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Cloning of *cryIA* fragment encoding toxin domain using pGEM-T vector

Kloning fragmen gen cryIA *penyandi domain toksin menggunakan vektor pGEM-T*

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ABSTRACT

Cry is one of the most widely used genes in plant genetic engineering for pest resistance. Cloning of this resistance-bearing gene from *Bacillus thuringiensis* has been conducted by screening the genomic library. The disadvantages of such a cloning approach are the complexity of the procedure and the screened clones are often not intact. They contain useless flanking regions or a part of open reading frame (ORF) is truncated. A simpler and direct cloning procedure is amplification of the gene using specific primer, and ligation of the amplification product into T-vector. This research aims to clone *cryIA* gene with that of direct procedure. The PCR product of *cryIA* gene fragment was ligated with pGEM-T vector, followed by transformation into *Escherichia coli* DH5α and JM109. Transformed cells were grown on selection media containing 50 mg/l ampicillin, 40 mg/l X-Gal, and 0.1 mM IPTG. Analysis of recombinant plasmid (5 kb) from white colonies were performed on agarose gel. Results showed that of the four *cryIA* fragments used, those from *B. thuringiensis* subsp. *kurstaki* of the FL and CD isolates produced 2 and 3 clones and were identified to carry pGEM/*cryIA(c)* recombinants.

[*Keywords*: Cloning; *cryIA* gene; pGEM-T vector; PCR product]

ABSTRAK

Cry adalah salah satu gen yang banyak digunakan dalam rekayasa genetika tanaman untuk ketahanan terhadap hama. Pada umumnya kloning gen penyandi sifat ketahanan yang diperoleh dari *Bacillus thuringiensis* dilakukan melalui seleksi pustaka genom. Kelemahan kloning dengan cara ini adalah prosedurnya relatif rumit dan klon yang diperoleh kadang-kadang tidak utuh, yaitu gen terpotong atau mengandung potongan gen yang tidak dikehendaki. Cara yang lebih sederhana adalah dengan mengamplifikasi gen yang diinginkan menggunakan primer spesifik, kemudian ligasi produk amplifikasi tersebut ke dalam vektor T. Penelitian ini bertujuan untuk mengklon gen *cryIA* secara langsung dengan menggunakan vektor pGEM-T. Gen *cryIA* produk PCR diligasikan ke dalam vektor kloning pGEM-T, kemudian ditransformasikan ke dalam *Escherichia coli* DH5α dan JM109. Sel hasil transformasi ditumbuhkan pada media seleksi mengandung 50 mg/l ampisilin, 40 mg/l X-Gal, dan 0,1 mM IPTG. Koloni putih yang tumbuh dianalisis ada tidaknya plasmid rekombinan (5 kb) pada gel agarosa. Hasil percobaan menunjukkan bahwa dari empat fragmen gen *cryIA* produk PCR yang digunakan, dua di antaranya yaitu yang berasal dari *B. thuringiensis* subsp. *kurstaki* isolat FL dan CD menghasilkan masing-masing 2 dan 3 klon yang mengandung rekombinan pGEM/*cryIA(c)*.

[*Kata kunci*: Kloning; gen *cryIA*; vektor pGEM-T; produk PCR]

INTRODUCTION

Bacillus thuringiensis-based biopesticides are used worldwide for controlling the larval forms of economically important pests. These toxins are considered to be environmentally safe. However, a problem related to the commercial *B. thuringiensis* preparation is their limited field stability (Bora *et al.*,1994). Therefore, expressing insecticidal crystal protein (ICP) genes of *B. thuringiensis* in organisms that are stable in the environment (Bora *et al.*, 1994; Herrera *et al.*, 1994; Lampel *et al.*, 1994) or expressing directly in plant to produce transgenic plants resistant to certain pest (Perlak *et al.*, 1991) maybe useful to overcome this problem.

Gene cloning is an important step in plant genetic engineering for pest resistance. Cloning of *cry* genes from *B. thuringiensis* has mostly been conducted through screening of genomic library using labelled probes (Schnepf *et al.*, 1981; Donovan *et al.*, 1988; Von Tersch *et al.*, 1991; Bora *et al.*, 1994; Herrera *et al.*,

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1994). The screening requires both the construction of a genomic library and the availability of the probe. Information on either the amino acid sequence of a specific polypeptide or the nucleotide sequence of DNA template that enables the synthesis of the probe is inevitable. Therefore, a sequencing experiment is necessary.

