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Detection of *cry*II gene from *Bacillus thuringiensis* using Polymerase Chain Reaction (PCR)

F D Wahyuni[1]*, Seprianto[1], H Saraswati[1]

[1] Biotechnology Department, Faculty of Health Sciences, Universitas Esa Unggul, Jakarta 11510, Indonesia

*febriana@esaunggul.ac.id

Abstract. *Bacillus thuringiensis* is one species of bacteria that has been applied as a microbiological control agent for pests and a vector of plant disease. The availability of *Cry* proteins in *B. thuringiensis* can be acted as a specific insect exterminator that only toxic to certain insects. The *cry*II gene is an example of a type of *cry* gene that encodes a *Cry*II Protein. The *Cry*II protein is toxic to Lepidoptera insects which can attack *Helicoverpa armigera* species which is a corn borer. Polymerase Chain Reaction (PCR) is a general method that can be used to amplify the gene. This research purposed to design a good primer candidate for *cry*II gene amplification from *B. thuringiensis*. *In silico* analysis for designing cryII primer was carried out using some software, such as BLAST for searching *cry*II gene sequence, Bioedit for sequences alignment, and DINAmelt for analyzing dimer structure of primers. Ten primer candidates were successfully obtained based on the result of the primer3 software. A pair of primer was selected to amplify the *cry*II gene, with forward primer 5'-GGTAGTGGACCACAGCAGAC-3'and reverse primer 5'-TCTTCTGGCGCCAAATGGAT-3'. This primer has fulfilled good primer characteristics because it does not cause dimer structure and the resulting amplicons do not form secondary structures. Amplification of the *cry*I gene by PCR method using selected primer resulting in a PCR product with a length of approximately 800 bp.

1. Introduction

Insect pests and plant pathogens are barriers in the production of a commodity in tropical and humid countries. Even in certain plants such as rice, pest insects are still a major barrier and become a serious problem, i.e brown planthopper and stem borer [1]. One of control effort that is routinely done by farmers is spraying insecticides. The utilize of insecticides has allegedly affected the environment and caused resistance to target insects. Concerns about the negative effect of the utilize of insecticides have increased public concern to bioinsecticides as an alternative to degrading pest populations [2].

Bacillus thuringiensis (Bt) is a gram-positive bacteria that can produce crystal proteins during sporulating [3]. *B. thuringiensis* bioinsecticides constitute 90-95% of the bioinsecticides commercialized for utilization by farmers in many countries [2]. The benefit of using this bacterium as a biopesticide, include a protein produced by *B. thuringiensis* which is a specific buffer and is called a *Cry* protein or also called as δ-endotoxin. *Cry* protein is only toxic to certain types of insects [3]. There are many kinds of cry gene from *B. thuringiensis*. One of them is *cry*II gene, known to be able to kill insects from the Lepidoptera group [4]. Insects of this type Lepidoptera are pests that attack *Helicoverpa armigera* species which is a corn borer [5]. The expansion of recombinant DNA technology is now probable to execute the transformation of *cry* genes into plant cells. The

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introduction of *cry* genes into plant genomes is expected to produce plants capable of expressing endotoxins that can induce pest insect death. In this way, it is also expected to degrade the utilize of insecticides that can pollute the environment.

In the present study, the genetic diversity and toxicity of *B. thuringiensis* isolates were investigated by the characterization of native isolates originating from the soil, fig leaves, and fruit [6]. Advance in technology in the area of molecular biology has encouraged scientists to isolate chromosomes or plasmids that comprise *cry* genes. The *cry* gene is a DNA sequence that codes for the expression of *Cry* proteins [3]. Many studies have been carried out for *cry* gene amplification [7,8]. Polymerase Chain Reaction (PCR) is one method that can be used for *cry* gene amplification which will be implemented in this study by applying a discipline of bioinformatics.

Bioinformatics is an interdisciplinary field as a composite of biology, especially in molecular biology and computing using computer and software support [9]. One of the most principal purposes of bioinformatics is to design a primer sequence. Primer is a component that plays a primary criterion in the PCR process [10]. A good primer can identify a specific sequence on a genome or template and restricted to a target sequence. Unspecified primers may cause amplification of other regions in the genome that are untargeted or otherwise there are no amplified genome regions. To obtain a primer that fulfills the good characteristic for amplification, in silico design is carried out [9]. The purpose of this study was to analyze the composite of primer pairs used in the PCR process to amplify the *cry*II gene from *B. thuringiensis*. The *cry*II gene that will be amplified is a domain of the total *cry*II gene sequences that already exist in the genebank.

