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# CONSTRUCTING EXPRESSION VECTOR CARRYING THE *CRY1AC* GENE DERIVED FROM *BACILLUS THURINGIENSIS* (BT) AND EXPRESSING IN *PSEUDOMONAS FLUORESCENS*

*Pham Van Hieu*, Dinh Thi Sau, Nguyen Xuan Dung, Nguyen Quoc Binh***

***Address(es):*** M.Sc. Pham Van Hieu,
Biotechnology Center of Ho Chi Minh City, Department of Plant Biotechnology, No. 2374 Do Muoi, Trung My Tay ward, Ho Chi Minh City 70000, Vietnam, (84-028) 371 53792.

*Corresponding author: hieupvbio@gmail.com; pvhieu.shtp@tphcm.gov.vn

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**ABSTRACT**

*Bacillus thuringiensis* (Bt) is widely recognized as a biological insecticide employed globally due to its environmentally friendly alternative. The *cry1Ac* gene, which encodes a pesticidal crystal protein derived from *Bacillus thuringiensis*, was introduced into *Pseudomonas fluorescens* to enhance the stability of the toxin. In this study, the expression vector pUDBtAc was constructed to carry the gene encoding the Cry1Ac protein (65 kD) from Bt, under the control of the *tac* promoter and a kanamycin resistance gene as a selectable marker. The recombinant protein produced in the *Pseudomonas fluorescens* expression system under induction with 200–400 µM indole-3-acetic acid (IAA) was subsequently validated using anti-Cry1Ac antibodies. The results confirmed that the fusion *cry1Ac* gene was successfully designed and expressed in *Pseudomonas fluorescens.* Moreover, an analysis of insecticidal activity at a laboratory scale was also conducted. The findings demonstrated that the recombinant protein exhibited larvicidal effects against on caterpillar worm (*Spodoptera litura*) and this hold indicating promising potential for future applications in agricultural pest control.

**Keywords:** *cry1Ac* gene, *Bacillus thuringiensis, Pseudomonas fluorescens, tac* promoter

## INTRODUCTION

*Bacillus thuringiensis* (*Bt*) is a spore-forming, Gram-positive bacterium that is aerobic or facultatively anaerobic. The cells are typically rod-shaped, measuring approximately 3 × 6 µm, motile due to the presence of peritrichous flagella, and can exist either as single cells or in chains. In previous studies, this bacteria is widely distributed in nature and has been easily isolated from diverse environmental sources, including soil, aquatic habitats, phylloplane surfaces, seed dust, insect cadavers, and even insect feces (**Federici, 1999; Ayyadurai *et al*., 2007**). Optimal growth conditions for Bt include temperatures ranging from 28 to 30 °C and a neutral pH of 6.8–7.2. Historically speaking, the bacterium was first discovered in 1901 by Japanese biologist *Shigetane Ishiwata*, who isolated it as the causative agent of a sudden disease in silkworm larvae, initially naming it *Bacillus sotto*. In 1915, a German microbiologist called Ernst Berliner, when isolated at a flour mill in Thuringia, found a parasitic bacteria in the body of insects, with very strong insecticidal power, named it *Bacillus thuringiensis*. It has been found that the life cycle of *Bt* bacteria is very simple and can be divided into 4 consecutive main developmental stages (1) nutritional growth; (2) onset of sporulation; (3) spore formation; (4) spore maturation and dissolution (**Berbert-Molina *et al*., 2008**). Under favorable environmental conditions, Bt spores can germinate to continue growing and dividing. However, in response to adverse conditions such as nutrient depletion, elevated temperature, or desiccation, the bacterium initiates sporulation. During this process, Bt synthesizes parasporal crystalline inclusions composed of insecticidal crystal proteins (ICPs), which are toxic to specific insect species (**Xia *et al*., 2005; Boonserm *et al*., 2005; Pérez -García *et al*., 2010**). These ICPs vary in size, shape, and insecticidal specificity, and are released into the environment upon cell lysis, along with mature spores. The crystals typically measure around 0.6 × 0.2 µm and may account for up to 30% of the dry weight of the bacterial cell. Both the spores and crystals can be visualized using electron microscopy (**Hofte & Whiteley, 1989; Li *et al*; 1991; Guo *et al*., 2009**).