In addition to the complexity of the procedure, the other disadvantage of using genomic library screening method to clone *cry* gene is that clones selected are frequently not intact. They often carry flanking regions which have to be removed from the gene. Sometimes, the clones have truncated open reading frame (ORF).

An easier and more direct approach to clone *cry* gene from *B. thuringiensis* is through amplification of *cryIA* fragment using specific primer. Amplification product of *cry* gene fragment directly ligated into cloning vector followed by transformation into *Escherichia coli.* Taking advantage of the tendency that a PCR product has an extra adenine on the 3' ends of each strands, an amplified *cry* fragment shall be efficiently ligated with T-vector. T-vector is constructed by enzymatically adding T on the 3' ends of a blunt-ended cloning vector such as pUC and pGEM (Marchuk *et al.*, 1990). Available nucleotide sequence of *cry* genes (Honee *et al.*, 1988; Hofte *et al.*, 1990; Perlak *et al.*, 1991) which have some conserved regions (Hofte and Whiteley, 1989) make application of this cloning strategy possible.

This report describes cloning of *cryIA* genes amplified from several *B. thuringiensis* DNAs using pGEM-T vector. This work was part of a research project aimed to produce estate crops resistant to Lepidopteran.

MATERIALS AND METHODS

Bacterial strains of JM109 and DH5α utilized in the cloning of the plasmid DNA were obtained from Promega and Gibco BRL, respectively. Cloning vector of pGEM-T, enzymes, and DNA purification kit were from Promega. *Taq* DNA polymerase and PCR reagents were from either Promega or Boehringer Mannheim Indonesia. Specific DNA primers were ordered from Operon Technologies, Inc. These DNA primers were specifically designed to amplify *cryIA* fragments encoding toxin domain of δ-endotoxin (Budiani *et al.*, 1996) based on *cryIA*(*b*) and *cryIA*(*c*) sequences (Perlak *et al.*, 1991; Von Tersch *et al.*, 1991). Isolates of *B. thuringiensis* were obtained from the collections of Bogor Research Institute for Estate Crops (BRIEC) and Indonesian Sugar Research Institute (ISRI), Indonesia. Quantum Preps kit for plasmid preparation was bought from Bio-Rad.

Isolation of cryIA fragments

CryIA fragments encoding the toxin domain of δ-endotoxin were isolated from *B. thuringiensis* using PCR technique followed by purification using DNA purification kit as described previously (Budiani *et al.*, 1996). A pair of primers synthesized based on *cryIA(c)* sequence (Von Tersch *et al.*, 1991) were used for amplifying *cryIA(c)* fragment. The sequences of both primers were 5'-CGCATGTTTGACTTTCTCGG-3'. DNA amplification was conducted with 35 reaction cycles, 100 μM dNTPs and 90 minutes extension time.

Cloning with pGEM-T vector

The gel purified 2-kb *cryIA* fragment of about 33 ng was ligated with 50 ng of pGEM-T vector according to the manufacture instruction. Ligation reaction was performed at 4°C for overnight in a reaction volume of 20 μl. The ligation mixture was transformed into the competent cells by heat shock treatment (Doyle, 1996). After induction for antibiotic resistance, the cultures were plated on Luria Bertani agar (LB) media containing 0.1 mM isopropyl-b-thiogalactopyranoside (IPTG), 40 g/l 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), and 50 mg/l ampicillin and incubated overnight at 37°C to select for *E. coli* transformans.

The white colonies grown on the selection media, which were putatively transformed cells carrying recombinant plasmids of pGEM/*cryIA*, were examined for the presence of true recombinants. Single colonies were cultured in 2,5 ml LB media (Sambrook *et al.*, 1989) containing 50 g/l ampicillin for an overnight at 37°C. DNA plasmids were isolated from the liquid cultures using the Quantum Prep method and electrophoresis on 1% agarose gel to identify the presence of a 5-kb DNA band representing pGEM/*cryIA* true recombinant.

RESULTS AND DISCUSSION

The *cryIA* fragments produced from PCR have been cloned with pGEM-T vector in bacterial strains of both JM109 and DH5α. Table 1 shows that the number of

Table 1. Transformation of pGEM/*cryIA* construct into competent cells of JM109 and DH5α.