2. Methods

2.1. Bacterial DNA Extraction

Bacteria culture of *B. thuringiensis* was obtained from the Indonesian Culture Collection (InaCC) from Indonesian Institute of Sciences in Cibinong with code number B432 and B327. *B. thuringiensis* DNA extraction method was used the gSYNC DNA Extraction kit from *Geneaid*.

2.2. Design of Primer

As a template for designing primers, the *cry*II *B. thuringiensis* gene (accession number AF047038.1) downloaded from NCBI was used. These sequences were obtained from the NCBI website (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov). The gene then was analyzed with Basic Local Alignment Search Tools (BLAST) software with other *B. thuringiensis* strains so that identity values from the highest to the lowest were obtained. Gene sequences that have high identity values are used in the multiple alignment method to obtain conserved sequences. The assignment of conserved sequences is determined using the Bioedit program. The sequence of bases obtained is aligned with the ClustalW Program and then the one which has the most similarity in its constituent bases is similar to 90-100%. As a basis for tracking primary attachments.

Determination analysis of DNA primer was performed with Primer3 software, and manually selected the most sustainable region by considering the region is the cipher sequence, with general requirements, including the number of nucleotides, GC content and there is no possibility of complementarity between bases in one primary chain or between one primer with another.

Occasionally the primers designed can identify the sequence of themselves, bind to one another to establish a structure called a dimer. This can be a matter because primers will tend to stick together, not with the target gene and this can degrade DNA concentration. For this reason, it is important to do a dimer prediction analysis using available software to predict the appearance of dimers in primary candidates, namely DINAmelt (http://unafold.rna.albany.edu/?q=DINAMelt) made by NM Markham and Michael Zucker of Rensselaer Polytechnic Institute [11,12].

2.3. *Amplification of the cryII Gene by PCR*
Amplification of the *cry*II gene using specific primer cry2 forward 5'-GGTAGTGGACCACAGCAG AC-3' and cry2 reverse 5'-TCTTCTGGCGCCAAATGGAT-3' with a total volume of 25 µl containing 1 µl DNA genome, 12.5 µl PCR HS redmix master mix (Bioline), 1 µl for each primer and 9.5 ddH2O. PCR amplification was carried out as many as 35 cycles using Sensoquest. One cycle consists of 3 stages, namely denaturation, attachment, and elongation. The pre-denaturation stage is carried out for 3 minutes at a temperature of 94°C once, the denaturation stage is 1 minute, and the primer attachment stage is at 54°C for 30 seconds, the extension of the DNA chain at a temperature of 72°C for 1 minute. In the last cycle, the chain was extended longer at 72°C for 7 minutes.

2.4. *PCR Electrophoresis*
The PCR products were migrated into 1% agarose gel under 100 volt for 30 minutes. A 1 kb DNA ladder is used as a marker. Fluorosafe DNA stain was used for gel staining which is directly added to the agarose gel. A Gel containing DNA fragments was visualized using UV Trans Illuminators and documented using the Digibox Camera Documentation System gel.