Since the late 1920s, *Bt* has been utilized as a biological pesticide, with early applications reported in Hungary and Yugoslavia (**Husz 1928**). However, The first commercial Bt-based formulation, known as Sporeine, was developed in France in 1938 (**Aronson *et al*., 1986**). *Bt* also began to be commercialized in the US in 1958 and in 1961 was licensed by the Environmental Protection Agency (EPA) in 1961. A significant advancement occurred in 1970 when the Abbott Laboratories launched Dipel, a commercial biopesticide formulated with a novel subspecies, *B. thuringiensis* subsp. *kurstaki*, which exhibited broad-spectrum activity against various lepidopteran larvae (**Dulmage, 1970**). By 1977, only 13 strains of *Bt* bacteria had been discovered, each of which was exhibiting specificity primarily against larvae of certain Lepidopterans. The 1980s saw a rapid expansion in the commercialization of Bt-based biopesticides, with numerous biotechnology companies registering for production and distribution. By the early 1990s, several countries, including Cuba, China, and the United States, had initiated large-scale industrial production of Bt formulations (**Chen *et al*., 2011**). Today, Bt-based biopesticides are extensively employed worldwide for the management of agricultural pests, particularly larvae belonging to the orders Lepidoptera, Diptera, and Coleoptera. These microbial insecticides are favored due to their specificity, minimal environmental impact, and suitability as sustainable alternatives to chemical pesticides (**Ahmedani *et al*., 2007; Ali *et al*., 2010; Barton *et al*., 1987; Rosas-García, 2009).**

The mechanism of action of *Bacillus thuringiensis* (Bt) Cry and Cyt toxins involves the disruption of midgut epithelial cells in susceptible insect larvae. Upon ingestion, Bt spores germinate, and the protoxins are solubilized and activated in the alkaline environment of the insect gut. The activated toxins bind to specific receptors on the apical membrane of midgut epithelial cells, leading to the formation of ion-permeable pores. This pore formation disrupts cellular ion homeostasis, resulting in osmotic lysis and ultimately, cell death (**Knowles & Ellar, 1987; Rausell et al., 2004**). In addition to this classical pore-formation model, an alternative hypothesis has been proposed to explain the mechanism of action of Cry toxins. This model suggests that Cry toxins trigger $Mg^{2+}$-dependent signaling pathways. Each of the three structural domains of the Cry toxin interacts independently with specific receptors, including the cadherin-like protein BT-R1, leading to the activation of signaling cascades (**Zhang et al., 2006; Pigott & Ellar, 2007; Soberón et al., 2009**). Activation of these $Mg^{2+}$-dependent pathways induces a sequence of intracellular events, such as membrane blebbing, cellular swelling, and eventual lysis of the target cells (**Zhang et al., 2006**). Notably, $Mg^{2+}$-dependent signaling responses triggered by Cry toxins are analogous to those observed with other pore-forming toxins when administered at sublethal (nanomolar) concentrations. These shared characteristics suggest that Cry toxins may exert both direct pore-forming activity and secondary effects through host cellular signaling modulation (**Nelson et al., 1999; Porta et al., 2011**).

In addition, recent advances have demonstrated that the heterologous production of *Bacillus thuringiensis* (Bt) toxins in *Pseudomonas fluorescens* offers several practical advantages over traditional Bt-based production systems. *P. fluorescens* has been shown to yield higher levels of δ-endotoxins compared to native Bt strains. Notably, these δ-endotoxins remain encapsulated within the *P. fluorescens* cells even after sterilization, which facilitates easier recovery and handling of the

bioinsecticidal protein. Moreover, the dead *P. fluorescens* cells serve as a protective matrix, shielding the endotoxin from ultraviolet degradation and thereby enhancing its environmental persistence. Importantly, unlike Bt, *P. fluorescens* does not produce a wide range of other secondary metabolites or toxins, reducing the risk of unintended effects on non-target organisms (**Peng *et al*., 2003; Bakker & Schippers, 1987; Donegan *et al*., 1995; Bangera & Thomashow, 1999; Haas & Defago, 2005; Hernández-Rodríguez *et al*., 2013**).