E. coli strain	Isolate source	No. of colonies[1]			Positive colonies
		Total	White	Blue	
JM109	Control (+)	8	3	5	nt
JM109	Kurstaki BP	4	2	2	0
JM109	Kurstaki CD	4	2	2	0
JM109	Kurstaki FL	15	5	10	2
JM109	Kurstaki DP	4	2	2	0
JM109	Control (-)	4	1	3	nt
DH5α	Control (+)	52	45	7	nt
DH5α	Kurstaki BP	23	15	8	0
DH5α	Kurstaki CD	44	4	40	3
DH5α	Kurstaki FL	10	3	7	0
DH5α	Kurstaki DP	5	2	3	0
DH5α	Control (-)	21	1	20	nt

[1]Average from three replicates
nt = not tested
Control (+): Positive control (pGEM-T was ligated with DNA control)
Control (-) : Negative control (no insert DNA in ligation mixture)

colonies grown on selection media were low either for white or blue colonies. Plasmid analysis showed that not all the white colonies were recombinant clones. Furthermore, the percentage of pGEM-T/*cryIA* true recombinant gained was considered low compared to the corresponding white colonies formed on the selection media which was about 6% in JM109 and 4.3% in DH5α (Table 1). Of the four *B. thuringiensis* isolates positively tested for the presence of *cryIA* gene, only those amplified from the *B. thuringiensis* isolates of FL and CD produced true recombinant of the pGEM/*cryIA*.

The pGEM-T vector used was linearized by restricted digestion on its *lacZ* region, therefore the white colonies could represent both transformed cells with pGEM-T or pGEM/*cryIA*. On the other side, blue colonies represented transformed cell with pGEM-T which might lose its T-overhang, allowed religation of the vector, therefore the *lacZ* gene was expressed.

There are several factors affecting ligation process. One of that factors is insert:vector ratio. The low yield of recombinant clones in this experiment was possibly due to the ligation process of *cryIA* fragment with pGEM-T vector had occurred inefficiently. Perhaps, the ratio of insert:vector was too low. Therefore there were many white colonies containing only vector 3 kb, instead of the 5-kb recombinant plasmid. The choice of 1:1 insert-vector ratio in the experiment was based on the optimum ratio for ligation of pGEM-T with DNA control. According to Doyle (1996), the optimum molar ratio of insert:vector for 50 ng of pGEM-T vector with DNA control is 1:1. However, the optimum ratio in a DNA ligation experiment might be different from one another and it can be somewhere between 1:8 to 8:1 (Doyle, 1996). In the case of ligation between *cryIA* fragment and pGEM-T, the optimum ratio should be higher than that used in this experiment. Furthe. research needs to try several ratios of pGEM-T/*cryIA*, to produce a high number of recombinant plasmids.

The presence of recombinant plasmids in all white colonies was determined through a screening of the DNA plasmids prepared quickly from the colonies by using the method of Speedy (Schnable, 1991) on an agarose gel electrophoresis. Of the white colonies produced, five indicated to have a 5-kb DNA band. These five clones were further examined by preparing plasmids using Quantum Preps method which resulted in a purer DNA plasmids. Electrophoretic analysis confirmed the presence of the recombinant pGEM/*cryIA*. Results of this analysis are presented in Fig. 1. All the tested colonies demonstrated to have 5-kb DNA band meaning they contained the recombinant plasmids.

To genetically engineer crops resistant to a specific pest using *cryIA* gene, it is required to utilize *cry* gene encoding a toxin specifically lethal to the pest. To examine if the *cryIA* of the pGEM/*cryIA* recombinant expressing toxin specific to a lepidopteran pest, the *cryIA* will be subcloned from the pGEM vector to express vector of pET5a. The gene will be cloned at the *NdeI* site of the pET5a so that it will produce a non-fusion protein. For this subcloning purposes, the orientation of the *cryIA* in the pGEM needs to be determined. The assay was carried out by double digestion of the pGEM/*cryIA* using *SalI*/*Eco*RI and electrophoresis on agarose gel.

Visualization of the digested and EtBr-stained DNA fragment on the gel under UV illumination is shown in Fig. 2A. In all four clones tested, each clone provides three DNA bands with size of 0.34, 0.73, and 3.93 kb. The DNA fragment analyses suggested that the pGEM/*cryIA* clones carrying *cryIA* having a forward orientation with respect to the following expression vector pET5a (Fig. 2B). Such orientation enables direct subcloning and the *cryIA* gene might be expressed directly in appropriate host cells such as JM109 (DE3) or BL21 (DE3) which has RNA polymerase gene required by the promoter T7 of the pET5a for the activation.