3. Results

3.1. *Sequence of cryII Gene*
Based on the search results of the *cry*II gene sequences in genebank with accession number AF047038.1 (Figure 1.), *cry*II gene sequences were obtained from *B. thuringiensis* consisting of 1937 bp. The BLAST process was carried out using *B. thuringiensis cry*II gene for insecticidal crystal protein as a determinant strain with other *B. thuringiensis* strains. It is because it has a long DNA sequence. BLAST results obtained an identity value of 100%-95% *cry*II gene against several strains of *B. thuringiensis*.

```
   1 ggatcccata tgaatagtgt attgaatagt ggaagaacaa ctatttgtga tgcgtataat
  61 gtagtagccc atgatccatt tagttttgaa cataaatcat tagataccat ccaaaaagaa
 121 tggatggagt ggaaaagaac agatcatagt ttatatgtag ctcctgtagt cggaactgtg
 181 tctagttttt tgctaaagaa agtggggagt cttattggaa aaaggatatt gagtgaatta
 241 tgggggataa tatttcctag tggtagtaca aatctaatgc aagatatttt aagggagaca
 301 gaacaattcc taaatcaaag acttaataca gataccettg ctcgtgtaaa tgcagaattg
 361 atagggctcc aagcgaatat aagggagttt aatcaacaag tagataattt tttaaaccct
 421 actcaaaacc ctgttccttt atcaataact tcttcggtta atacaatgca gcaattattt
 481 ctaaatagat taccccagtt ccagatacaa ggataccagt tgttattatt acctttattt
 541 gcacaggcag ccaatatgca tctttctttt attagagatg ttattcttaa tgcagatgaa
 601 tggggtattt cagcagcaac attacgtacg tatcgagatt acctgagaaa ttatacaaga
 661 gattattcta attattgtat aaatacgtat caaactgcgt ttagagggtt aaacacccgt
 721 ttacacgata tgttagaatt tagaacatat atgtttttaa atgtatttga atatgtatcc
 781 atttggtcat tgtttaaata tcagagtctt atggtatctt ctggcgctaa tttatatgct
 841 agcggtagtg gaccacagca gacacaatca tttacagcac aaaactggcc atttttatat
 901 tctcttttcc aagttaattc gaattatata ttatctggta ttagtggtac taggctttct
 961 attaccttcc ctaatattgg tggtttaccg ggtagtacta caactcattc attgaatagt
1021 gccagggtta attatagcgg aggagtttca tctggtctca taggggcgac taatctcaat
1081 cacaacttta attgcagcac ggtcctccct cctttatcaa caccatttgt tagaagttgg
1141 ctggattcag gtacagatcg agagggcgtt gctacctcta cgaattggca gacagaatcc
1201 tttcaaacaa ctttaagttt aaggtgtggt gcttcttcag cccgtggaaa ttcaaactat
1261 ttcccagatt attttatccg taatatttct ggggttcctt tagttattag aaacgaagat
1321 ctaacaagac cgttacacta taaccaaata agaaatatag aaagtccttc gggaacacct
1381 ggtggagcac gggcctattt ggtatctgtg cataacagaa aaaataatat ctatgccgct
1441 aatgaaaatg gtactatgat ccatttggcg ccagaagatt atacaggatt tactatatcg
1501 ccaatacatg ccactcaagt gaacaatcaa actcgaacat ttatttctga aaaatttgga
1561 aatcaaggtg attccttaag atttgaacaa agcaacacga cagctcgtta tacgcttaga
1621 gggaatggaa gtagttacaa tctttattta agagtatctt caataggaaa ttcaactatt
1681 cgagttacta taaacggtag agtttatact gtttcaaatg ttaataccac tacaaataac
1741 gatggagtta atgataatgg agctcgtttt ccagatatta atatcggtaa tatagtagca
1801 agtgataata ctaatgtaac gctagatata aatgtgacat taaactccgg tactccattt
1861 gatctcatga atattatgtt tgtgccaact aatcttccac cactttatta aggtttgagt
1921 aggatccgta cggatcc
```
Figure 1. *cry*II gene sequence from NCBI

3.2. *DNA primer cryII gene*

The design of the DNA primer of the *cry*II gene from *B. thuringiensis* uses primer3 software that has passed the BLAST process and multiple alignments (Table 1). Each primer consists of 20 base pairs. This primer has a percentage of G and C around 50-60%. Forward and reverse primer has Tm difference of around 5°C and having a low self 3'complementarity. The relative position of the primers with the *cry*II gene can be seen in Figure 2.

Table 1. cry2 Primer design from *B. thuringiensis*

Primer (*cry*2)	Sequence (5'-3')	Tm (ºC)	GC%	Self 3' complementarity
Forward	GGTAGTGGACCACAGCAGAC	60	60	1
Reverse	TCTTCTGGCGCCAAATGGAT	65	50	2

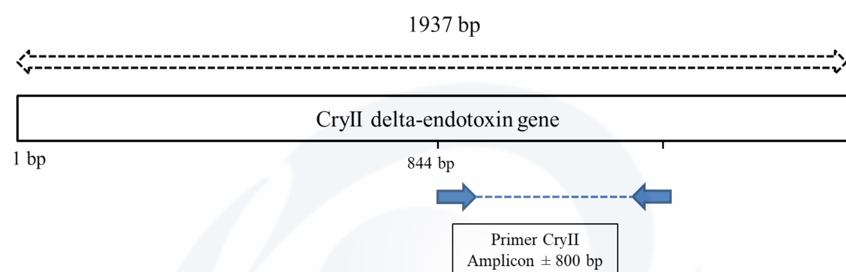

Figure 2. The relative position of primer to *cry*II gene

3.3. *Dimer structure in primer candidate*

The results of the analysis with DINAmelt software, it can be seen that the primers have been designed only have 2 G-C bonds (figure 3). This is advantageous because it can increase the concentration of PCR products. On the other hand, the possibility for the primer to bond to each other is very small.