In the present study, a recombinant expression vector, pUDBt, was constructed for the cloning and expression of the *cry1Ac* gene from *B. thuringiensis*. The gene was placed under the control of the tac promoter, a synthetic hybrid of the *trp* and *lac* promoters, which enables strong inducible expression in bacterial hosts. The promoter was synthesized using PCR extension techniques, and a kanamycin resistance gene was included as a selectable marker. The engineered plasmid was introduced into *P. fluorescens* to express the Cry1Ac protein as a bioinsecticidal agent. Additionally, preliminary evaluations of insecticidal activity were conducted at the laboratory scale to assess the efficacy of the recombinant strain.

## MATERIALS AND METHDS

### Materials

The *E. coli* DH5∝ strain was used for cloning purposes. The *Pseudomonas fluorescens* strain utilized for gene expression was provided by the Department of Microbiology at the Biotechnology Center of Ho Chi Minh City. The pUDBt vector carrying the target *Bt -Cry(1Ac)* gene is referenced from GenBank (ID:M11068) with a length of 1848 bp, was obtained by through artificial gene synthesis. The pUCIDT vector was used to supply the terminator segment. The pK7GWIWG2(II)/CP-CyMV vector carrying the kanamycin resistance gene was provided by the Department of Plant Biotechnology.

### Methods

The designed expression vector carrying the *cry1ac* gene derived from *Bacillus thuringiensis* (Bt) was shown in Figure 1.

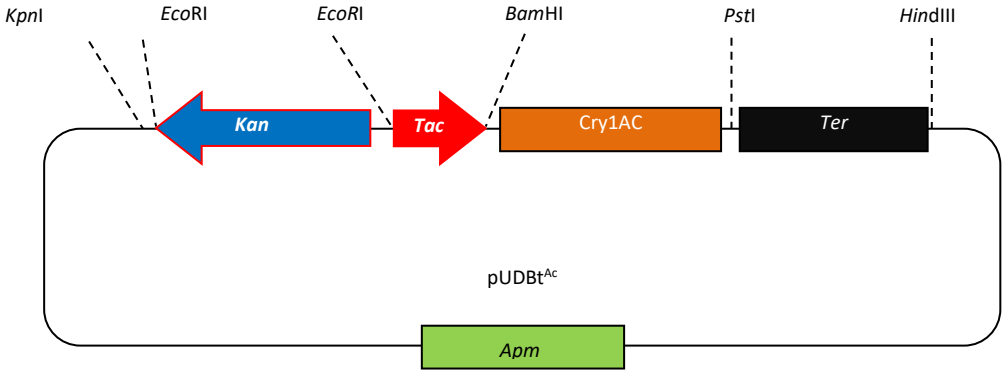



**Figure 1** Schematic representation of the pUDBtAc expression vector carrying the *Bt* gene. Key features include the K*an* gene (kanamycin resistance), *Tac* promoter (inducible expression), *Ter* (transcription terminator), and A*pm* gene (ampicillin resistance).

### Synthesizing, testing *Tac* promoter and cloning into pJET vector

The *Tac* promoter (63 bp) was synthesized using the PCR extension method with a primer pair designated Ftac (forward) and Rtac (reverse) (Figure 2). The primers were designed to include the -35 and -10 regions, as well as an overlapping region. Additionally, the primers were engineered to contain restriction enzyme recognition sites for *KpnI* (GGTACC) and *BamHI* (GGATCC). An *EcoRI* site (GAATTC) was also included downstream of the *KpnI* site to facilitate subsequent insertion of the kanamycin resistance gene.