More than 40 *cry* genes have been cloned and sequenced (Udayasurian *et al.*, 1994). However, there

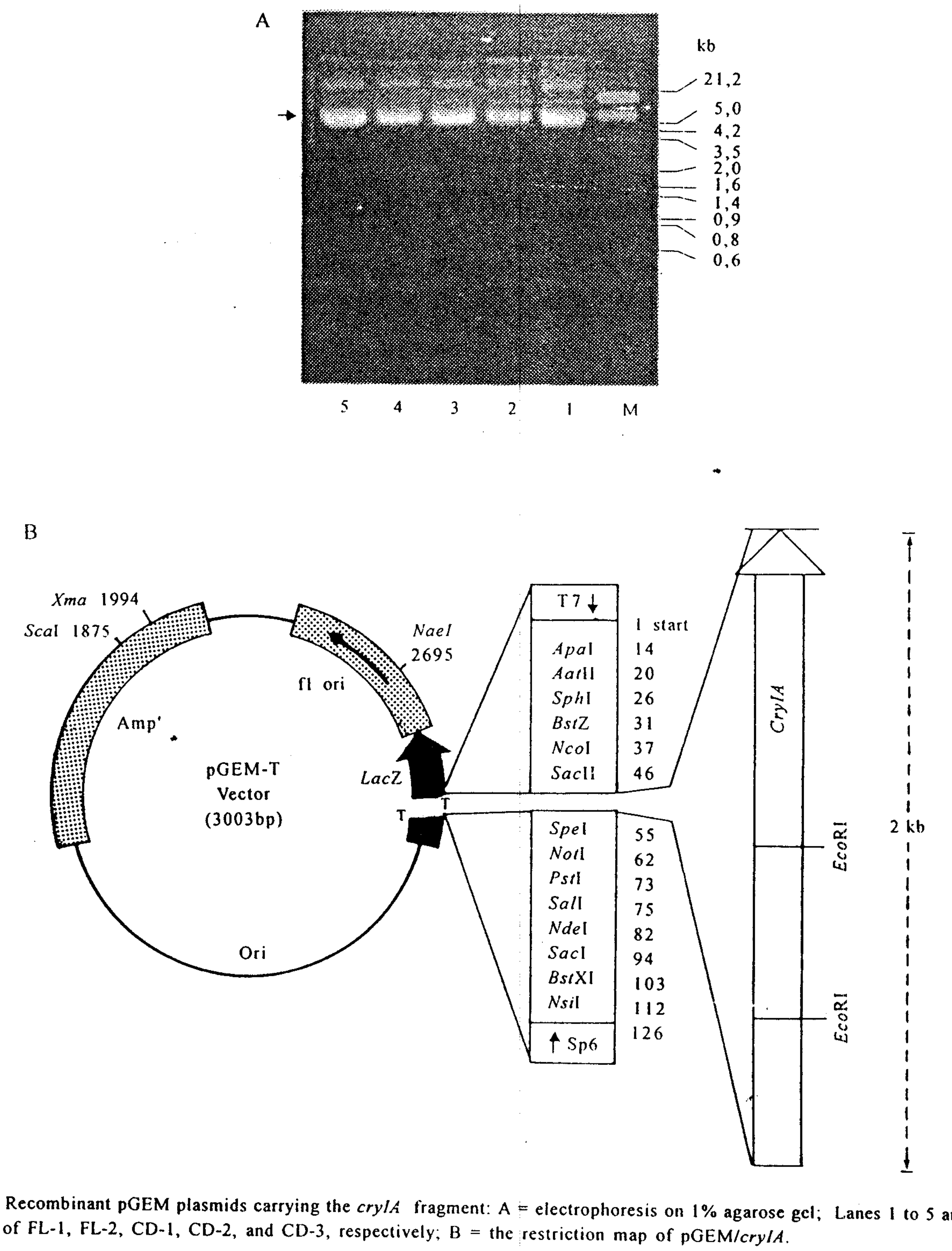

Fig. 1. Recombinant pGEM plasmids carrying the *cryIA* fragment: A = electrophoresis on 1% agarose gel; Lanes 1 to 5 are the clones of FL-1, FL-2, CD-1, CD-2, and CD-3, respectively; B = the restriction map of pGEM/*cryIA*.

was limited report, if any, *cry* gene cloning directly from PCR product using T vector. Sismindari and Sudjadi (1996) reported their work on cloning of coat protein gene of soybean mozaic virus (SMV) with the PCR approach. The amplification product (0.8 kb) was treated by S1 nuclease to make blunt ends followed by ligation into pUC18 cloning vector which has been digested by *Sma*I restriction enzyme. It is obvious that technically, our cloning procedure using pGEM-T vector was simpler compared with cloning of PCR product with blunt ends.