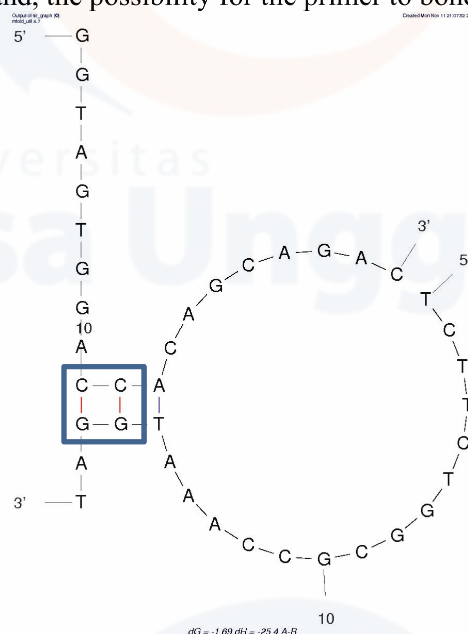

Figure 3. This figure shows DINAmelt analysis results to predict dimer structure on primer pair previously designed

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3.4. *PCR Electrophoresis*

The DNA visualization results of the PCR product that amplified using cry2 forward and cry2 reverse primers showed DNA bands with a size of approximately 800 base pairs (Figure 4).

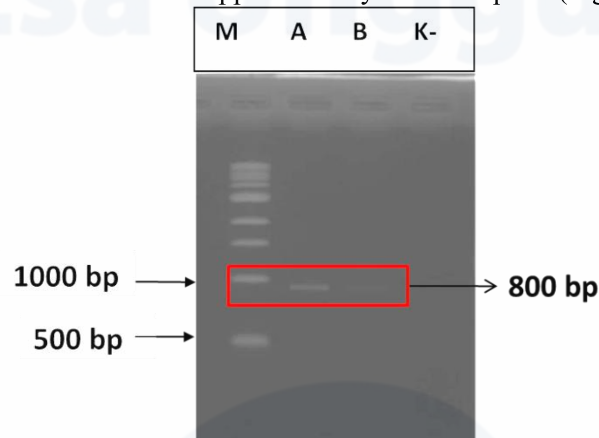

Figure 4. Amplification of *cry*II gene (M: DNA ladder 1 kb, A: isolate 432 and B: isolate 327 using spesific primer; K-: negative control)

4. Discussion

The first step which determines the success of DNA amplification by the PCR method is the design of primers [13]. primers used to amplify the *cry*II gene were designed using a primer3 program and Bioedit with DNA sequences of *cry*II genes was obtained from Genbank (accession number AF047038.1). The characteristics to be considered in the selection of primers include the length of the primer, temperature melting (Tm), GC content and bonds at the end of 3'. A good primer has a range from 18-30 base pairs. Primers that have a length of more than 30 base pairs will induce the unspecific attachment. A pair of selected primers from the design in this research has a length of 20 base pairs for each primer, with forward primer 5'-GGTAGTGGACCACAGCAGAC-3'and reverse primer 5'-TCTTCTGGCGCCAAATGG AT-3'. The primer can amplify the nucleotide bases with a PCR product size of 800 base pairs.

The second criterion to consider in primer selection is Tm. The ideal Tm criteria are 55°-65°C. A pair of good primers have a Tm difference of around 5°C between forward and reverse. This is intended to prevent a decrease in the amplification process. In this study, the temperature Tm difference between primer is 5 degrees. This temperature will affect the annealing temperature in the PCR process.

The percentage between bases G and C also needs to be considered because the content of the number of bases G and C can affect the Tm of a primer [14]. A good primer has a percentage of G and C around 40-60%. Primers with low GC are not expected to be able to compete to stick effectively to the target so it will reduce the efficiency of the PCR process. On the other hand, a high GC percent will increase Tm and PCR annealing temperature. Another criterion for good primers is having a low self 3'complementarity so that there is no attachment between primer pairs and forms a structure called a hairpin [15].

The forward and reverse primers should not have dimers or hairpin. Dimers are structures that formed between primer pairs, they are united because they have a complementary base. Dimers indicate hybridization between identical primer base due to the complementary sequence at the 3' end. This affects the efficiency of the attachment process on the target. From the analysis using DINAmelt, it can be seen that the primer forms a dimer with two GC

bonds. The bond between bases G and C is a strong bond because it consists of 3 hydrogen bonds. That will build the primer easier to put together.

5. Conclusion

The primary design with forward primer 5'-GGTAGTGGACCACAGCAGAC-3'and reverse primer 5'-TCTTCTGGCGCCAAATGGAT-3' successfully amplified the c*ry*II gene and showed a DNA band with molecular size approximately 800 base pairs.

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