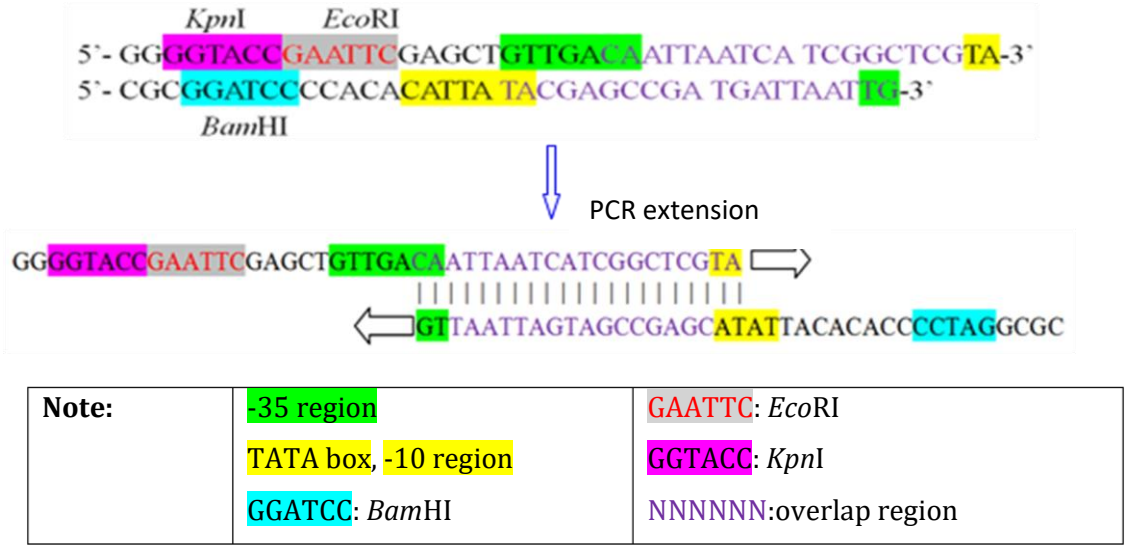



**Figure 2** Extension process from both sides when conducting PCR

Following PCR extension, the 63 bp Tac promoter fragment was purified via ethanol precipitation using 5 M ammonium acetate ($CH_3COONH_4$) and 96% ethanol. The precipitated product was verified by electrophoresis on a 2% agarose gel. The synthesized *Tac* promoter fragment was then ligated into the pJET1.2 vector using the Clone JET™ PCR Cloning Kit (Fermentas). The recombinant vector containing the *Tac* promoter (pJet-Tac) was subsequently introduced into *E. coli* DH5α via chemical transformation (**Sambrook et al., 1989**).

### Isolation of antibiotic resistance kanamycin (*Kan*) gene from vector pK7GWIWG2(II)/CP-CyMV

In this experiment, the *kan* gene (~1219 bp) was amplified by PCR using the primer pair Fkd/Rkd from the vector pK7GWIWG2(II)/CP-CyMV, based on the *kan* gene sequence available in GenBank (accession number: AF234296). Additionally, *Eco*RI restriction sites were incorporated at the 5' ends of both the forward and reverse primers. The sequences of the primers were as follows: Fkd:5'-GGAATTCCCAGCCAGCCAA-3' and Rkd: 5'-GGAATTC CTAAAACAATTCATCCAGT-3'. Following amplification, the *kan* gene was ligated into the pJet-Tac vector using *Eco*RI restriction sites at both ends to construct the recombinant vector pJET-Kan-Tac. The recombinant clones

harboring this construct were transformed into *E. coli* DH5α using the chemical transformation method and selected on Luria-Bertani (LB) agar plates supplemented with 50 mg/L kanamycin.

**Isolating the *Terminator (Ter)* gene segment from pUCIDT vector and attaching the *Terminator* gene segment to pUCBt vector**

The *Ter* gene fragment (~170 bp) was isolated by PCR using the primer pair Fter/Rter to amplify the *Ter* sequence from the pUCIDT vector, based on the *Ter* gene sequence from GenBank (accession number: KJ870100.1). The primers were designed to include restriction sites for *Pst*I and *Hin*dIII, respectively. The sequences were as follows: Fter: 5'-AACTGCAGTGCTGCTAGCGCGGTGTAGCG-3' (*Pst*I site) Rter: 5'-CCCAAGCTTTGCAAGGCCCAGTCTTTCGACTG-3' (*Hin*dIII site). Following amplification, the *Ter* fragment was ligated into the pUCBt vector using *Pst*I and *Hin*dIII restriction enzymes at both ends to construct the recombinant vector pUCBt-Ter.