The *cryIA* gene cloned in this experiment will be sequenced and modified prior to transformation into plant cell to produce transgenic plant resistant to

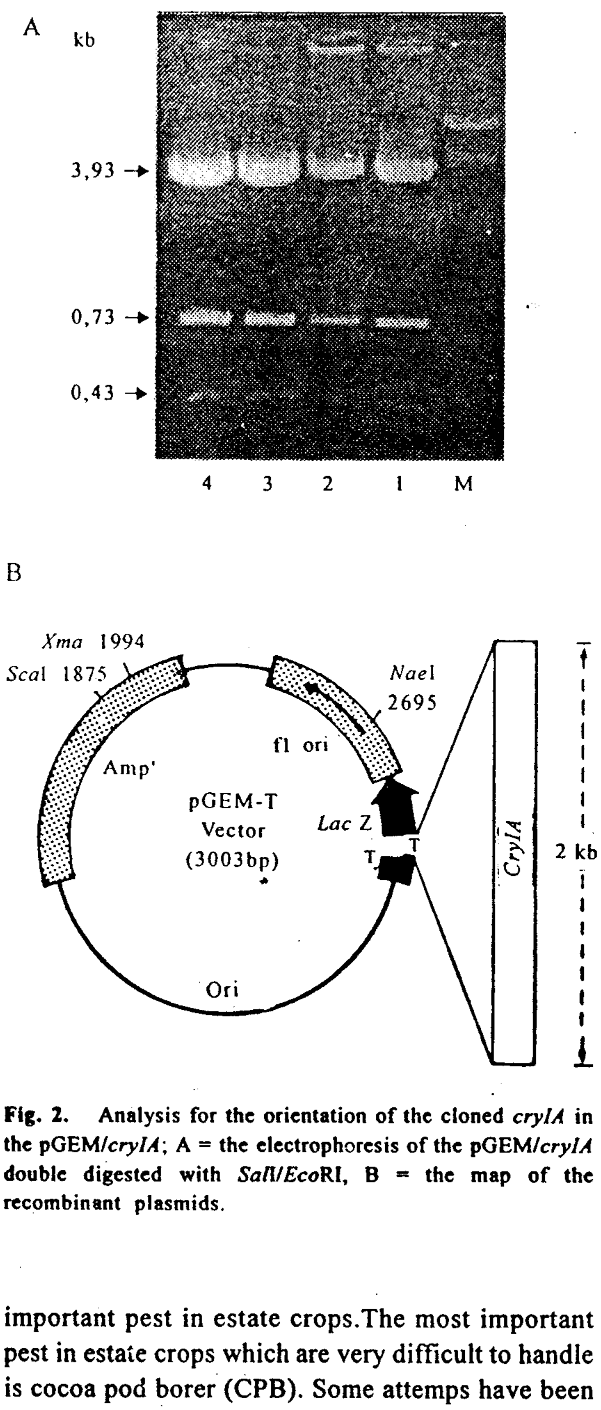

Fig. 2. Analysis for the orientation of the cloned *cryIA* in the pGEM/*cryIA*; A = the electrophoresis of the pGEM/*cryIA* double digested with *SalI*/*Eco*RI, B = the map of the recombinant plasmids.

important pest in estate crops.The most important pest in estate crops which are very difficult to handle is cocoa pod borer (CPB). Some attemps have been done to overcome this problem, but none was effectively worked out. Another important insect is *Setothosea asigna*, that attack oil palm leaf. Both insects are Lepidoptera. Therefore, transformation of the *cryIA* gene into cacao or oil palm cell would be very useful to overcome the problem.

CONCLUSION

PCR-amplified *cryIA* fragment encoding toxin domain of the δ-endotoxin has been cloned directly using pGEM-T vector. With insert:vector molar ratio of 1:1, the ligation was considered low. Of the four *B. thuringiensis* isolates used, only CD and FL isolates yielded positive clones with pGEM/*cryIA* of 4.3 and 6%, respectively.

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