Conference Paper

Detection of cryIII 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 cryIII 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 cryIII gene from local isolate of B. thuringiensis. The method that can be used for cryIII 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 Cry3B forward 5’-AAAGTGCGGCT A TTCGACCA-3’ and Cry3B reverse 5’-CACTTCA TCCTGTGACGCCCT-3’. This primer pair successfully amplified cryIII gene and showed a DNA band with molecular size approximately 914 base pairs. Gradient PCR needs to be done for optimizing specific amplification of cryIII gene.

Keywords: PCR, primer, sweet potato

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 crystal) 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, CryIII, 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 cryIII 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 cryIII gene from B. thuringiensis. The cryIII gene that will be amplified is a domain of the total cryIII 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. cryIII gene Bacillus thuringiensis

As a template for designing primers, the cryIII 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 cryIII 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 cryIII Gene

To find out the restriction sites contained in the cryIII 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 cryIII gene by PCR

Amplification of the cryIII gene using specific cry3B forward 5’- AAAGTGCGGCTATTCGACCA-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 ddH₂O. 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°C, followed by denaturation for 1 min, annealing at 52°C for 1 min, and lastly a DNA elongation step at 72°C for 1 min. Final elongation at 72°C 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

Primer candidates 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.](image)

3.3. Map of cryIII Genes

The results of the SnapGene analysis in Figure 2 showed that the amplified cryIII 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.

| Primer Pair | Sequence (5’-3’) | Length | Tm  | GC% | Self 3’ complementarity |
|-------------|------------------|--------|-----|-----|-------------------------|
| 1           | Forward: GCTCGAGCCGCTAAACAATGG | 20     | 59.97 | 55  | 2.00                    |
|             | Reverse: TCGACTTCCGCGCATTCAAAA | 20     | 59.96 | 50  | 2.00                    |
|             | Product length 529 |        |      |     |                         |
| 2           | Forward: GGCTCGAGCCGCTAAACAATGG | 20     | 59.97 | 55  | 3.00                    |
|             | Reverse: TGGCTGAATAGGCCGACTTTT | 20     | 60.04 | 50  | 1.00                    |
|             | Product length 243 |        |      |     |                         |
| 3           | Forward: AAAGTGCGGCTACATTGCACCA | 20     | 60.04 | 50  | 2.00                    |
|             | Reverse: CACTTCATCTCGTGACGCCT | 20     | 60.04 | 55  | 2.00                    |
|             | Product length 914 |        |      |     |                         |
| 4           | Forward: TCTTGGACACATCGAGGAGTGC | 20     | 60.04 | 55  | 3.00                    |
|             | Reverse: TTCCGATGCGGTCCGTTAAA | 20     | 60.04 | 50  | 3.00                    |
|             | Product length 163 |        |      |     |                         |
| 5           | Forward: GAAGTGCGGCTACATTGCACCA | 20     | 59.90 | 60  | 1.00                    |
|             | Reverse: GCACTTCGCTGTAAGCCAGA | 20     | 60.04 | 55  | 1.00                    |
|             | Product length 606 |        |      |     |                         |
| 6           | Forward: CCATAATTCCGGGAGAGCAAGT | 20     | 59.96 | 55  | 1.00                    |
|             | Reverse: CTCCCCCTGATATACACCCGC | 20     | 59.89 | 60  | 2.00                    |
|             | Product length 77 |        |      |     |                         |
| 7           | Forward: AGGCTCGAGGCTCTAAAAACAT | 20     | 60.11 | 50  | 2.00                    |
|             | Reverse: ACGTGCCCCCTTGTATATTGG | 20     | 59.96 | 55  | 0.00                    |
|             | Product length 812 |        |      |     |                         |
| 8           | Forward: CAAGTATGAGTAAGGCCGC | 20     | 59.90 | 55  | 3.00                    |
|             | Reverse: TCCGACTTCGCTAAGCCGAGT | 20     | 59.89 | 50  | 2.00                    |
|             | Product length 271 |        |      |     |                         |
| 9           | Forward: GCAAGATGAGTAAGGCCGC | 20     | 59.90 | 55  | 4.00                    |
|             | Reverse: TCCGACTTCGCTAAGCCGAGT | 20     | 59.89 | 50  | 5.00                    |
|             | Product length 273 |        |      |     |                         |
| 10          | Forward: GGACACATCGAGGAGTGCAGT | 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 cry3B forward and cry3B reverse primers show DNA bands with a size of about 1000 bp (Figure 3).
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°C. 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 cryIII gene in both Bt isolates used, where a PCR product of around 1000bp was obtained, similar to the predicted size of cryIII gene amplified using this primer pair (914 bp). Annealing temperature optimization is needed to get a more specific amplicon.

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References

[1] Saraswati NPA. Deteksi dan Identifikasi Gen Cry4 pada Isolat Bacillus thuringiensis Daerah Bogor dan Sekitarnya. Inst Pertan Bogor, Bogor. 2007.
[2] Bahagiawati. Penggunaan Bacillus Thuringiensis sebagai biolarvasida. *Bul AgroBio*. 2003;5(1):21-28.

[3] Suryanto D. Amplifikasi gen Cry1 dan analisis genom isolat Bacillus thuringiensis lokal. *J Biol Res*. 2017;15(1):1-4. doi:10.23869/bphjbr.15.1.20091

[4] Malik K, Muhammad M, Taipur A. Cloning and Expression of a cry III Gene Isolated from the Local Habitat into a Modified Strain of Bacillus thuringiensis. *IOSR J Pharm Biol Sci*. 2013;7(5):87-95.

[5] Ceron J, Ortiz A, Quintero R, Guereca L, Bravo A. Specific PCR primers directed to identify cry1 and cryIII genes within a Bacillus thuringiensis strain collection. *Appl Environ Microbiol*. 1995;61(11):3826-3831.

[6] Suryadi PT, Ratnayani K, Yowani SC. Desain Primer untuk Amplifikasi Gen katG Multidrug Resistance Tuberculosis (MDR-TB) dengan Metode Polymerase Chain Reaction (PCR). *J Kim*. 2014;8(1):77-82.

[7] Septiari IGAA, Yustiantara PS, Yowani SC. Analisis Primer untuk Amplifikasi Promoter inhA Multidrug Resistance Tuberculosis (MDR-TB) dengan Metode Polymerase Chain Reaction (PCR). *J Kim*. 2015;9(1):117-123.

[8] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*. 2003;31(13):3406-3415. doi:10.1093/nar/gkg595

[9] Suparman, Ahmad H, Ahmad Z. Desain Primer PCR Secara in silico untuk Amplifikasi Gen COI pada Kupu-kupu Papilio ulysses Linnaeus dari Pulau Bacan. *J Pendidik Mat dan IPA*. 2016;7(1):14-25.

[10] Dewi RW, Dewi VR, Yowani SC, Yustiantara PS. Desain Primer untuk Amplifikasi Regio Promoter Gen inhA Isolat P016 Multidrug Resistance Mycobacterium tuberculosis dengan Metode Polymerase Chain Reaction. *J Farm Udayana*. 2018;7(1):34-39.

[11] Safitri TA, Nurul D, Patty J, Saraswati H. Gen L1 HPV 16 dan 18 Sebagai Dasar Dalam Desain Primer untuk Deteksi Kanker Leher Rahim dengan in-House Multiplex PCR. *Indones J Biotechnol Biodivers*. 2018;2(2):67-71.