**Creating expression pUCBt$^{Ac}$ vector conferring target Cry1Ac gene and transforming into *Pseudomonas fluorescens***

The recombinant *Kan-Tac* gene fragment was obtained by digesting the pJET-Kan-Tac vector with the restriction enzymes *Kpn*I and *Bam*HI, followed by gel purification using the QIAquick Gel Extraction Kit (QIAGEN). The purified fragment was then ligated into the pUCBt-Ter vector, which had been digested with the same enzymes (*Kpn*I and *Bam*HI), using T4 DNA ligase (Fermentas) to construct the expression vector pUCBtAc. The recombinant pUCBtAc vector was introduced into *Pseudomonas fluorescens* under electroporation conditions of 2.5 kV, 25 µF, and 200 Ω.

The transformed bacterial suspension plated on LB agar supplemented with kanamycin (50 mg/L) and ampicillin (50 mg/L) were screened by PCR using the primer pair Fkd/Rkd. Cultivation was carried out at 37°C for 24–30 hours. Colonies that tested positive by PCR were inoculated into LB broth containing 50 µg/mL ampicillin and 50 µg/mL kanamycin and grown overnight. Bacterial cells were harvested by centrifugation at 13,000 rpm for 1 minute, and plasmid DNA was extracted using the DNA-Spin™ Plasmid Purification Kit (Intron) following the manufacturer's instructions. The resulting recombinant vectors were subsequently verified by restriction digestion using the enzyme pairs *Kpn*I/*Hin*dIII and *Bam*HI/*Hin*dIII.

**Induction of Cry1Ac protein expression under shaking culture conditions**

*Pseudomonas fluorescens* colonies harboring the expression vector were cultured in 3 mL of liquid LB medium supplemented with kanamycin (50 mg/L) and ampicillin (50 mg/L), incubated overnight at 37°C with shaking at 250 rpm. Subsequently, 300 µL of the overnight culture was transferred into 30 mL of fresh LB medium containing the same antibiotics and incubated under the same conditions (37°C, 250 rpm) until the cell density reached an optical density (OD) of 0.6–0.7. At this point, indole-3-acetic acid (IAA) was added at final concentrations ranging from 50 to 500 µM to induce expression, and the cultures were incubated for an additional 48 hours. Following induction, the cultures were centrifuged and the supernatant discarded to collect the bacterial cell biomass.

**Examining proteins after expression by SDS-PAGE and DAS-ELISA methods**

Bacterial biomass was harvested by centrifugation at 8000 rpm for 15 minutes at 4°C. The cell pellet was washed with buffer (50 mM $NaPO_4$, containing $NaH_2PO_4$, $Na_2HPO_4$, 300 mM NaCl, and 10% glycerol, pH 7.8). Cell disruption was performed using sonication in a lysis buffer (50 mM $NaPO_4$, containing $NaH_2PO_4$, $Na_2HPO_4$, 300 mM NaCl, pH 6.0) for 10 minutes at 16 W. The resulting lysate was centrifuged at 10,000 rpm for 10 minutes, and the supernatant containing soluble proteins was collected, while the pellet was discarded. The collected protein samples were quantified using the Bradford assay before being subjected to SDS-PAGE on a 15% polyacrylamide gel following the Laemmli method (Laemmli, 1970). Detection of the Cry1Ac protein was performed using the DAS-ELISA method with specific anti-Cry1Ac antibodies, according to the manufacturer's instructions (Bt-Cry1Ab/1Ac ELISA Kit PSP 06200/0096 1, Agdia, USA).

**Analysis of insecticidal activity at laboratory scale**

*Pseudomonas fluorescens* biomass was obtained from cultures induced with 400 µM indole-3-acetic acid (IAA) for Cry1Ac expression. Cultures were heat-inactivated at 100 °C for 5 minutes and centrifuged; the resulting biomass from 100 mL culture was resuspended in 10 mL distilled water. Serial dilutions (1, 1:10, 1:100, 1:1000; designated X2–X5) were prepared. Second- to third-instar *Spodoptera litura* larvae, collected from vegetable fields, were used for bioassays. Thirty larvae per treatment (10 larvae per replicate, three replicates) were fed on a pre-cooked diet mixed with the respective bacterial suspensions.

