Cloning and expression of *Bacillus thuringiensis* cry1B in *Escherichia coli* strain NiCo21

S Nugroho*, E R Sembiring, A Rachmat, and W Koesharyoto

Research Center for Biotechnology-Indonesian Institute of Sciences [LIPI], CSC-BG, Jl. Raya Bogor Km 46, Cibinong, Kabupaten Bogor 16911, Indonesia

Email: nugroho_satya@yahoo.com

Abstract. *Bacillus thuringiensis* produces crystal proteins, known as Cry proteins, that are toxic to certain target insects. The cry1B gene from *B. thuringiensis* was reported to be effective against the rice yellow stem borer [YSB, *Scirpophaga incertulas*]. The gene has been introduced into a Javanica rice cv Rojolele, by Agrobacterium mediated method, to improve its resistance against the Lepidopteran insect pest. To comply with the regulation for future release, food, and feed safety tests need to be performed, which involves characterization of the toxicity and allergenicity of the introduced Cry1B protein. In this experiment, the cry1B gene was cloned into pJ804:77539 expression vector to produce Cry1B protein under the pRHA promoter fused to a 6xHis tag to produce pJ804: cry1B. Expression of Cry1B protein was performed in *Escherichia coli* strain NiCo21 and was able to be detected by Western Blot using the anti-rabbit Cry1B polyclonal antibody and anti-His Detector. The results indicated that plasmid pJ804: cry1B expressed Cry1B in *E. coli* Nico21 and the system could be used to produce Cry1B protein for future studies.

Keywords: *Bacillus thuringiensis*, cloning, cry1B, *Escherichia coli*, expression

1. Introduction

*Bacillus thuringiensis* [Bt] is a Gram-positive, soil and water-dwelling bacteria, producing insecticidal crystal protein [sp-endotoxin] known as Cry or Cyt toxins. Cry toxins are effective against insect pests and also mosquitoes which are vectors to many human diseases [1]. After its discovery by Shigetani Ishiwatari in 1901, Bt has been studied extensively and widely applied in agriculture to control insect pests. The first Bt toxins bioinsecticides formulation was developed by Berliner in 1938 [2]. Although toxic to the limited numbers of insect species, Bt toxins are known to be safe for humans, animals, and environments [2].

Due to their effectiveness against insect pests, in particular those Lepidopteran chewing insect pests, an approach by introducing *Bt* genes to crop can be seen as an alternative to improve crops’ resistance. This approach has been successful in developing transgenic crops against insect pests, such as *Bt* corn, *Bt* soybean, *Bt* cotton, and *Bt* eggplant [3] [4] [5]. In rice, *Bt* transgenic events have also been reported, overexpressing cry2Aa gene [6], synthetic cry1Ca [7], synthetic cry1Ab and cry1Ac genes [8], synthetic cry1Ab [9], cry1Ab-1Ac and cry1B-cry1Aa fusion proteins [10] [11] [12] [13] and stacking of cry1Ac and CpTT [cowpea trypsin inhibitor] [14].

We have been developing *Bt* rice to target YSB overexpressing cry1B driven by the wound-inducible promoter mpi [maize proteinase inhibitor] [15] [16] [17] and cry1B-cry1Aa fusion gene controlled by maize ubiquitin [18], in indica rice cultivars IR64 and Cisadane and Javanica rice cultivar Rojolele backgrounds [19] [20] [21]. According to Government Regulation no 21/2005 on the
biosafety of genetically modified products, biosafety assessments need to be performed. To perform food and feed safety studies, especially in the aspects of toxicity and allergenicity of the transgenes, a sufficient amount of transgene protein products needs to be obtained. In this article, we described the cloning and expression of *B. thuringiensis cry1B* gene into *Escherichia coli* for Cry1B protein production.

2. Materials and Methods

2.1. Materials

The plasmid pCAMBIA1300 Ubi*cry1B-cry1Aa* and the sequence of the *cry1B* gene used to isolate *cry1B* [2031 bp] were obtained from CIRAD, France. The expression plasmid used was pJ804:77539 [Figure 1B]. *Escherichia coli* NiCo21 [NEB] was used in this study. HisDetector™ Ni-HRP Conjugate [KPL Inc, USA] and Cry1B antibody [GenScript, USA] were used in the Western Blots.

