Conference Paper

Detection of *cry*III gene in Local Isolate of *Bacillus thuringiensis* Using Polymerase Chain Reaction (PCR)

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Abstract

Bacillus thuringiensis is a biological biopesticide that was used for defense against pests. In *B. thuringiensis* there is a *Cry* protein that is only toxic to certain insects. This *Cry* protein is encoded by *cry* gene. There are many types of *cry* genes that have been identified, one of which is *cry*III gene that is toxic to insects from the Coleoptera group as pests in sweet potato (*Ipomoea batatas*). The aim of this study was to amplify the *cry*III gene from local isolate of *B. thuringiensis*. The method that can be used for *cry*III gene amplification is Polymerase Chain Reaction (PCR). The primer pair is one of the important factors that determine the success of PCR. From a number of designed primers, the primer pair selected to be used in this study is *Cry*3B forward 5'-AAAGTGCGGCTATTCGACCA-3' and *Cry*3B reverse 5'-CACTTCATCCTGTGACG CCT-3'. This primer pair successfully amplified *cry*III gene and showed a DNA band with molecular size approximately 914 base pairs. Gradient PCR needs to be done for optimizing specific amplification of *cry*III gene.

Keywords: PCR, primer, sweet potato

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1. Introduction

Bacillus thuringiensis (Bt) is a protein-producing bacterium that is toxic to insects and nematodes during sporulation [1]. Bt bioinsecticides constitute 90-95% of the bioinsecticides commercialized for use by farmers in various countries [2]. One of the advantages of using this bacterium as a biopesticide include a protein produced by *B. thuringiensis* that is a specific buffer and is called a *Cry* protein (from the word *cry*stal) or also known as δ-endotoxin. *Cry* protein is only toxic to certain types of insects and is not toxic to useful insects or to other organisms [1]. One type of *Cry* protein, *Cry*III, is known to be able to kill Coleoptera [3]. Insects belonging to Coleoptera are pests that attack sweet potato (*Ipomoea batatas*).

Advances in technology in the field of molecular biology have encouraged scientists to isolate chromosomes or plasmids that contain *cry* genes. The *cry* gene is a DNA sequence coding for the formation of *Cry* proteins [1]. Several studies have been carried

out for *cry*III gene amplification [4, 5]. One method that can be used for *cry* gene amplification is the Polymerase Chain Reaction (PCR) which will be carried out in this study by applying a discipline of bioinformatics.

Bioinformatics is an interdisciplinary field which is broadly defined as a combination of biology (molecular biology) and computing using computer and software [6]. One of the most significant roles of bioinformatics is to design and produce primary sequences. Primers are a component that plays an important role in the PCR process [7]. A good primer is a specific primer. Unspecific primers can cause amplification of other regions in the genome that are not targeted or otherwise there are no amplified genome regions. To get a primer that fulfills good primary criteria for amplification, an *in silico* design is carried out [6]. The purpose of this study was to analyze the combination of primer pairs used in the PCR process to amplify the *cry*III gene from B. thuringiensis. The *cry*III gene that will be amplified is a domain of the total *cry*III gene sequences that already exist in GeneBank.

2. Method

2.1. *B. thuringiensis* isolates

B. thuringiensis strains were obtained from the Indonesian Cultur Collection (InaCC) from Indonesian Institute of Sciences in Cibinong with code number B432 and B327.

2.2. Bacterial DNA extraction

B. thuringiensis DNA extraction method was performed using the gSYNC DNA Extraction kit (Geneaid) according to the manufacturer's instructions.

2.3. *cry*III gene Bacillus thuringiensis

As a template for designing primers, the *cry*III *B. thuringiensis* gene (code JN675717.1) downloaded from NCBI was used.

2.4. Primer Specificity Analysis

Primer specificity must be considered so that the primer can recognize and stick to the desired target gene. For the analysis, BLAST from NCBI (https://blast.ncbi.nlm.nih.

gov/Blast.cgi) was used. By using this software, the sequence of primary candidates will be compared with a set of database sequences from various organisms in NCBI. The similarities between the primers and genes of certain organisms will be shown in percentage identities. The higher this percentage value, the more gene specific primers. If there were a primer that can recognize other genes than the *cry*III gene with high specificity, we would not choose this primer.

2.5. Primer Dimer Analysis

Sometimes the designed primers recognized the sequences from themselves, bind to one another to form a structure called a dimer. This can be a problem because primers will tend to stick together, not with the target gene and this can reduce DNA concentration. For this reason, it is necessary to do a dimer prediction analysis using available software to predict the presence of dimers in primary candidates, namely DINAmelt (http://unafold.rna.albany.edu/?q=DINAMelt) made by NM Markham and Michael Zucker [8] of Rensselaer Polytechnic Institute. From the results of this analysis using DINAmelt, it can be seen whether the dimers are formed in the primer, how much the formation of G-C bonds, and the existence of the 3 'end of the complement.