Positive controls included two commercial *Bacillus thuringiensis*-based biopesticides: Vi-BT 32000WP (X6; 32,000 IU/mg, Vietnam Fumigation JSC) and BICILUS 18WP (X7; 18,000 IU/mg, KING ELONG Co., Ltd.). Negative controls consisted of distilled water (X1) and culture supernatant from *P. fluorescens* lacking the Bt gene (X8). Larval mortality was assessed after 7 days. All treatments were performed in triplicate, and data were statistically analyzed using SAS v9.1.3 to evaluate insecticidal efficacy.

The effectiveness of the drug will be calculated based on the formula of Abbott W.S. 1925

$$The\ effectivness\ of\ the\ drug\ (\%) = \left(1 - \frac{A}{B}\right) x\ 100$$

In which, T: drug concentration

Co: Control

A: Total number of worms after treatment with T concentration

B: Total number of worms after Co concentration treatment

**RESULTS AND DISCUSSION**

**Synthesizing, testing *Tac* promoter and cloning into PJET vector**

The PCRextension of the *tac* promoter yielded a ~63 bp product and indicated a band below 100 bp in 2% agarose gel electrophoresis (Figure 3). The purified product, precipitated with 5 M $NH_4OAc$, retained the expected size (Figure 4). The *tac* promoter was ligated into the pJet1.2/blunt vector to construct the pJet-Tac plasmid, which was transformed into *E. coli* DH5α. Colonies selected on ampicillin-containing plates were screened via PCR using pJet1.2F/pJet1.2R primers (Figure 5).

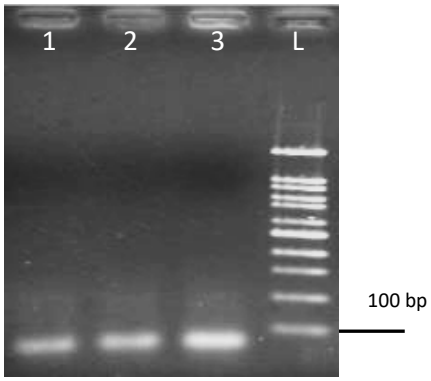



**Figure 3** Agarose gel electrophoresis of the *Tac* promoter synthesized by PCR extension. L: DNA ladder; Lanes 1–3: PCR products corresponding to the *Tac* promoter.

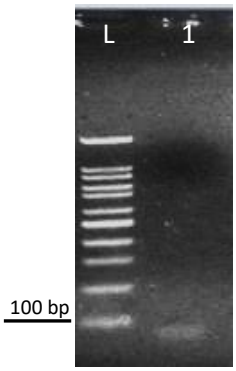



**Figure 4** Agarose gel electrophoresis of the *T*ac promoter following DNA precipitation. L: DNA ladder; Lane 1: Precipitated *T*ac promoter fragment.

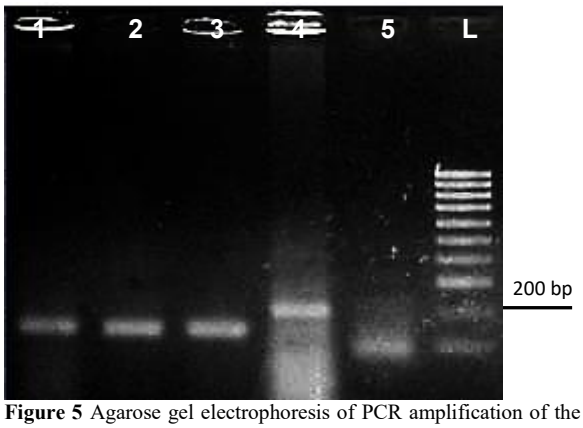



**Figure 5** Agarose gel electrophoresis of PCR amplification of the *T*ac promoter using primer pair pJet1.2F/pJet1.2R after cloning into the pJet1.2/blunt vector. L: DNA ladder; Lanes 1–4: PCR products from putative positive colonies; Lane 5: Positive control.