2.2. Methods

2.2.1. Cloning of *cry1B*

Primers were synthesized with an *NdeI* [ForIB: CTA CAT ATG GTT ACC TCC AAC CGT AAG AAC GAG AAC], and an *XhoI* and 6His [RevIB: ATT CTC GAG TTA ATG GTG ATG GTG ATG ATG GAA CTG GTC AAT GTG GTA GTC GTG GAC ATC]. PCR was conducted with KAPA LongRange [Sigma-Aldrich, USA] to isolate the 2031 bp *cry1B* fragment as described by the manufacturer. PCR fragment was isolated and purified [Silica Bead DNA Gel Extraction Kit, Thermo Scientific, USA], double digested with *NdeI* and *XhoI* [Thermo Scientific], and purified [Silica Bead DNA Gel Extraction Kit, Thermo Scientific]. The fragment was ligated [T4-DNA Ligase, Promega, USA] with *NdeI* and *XhoI* digested pJ804:77539 to produce plasmid pJ804:cry1B. Digestions with *NdeI* and *XhoI* were performed to confirm the insert.

2.2.2. *Cry1B* protein production

*E. coli* NiCo21 was transformed with pJ804:cry1B by heat shock [22], and selected on solid LB containing 100 μg/mL ampicillin. Transformed cells were cultured in 200 ml LB broth containing ampicillin and 0.2% glucose, and shaken at 150 rpm, RT until OD600 = 0.3-0.4. The culture was then induced with 1mM L-rhamnose at room temperature 150 rpm overnight until OD600 = 0.8.

2.2.3. Protein isolation and purification

Cells were harvested under 4000×g, 4°C for 10 minutes and resuspended in lysis buffer containing 1 mM PMSF [23]. Protein fractions were isolated by sonication and collected by 25,000×g centrifugation at 4°C for 10 minutes. Protein purification was performed using IMAC Co2+ with TALON™ matrix [Clontech Laboratories] with loading, equilibrium, washing, and elution buffers as described before [24] [25].

2.2.4. SDS-PAGE and visualization

Twenty-five μL protein samples were separated in SDS PAGE with 10% acrylamide gel [22] and stained with PAGE Blue™ Protein Staining Solution [Thermo Scientific] by incubating overnight with agitation.

2.2.5. Western Blot

Western blot with anti-His antibody was performed using HisDetector™ Ni-HRP Conjugate on 0.45 μm nitrocellulose membrane as described previously [KPL Inc., USA]. Detection was performed in the dark at RT 30 minutes after the addition of 4 ml TMB. Western blot with custom anti-rabbit Cry1B polyclonal antibody was performed on 0.45 μm nitrocellulose membrane following the previously
described protocols [22]. Detection was performed in the dark 15-30 minutes after the addition of BCIP/NBT.

3. Results and Discussions

The isolation of cry1B gene fragment from plasmid pCAMBIA1300Ubi cry1B-cry1Aa has been performed using the strategy that was shown in Figure 1. Cloning of cry1B in expression plasmid pJ804:77539 was facilitated with the addition of restriction sites at the 5’ end of the primers. The specific forward [ForIB] and reverse [RevIB] primers were added with restriction sites NdeI and XhoI, respectively. Hexa-His tag was added to the cry1B gene fragment by incorporating them in primer RevIB.

**A.**

| Restriction Site | Primer Design | Gene Size |
|------------------|---------------|-----------|
| NdeI             | ForIB         | cry1B = 2031 bp (677 aa/77kD) |

| NdeI site added to the forward primer [For1B]. | XhoI and 6xHis were added to the reverse primer [Rev1B]. |

**B.**

The specific forward [ForIB] and reverse [Rev1B] primers were added with restriction sites NdeI and XhoI, respectively. Hexa-His tag was added to the cry1B gene fragment by incorporating them in primer RevIB.

**Figure 1.** A. Primer designs to isolate the cry1B gene. The annealing positions were indicated in yellow. NdeI site added to the forward primer [For1B]. XhoI and 6xHis were added to the reverse primer [Rev1B]. B. The map of plasmid pJ804:77539 was used in this study. The plasmid has NdeI and XhoI cloning sites, driven by the pRHA promoter and ampicillin resistance selectable marker gene.