2.6. Analysis of Restriction Sites in the *cry*III Gene

To find out the restriction sites contained in the *cry*III gene, an analysis was performed using *Snapgene* software. The purpose of knowing the restriction site is so that genes can be cut with the desired endonuclease restriction enzymes.

2.7. Amplification of the *cry*III gene by PCR

Amplification of the *cry*III gene using specific *cry*3B forward 5'- AAAGTGCGGCTATTC GACCA-3 'primers and reverse 3'- CACTTCATCCTGTGACGCCT-3' primers with a total volume of 25 µl containing 1 µl genomic DNA, 12.5 µl PCR HS redmix master mix (Bioline) 1 µl for each primer and 9.5 ddH_2O. PCR amplification was carried out as many as 35 cycles using Sensoquest. A single pre-denaturation stage was carried out for 3 min at 95^0C, followed by denaturation for 1 min, annealing at 52^0C for 1 min, and lastly a DNA elongation step at 72^0C for 1 min. Final elongation at 72^0C for 5 min was performed in the end.

2.8. PCR Electrophoresis

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

3. Results

3.1. Primer Design

3.2. Dimer Structure in Primer candidates

Primers that are designed sometimes recognize the sequence of themselves, bind to one another to form a structure called a dimer. This can be a problem because primers will tend to stick together, not with the target gene and this can reduce DNA concentration. From the results of the analysis of prediction of dimers using DINAmelt software (Figure 1), it can be seen that the primary candidate number 3 has 3 G-C bonds.

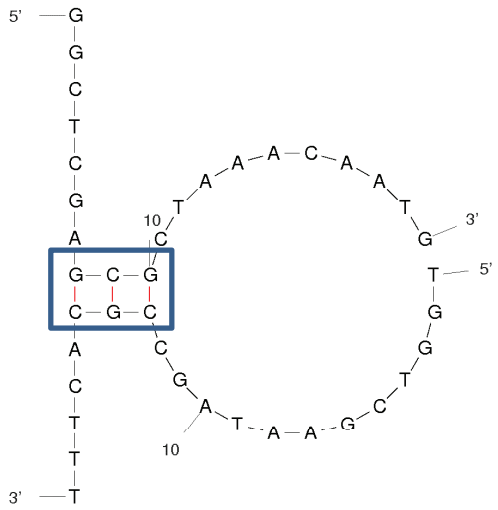

Figure 1: Dimer structure for Primer.

3.3. Map of cryIII Genes

The results of the SnapGene analysis in Figure 2 showed that the amplified *cry*III gene had 15 restriction sites. This information can be used as a basis for cloning.

Table 1: Primer Candidates designed using the sequence obtained from NCBI.

	Sequence (5'-3')	Length	Tm	GC%	Self 3' complementarity
	Primer Pair 1				
Forward	GCTCGAGCGCTAAACAATGG	20	59.97	55	2,00
Reverse	TCGACTTCCGGCATTCCAAA	20	59.96	50	2,00
	Product length 529				
	Primer Pair 2				
Forward	GGCTCGAGCGCTAAACAATG	20	59.97	55	3,00
Reverse	TGGTCGAATAGCCGCACTTT	20	60.04	50	1,00
	Product length 243				
	Primer pair 3				
Forward	AAAGTGCGGCTATTCGACCA	20	60.04	50	2,00
Reverse	CACTTCATCCTGTGACGCCT	20	60.04	55	2,00
	Product length 914				
	Primer pair 4				
Forward	TCTTGGACACATCGGAGTGC	20	60.04	55	3,00
Reverse	TTCCGATGCGGTCCGTTAAA	20	60.04	50	3,00
	Product length 163				
	Primer pair 5				
Forward	GGATCCACTCGGTGCAGTAG	20	59.90	60	1,00
Reverse	GCACTCCGATGTGTCCAAGA	20	60.04	55	1,00
	Product length 606				
	Primer pair 6				
Forward	CCAAATACCAGGGGGCAAGT	20	59.96	55	1,00
Reverse	CTCCCCCTGTATAACCACGC	20	59.89	60	2,00
	Product length 77				
	Primer pair 7				
Forward	AGGCTCGAGCGCTAAACAAT	20	60.11	50	2,00
Reverse	ACTTGCCCCCTGGTATTTGG	20	59.96	55	0,00
	Product length 812				
	Primer pair 8				
Forward	CAAGATGCAGTTAAGGCCGC	20	59.90	55	3,00
Reverse	TCCACTCTTCAAGCGCAGTT	20	59.89	50	2,00
	Product length 271				
	Primer pair 9				
Forward	GCAAGATGCAGTTAAGGCCG	20	59.90	55	4,00
Reverse	TTCCACTCTTCAAGCGCAGT	20	59.89	50	5,00
	Product length 273				
	Primer pair 10				
Forward	GGACACATCGGAGTGCAGAT	20	59.82	55	5,00
Reverse	AAGCCACTAAGTCTCCCCCT	20	59.88	55	5,00
	Product length 141				

3.4. Electrophoresis of PCR product

DNA visualization results that have been amplified with *cry*3B forward and *cry*3B reverse primers show DNA bands with a size of about 1000 bp (Figure 3).