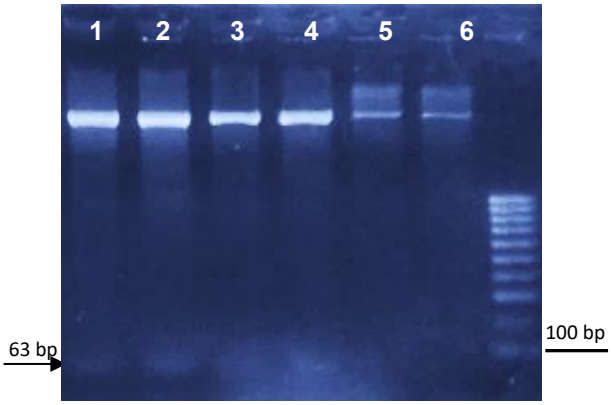



**Figure 6.** Agarose gel electrophoresis of restriction analysis of the pJet-Tac vector using *Kpn*I and *Bam*HI enzymes. L: DNA ladder; Lanes 1–4: pJet-Tac digested with *Kpn*I and *Bam*HI; Lanes 5–6: Undigested pJet-Tac vector

Lane 4 showed an amplicon of ~175 bp, consistent with successful *tac* promoter insertion (63 bp insert plus ~112 bp vector backbone). Lanes 1–3 showed ~112 bp products, indicating the presence of vector only. The negative control (Lane 5) produced non-specific amplification products. Restriction digestion of the pJet-Tac vector with *KpnI/BamHI* released a fragment <100 bp, confirming the insertion of the *tac* promoter (63 bp) (Figure 6). These results verify the successful cloning of the *tac* promoter into the pJet1.2/blunt vector, consistent with standard protocols (**Sambrook et al., 1989**).

**Isolation of antibiotic resistance kanamycin (Kan) gene from vector pK7GWIWG2(II)/CP-CyMV and cloning onto pJET-*Tac***

The *kan* gene (~1219 bp) was amplified from the pK7GWIWG2(II)/CP-CyMV vector using primers Fkd/Rkd (Figure 7), confirming successful amplification for downstream vector construction. The gene was ligated into the pJet-Tac backbone to generate the pJet-TK vector. Following transformation and antibiotic selection (kanamycin and ampicillin, 50 mg/L each), positive colonies were screened by PCR using primers pJet1.2F/pJet1.2R. Colonies in lanes 3–5 showed amplicons matching the expected 1219 bp product, while the negative control showed no amplification (Figure 8).
*Eco*RI digestion of pJet-TK released a ~1219 bp fragment, confirming the presence of *Eco*RI sites flanking the *kan* gene introduced via primer design (Figure 9). To assess the gene's orientation, PCR was performed with primer pairs Fkd/Rtac and Rkd/Rtac (Figure 10). No product was observed with Fkd/Rtac (Lane 2), while Rkd/Rtac (Lane 3) produced an amplicon consistent with reverse orientation of the *kan* gene relative to the *tac* promoter. An additional PCR with pJet1.2F/pJet1.2R showed a slightly larger amplicon for the positive control due to the inclusion of full *kan*, *tac*, and primer sequences. These results confirm the reverse insertion of *kan* upstream of the *tac* promoter, allowing for efficient downstream expression of the *Bt* gene while maintaining *kan* gene functionality.

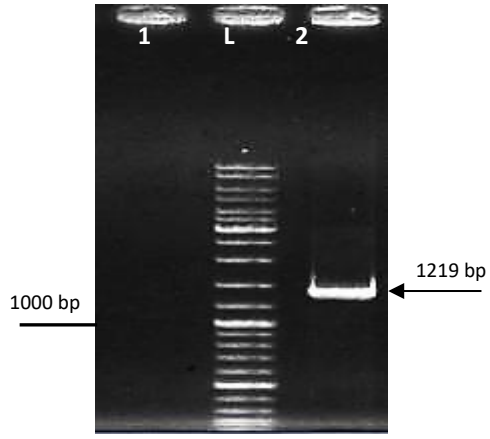



**Figure 7** Agarose gel electrophoresis of PCR amplification of the kanamycin resistance gene from the pK7GWIWG2(II)/CP-CyMV vector using the Fkd/Rkd primer pair. L: DNA ladder; Lane 1: Negative control (–); Lane 2: Amplified kanamycin resistance gene fragment.

