in two different amounts i.e. the high and low amount. The agar plates were then put into the incubator and were kept there at 37°C overnight. Next day, after 20 hours of incubation, the grown colonies were counted. Next, the single colonies of the particular samples from the agar plates were picked and were grown in overnight culture of 2.5 ml of total volume each, with the ampicillin (5 µl of the ampicillin stock solution per 2.5 ml of the culture, the concentration of the ampicillin stock solution was 50 mg / ml), at 37°C on the shaker. Next, the plasmids i.e. the pET-3a from the particular cultures, were isolated using the CTAB method [9], as mentioned earlier.The concentration of the plasmid DNA preparations was estimated by the spectroscopic measurement of the Absorbance of the samples at the λ=260 nm (OD260) and was calculated from the following formula:

OD260 of the sample x dilution (i.e. 500) x 50 =the concentration of the plasmid DNA

The multiplication by 50 is based on the fact that the 1 OD260 unit corresponds to about 50 µg / ml of double stranded DNA [14]. The identification of the particular bacterial colonies, containing the recombinant pET-3a plasmids i.e. containing the ORF I and II reflecting DNA fragments as the inserts, was performed by the digestion of the mini-preparations [9] of the plasmids with the Nde I and Bam HI. The composition of the Nde I reaction mixture was the following: 1) pET-3a DNA – about 12 µg in the volume of 16.5 µl, 2) 10 x buffer H (of Boehringer Mannheim, Germany) – 2 µl, 3) Nde I (from Boehringer Mannheim) – 15 units i.e. 1.5 µl. The total reaction volume was 20 µl. The Nde I digestion was performed by incubation of the above reaction mixture of the particular samples at 37°C in water-bath for 30 minutes. Then additional 1.5 µl of the Nde I was added to each reaction mixture and the incubation was continued for another 30 minutes at 37°C. After the reaction, the DNA was precipitated. It was done first by increasing the volume to 300 µl by adding the sterile, redistilled H_2O and then 30 µl (i.e. 1 / 10 of the volume) of the 3 M sodium acetate pH 5.4 and 2.5 volume of the ethanol. The samples were kept at – 70°C in

the freezer for 1 hour. The precipitated DNA was collected by centrifugation for 30 minutes at 10 000 x g in the cold room. The supernatant of each sample was discarded and the pellet was washed with small amount (about 150 µl) of ethanol and was dried using the speed-vac, for 3 minutes. Next, the plasmid DNA was dissolved in 16 µl of the sterile, redistilled H_2O and was subjected to the Bam HI digestion. The composition of the Bam HI digestion mixture was the following: 1) pET-3a, after the Nde I digestion in 16 µl of sterile, redistilled H_2O, 2) 10 x buffer B (from Boehringer Mannheim, Germany) – 2 µl, 3) Bam HI (from Boehringer Mannheim) – 2 µl (10 units / µl). The total reaction volume was 20 µl. The samples were incubated at 37°C in water-bath for 1 hour. Finally, after the termination of the reaction, the Bam HI was inactivated by incubation at 65°C for 10 minutes in water-bath. The pET-3a plasmid mini-preparations, after the Nde I and Bam HI digestion were analysed by the agarose gel electrophoresis (2% gel, 1 x TBE buffer) [11] to indicate the presence of the inserts i.e. the ORF I and II reflecting DNA fragments. The recombinant pET-3a vectors carrying the ORF I and II reflecting DNA fragments, isolated from the *E. coli* XL-1 cells, as described above, were used (i.e. the whole plasmid constructs) to transform the final host cells i.e. the BL21(DE3) cells of *E. coli* [13,15]. This step was necessary because the competence of the BL21(DE3) cells was much smaller than the competence of the XL-1 cells of *E. coli*. The BL21(DE3) cells were prepared, as described earlier and so were aliquoted and frozen at -70°C. The frozen bacterial cells, before their transformation, were kept on ice in a wet ice bucket. The content of the transformation mixture was the following: 1) 200 µl of the bacterial cells (i.e the BL21(DE3) cells of *E. coli*), 2) 24 µl of the 10 x TCM buffer, 3) 20 µl of the recombinant constructs of the pET-3a i.e. containing the ORF I or II reflecting DNA fragments, as the inserts, in the amount of about 30 ng (the standard amount of the vector i.e. the pET-3a for testing the competence of these cells was 1 ng). These ingredients were put into the one Eppendorf tube, mixed a little bit and were kept on ice for 40 minutes. The 10 x concentrated TCM buffer was composed of: 1) 100 mM Tris-HCl pH 7.5, 2) 100 mM $MgCl_2$, 3) 100 mM $CaCl_2$. After the incubation on ice, the samples of the bacterial cells were transferred to 42°C water-bath for 2 minutes and then were put back on ice for 2 minutes. Finally 800 µl of the LB medium [11] was added to each of the bacterial samples and the samples were incubated for 60 minutes at 37°C in water-bath. Next, the bacterial cells samples were transferred to the previously prepared agar plates with the ampicillin (as described earlier), so that each sample was transferred to two agar plates at low and high density of the transferred and distributed cells, as described above. The agar plates were then put into the incubator and were kept there overnight, for 20 hours. Next, the single bacterial colonies were isolated and were transferred to the LB medium (2.5 ml total volume, with the ampicillin, as described earlier) and were grown overnight. Next, the mini-preparations of the pET-3a recombinant plasmids from these colonies were performed, according to the CTAB method [9], as mentioned earlier. The concentration of the plasmid DNA samples was estimated by the spectroscopic measurement at the λ=260 nm [14], as described in detail earlier. The pET-3a plasmid preparations were digested with Nde I and Bam HI to release the inserts i.e. the ORF I and II reflecting DNA fragments. So, after the digestion the plasmid samples were analysed using the agarose gel electrophoresis (2% gel, 1 x TBE buffer) [11] to confirm the presence of the inserts in the