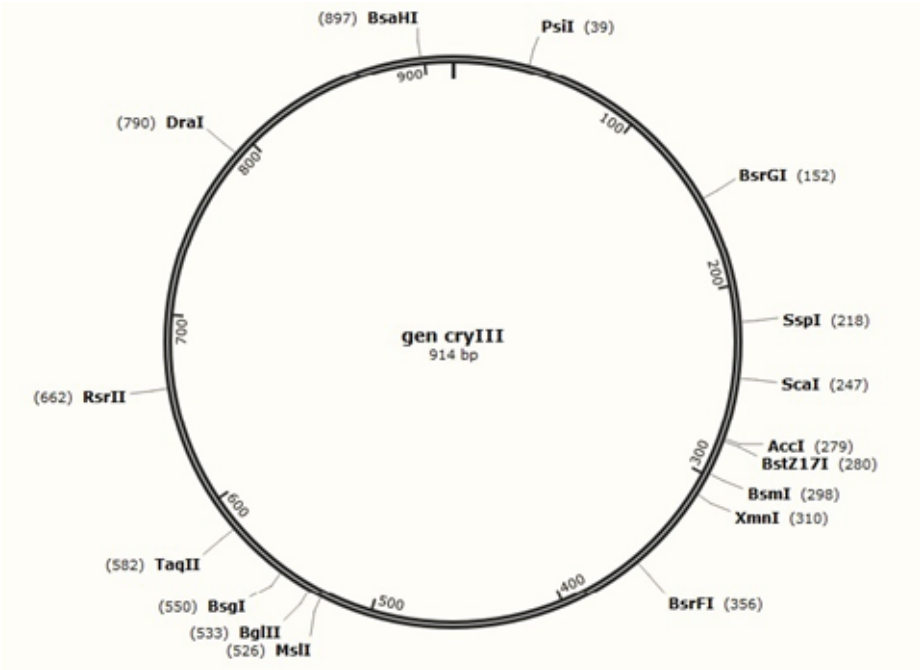

Figure 2: Mapping of Restriction Enzymes in *cry*III Genes with Snapgene software.

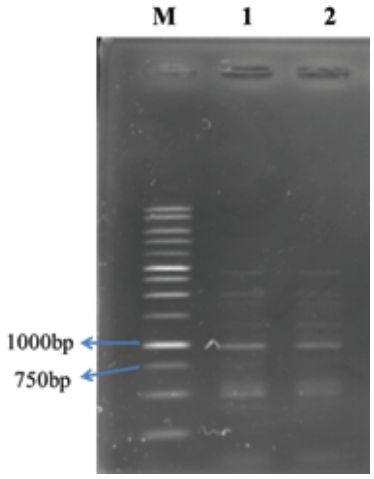

Figure 3: Amplification of *cry*III gene.

4. Discussions

The primer design is the first step that determined the success of DNA amplification by the PCR method [9]. Things that need to be considered in the selection of primers include the length of the primer, melting temperature (Tm), GC content and bonds at the end of 3 '. Good primers range from 18-30 base pairs. Primers which have a length of more than 30 base pairs will cause the primary attachment to be unspecified. The second characteristic to consider in primary selection is Tm. A good primer has a Tm difference of around 5^0C. This is intended to prevent a decrease in the amplification

process. The percentage between base G and C also needs to be considered because the amount of base G and C can affect the Tm of a primary [10]. A good primary has a percentage of G and C around 40-60%. Another criterion for good primers is having a low 3-complementarity self so that there is no attachment between primary pairs and forms a structure called a hairpin [11].

Dimers are structures formed between primary pairs, where they are united because they have a complementary basis. This process occurs at the appropriate attachment temperature, usually at low temperatures. By looking at the stages of the PCR process, it can be seen that the primary attachment to the printed DNA occurs at optimal annealing temperatures. This process can occur simultaneously with the formation of dimers. The problem that may arise is that primers have a tendency to stick to one another, and not stick to printed DNA. If this dimer bond is too strong, it will interfere with the DNA extension process and will result in low DNA concentration. From the analysis using DINAmelt, it can be seen that all primers form dimers. But there is one primer that has only one G-C bond. The bond between bases G and C is a strong bond because it consists of 3 hydrogen bonds. That will make the primer easier to put together. That is the reason for choosing a primer with a G-C bond to minimize bonding between primers.

5. Conclusions

The primer pair 3 designed was specific for *cry*III gene in both Bt isolates used, where a PCR product of around 1000bp was obtained, similar to the predicted size of *cry*III gene amplified using this primer pair (914 bp). Annealing temperature optimization is needed to get a more specific amplicon.

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