Plasmid pJ804:77539 was chosen since it has restriction sites with NdeI and XhoI. The resulting cloned gene is driven by promoter pRHA that has superiority against other inducible promoters. Promoter pRHA is inducible by L-rhamnose, which is a non-toxic, photostable, and non-metabolizable inducer. Promoter pRHA also gives a linear response to inducer concentration and crucially no basal transcription in the absence of inducer is observed [Kelly et al, 2018, 26]. The results of the cloning of the cry1B gene fragment into the expression plasmid pJ804:77539 were confirmed by restriction digests [Figure 2]. The results indicated that the cry1B gene fragment was successfully cloned to create the pJ804: cry1B expression vector driven by the pRHA promoter which is inducible by L-rhamnose. The pJ804: cry1B plasmid allows bacterial transformant selection using ampicillin in the growing media.
Figure 2. A. Plasmid pJ804::cry1B. B. Digestion results of pJ804::cry1B with NdeI and XhoI. M. 1 Kb Plus DNA Ladder; 1. Fragment cry1B [2,031 bp]; 2. Fragment cry1Aa [1,854 bp, control]; 3. pJ804 digested with NdeI and XhoI; 4-5. pJ804::cry1B digested with NdeI and XhoI.

The E. coli strain used for the expression of cry1B is NiCo21. NiCo21 was chosen due to its superiority as compared to other commonly used E. coli strains for protein expression, such as BL21(DE3). E. coli NiCo21 is deficient in proteases Lon and OmpT, resistant to phage T1 [fhuA2], and free of animal products. This strain has been reported to be used in many protein expression studies recently [27] [28] [29].

Based on the SDS-PAGE, we can see that the IMAC-Co2 column with TALON Matrix works well to isolate and purify the protein from most contaminants as was shown in Figure 3. The affinity chromatography column IMAC [Immobilized Metal Affinity Chromatography] with Co metal ion was designed to capture histidine-tagged protein and is commonly used for that purpose [30] [31] [32]. Likewise, the TALON matrix used which consists of highly cross-linked agarose beads with an immobilized chelating group precharged with Co2+ ions is also designed to capture histidine-tagged protein. As can be seen in Figure 3, most of the contaminants shown on lane 1 [crude extract] were removed [lanes 2 and 3] giving purer protein elutes as shown on lanes 6 to 15 [1st to 10th elutes]. Dominant protein bands were observed in the gel on lane 6 to 15. Among them, one most dominant protein band at approximately 77 kDa was predicted to be the Cry1B overexpressed protein, which was proven by the Western Blot analyses [Figure 4]. The other protein bands were most likely to be unspecific proteins that bound to the matrix column, which will need to be removed if a purer protein is required.

Nevertheless, the isolated Cry1B protein was able to be identified by both anti-histidine and anti-rabbit Cry1B antibodies [Figure 3]. The anti-histidine antibody identified a specific protein band at approximately 77 kDa, which was predicted to be the Cry1B purified protein. The results showed that
the cloning of *cry1B* in pJ804:*cry1B* and the fusion of the hexahistidine tag [6xHis] were successful. Cry1B protein was able to be produced fused to the 6xHis tag. The anti-rabbit Cry1B antibody showed an immunological reaction to a specific band, also the same size as previously identified using the anti-histidine antibody [approximately 77 kDa], which was predicted as the size of Cry1B proteins. The results indicated that the expression plasmid pJ804:*cry1B* was capable of expressing Cry1B protein fused to the 6xHis tag using the pRHA L-rhamnose inducible promoter in *E. coli* NiCo21 as expected.

**Figure 3.** SDS-PAGE of the purified protein from different fractions. M. Pre-stained Protein MW Marker [Thermo Scientific]; 1. Crude Sample; 2 & 3. Flow-through; 4 & 5. Washing; 6 – 8. 1st to 3rd Elusion fractions; 9 – 15. 4th to 10th Elusion fractions.

**Figure 4.** A. Western blot using anti-rabbit Cry1B antibody on the 1st to 9th elusion fractions. B. Western blot using HisDetectorTM Ni-HRP Conjugate [KPL] on the 1st to 4th elusion fractions. M. Prestained Protein MW Marker [Thermo Scientific].

**4. Conclusion**

The *cry1B* gene was cloned in expression vectors pJ804:*cry1B* is driven by the pRHA promoter. The successful cloning of *cry1B* fused to the 6xHis tag was validated by the expression of Cry1B protein in *E. coli* NiCo21 in the presence of L-rhamnose, which were detected by both anti-rabbit Cry1B and anti-His Detector antibodies. The results confirmed that Cry1B protein production attempts using the established system for future protein characterizations can be initiated.

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