pET-3a plasmids of the particular bacterial cultures. About 12 μg of the pET-3a plasmid DNA was used for the digestion with Nde I and Bam HI for each sample. The composition of these reaction mixtures, as well as the steps of the ethanol precipitation of the plasmid DNA with the sodium acetate, after the Nde I digestion and the preparation of such DNA sample for the Bam HI digestion, were described earlier. So, finally, the bacterial cells of BL21(DE3) strain of *E. coli* carrying the inserts i.e. the ORF I and II reflecting DNA fragments of the I-18 C gene of *Chironomus tentans* were obtained. The presence of these inserts in the recombinant pET-3a plasmids of the BL21(DE3) cells was confirmed by Nde I and Bam HI digestion with the agarose gel electrophoresis of the digestion reactions product, as described above. Certain amounts of these bacterial cells were frozen in the HMFM medium and were stored in the – 70°C freezer. The composition of the HMFM medium is as follows: 1) 3.6 mM K_2HPO_4, 2) 1.3 mM KH_2PO_4, 3) 2 mM sodium citrate, 4) 1 mM $MgSO_4 \cdot 7H_2O$, 5) 4.4% [v / v] glycerol. Finally, the obtained bacterial cells, containing the recombinant plasmids were used to express, at the translational level, the ORF I and II reflecting DNA fragments of the I-18 C gene of *Chironomus tentans* in the T7 RNA polymerase / promoter system [13,15].

III. LITERATURE

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KLONOWANIE FRAGMENTÓW DNA ODZWIERCIEDLAJĄCYCH OTWARTĄ RAMĘ ODCZYTU I I II GENU I-18 C *CHIRONOMUS TENTANS*. VIII. LIGACJE WSTAWEK Z WEKTOREM pET-3a I IDENTYFIKACJA KOLONII BAKTERYJNYCH, ZAWIERAJĄCYCH REKOMBINACYJNE PLAZMIDY

STRESZCZENIE

Przedstawiono technologię użytą do klonowania fragmentów DNA odzwierciedlających otwartą ramę odczytu I i II genu I-18 C *Chironomus tentans* w zakresie ligacji wstawek z wektorem pET-3a oraz identyfikacji kolonii bakteryjnych, zawierających rekombinacyjne plazmidy. Główne etapy obejmują: obliczenie stosunków molarnych wektora do DNA wstawki, reakcje ligacji, transformację komórek kompetentnych *E. coli* szczepu XL-1, izolację plazmidów, trawienie preparatów plazmidów Nde I i Bam HI w celu wskazania obecności wklonowanych do plazmidów wstawek, uwolnionych z tych plazmidów poprzez trawienie wymienionymi enzymami restrykcyjnymi. Analizę produktów trawienia poszczególnych próbek plazmidów wykonano przy użyciu elektroforezy w żelu agarozowym (2% żel, 1 x stężony bufor TBE). Następne etapy to transformacja komórek kompetentnych *E. coli* BL21(DE3) preparatami wyizolowanych zrekombinowanych plazmidów pET-3a i identyfikacja komórek bakteryjnych, zawierających plazmidy pET-3a z wklonowanymi wstawkami oraz przechowywanie tych komórek.