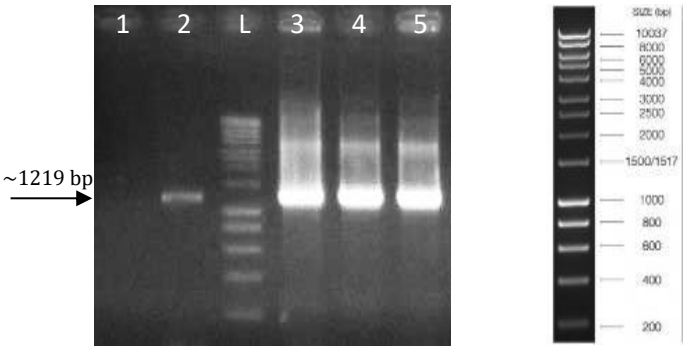



**Figure 8** Agarose gel electrophoresis of PCR amplification of the kanamycin resistance gene from putative positive colonies harboring the recombinant plasmid pJet-TK using the pJet1.2F/pJet1.2R primer pair. L: DNA ladder; Lane 1: Negative control; Lane 2: Positive control (purified kanamycin gene fragment); Lanes 3–5: PCR products from putative positive colonies.


**Figure 10** Agarose gel electrophoresis of PCR analysis to determine the orientation of the kanamycin resistance gene insertion into the pJet-Tac vector. L: Standard DNA ladder; Lane 1: Positive control amplified with primer pair pJet1.2F/pJet1.2R; Lane 2: PCR product using primer pair Fkd/Rtac; Lane 3: PCR product using primer pair Rkd/Rtac.

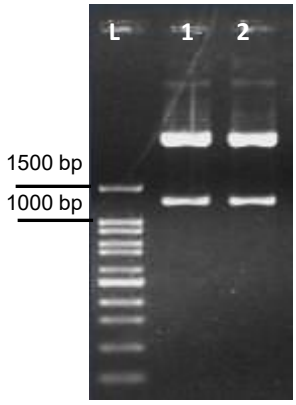



**Figure 9** Agarose gel electrophoresis of restriction digestion analysis of the pJet-TK vector containing the Kan + Tac gene segment. L: DNA ladder; Lanes 1–2: DNA fragments corresponding to the kanamycin resistance gene obtained after restriction digestion.

**Isolation of the *Terminator (Ter)* gene segment from pUCIDT vector and attaching the *Terminator* gene segment into pUCBt vector**

The *Ter* gene fragment (~200 bp) was amplified from the pUCIDT vector using primers Fter/Rter, which indicated in lanes 2 and 3 (Figure 11) and ligated into the pUDBt vector to generate the recombinant plasmid pUDBt-Ter. Following transformation into *E. coli* DH5α, PCR screening with Fter/Rter identified positive clones. Agarose gel analysis showed that the colony in lane 1 produced an amplicon of ~200 bp, matching the expected size, while lanes 2 and 3 displayed non-specific products (Figure 12). Restriction digestion with *HindIII/PstI* further confirmed the presence of the *Ter* gene, yielding a ~200 bp fragment consistent with the DNA ladder and control (Figure 13). These results validate the successful construction of the pUDBt-Ter plasmid.

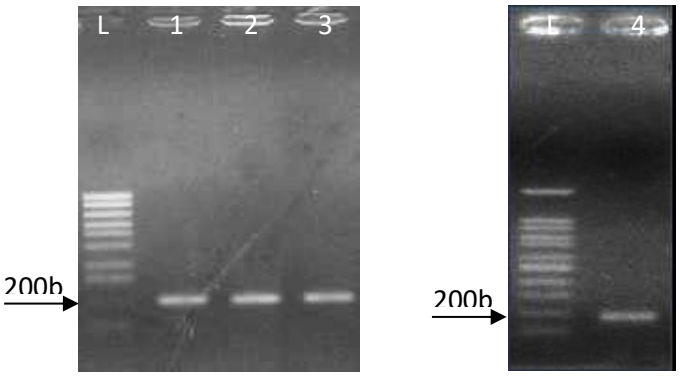



**Figure 11** Agarose gel electrophoresis analysis of PCR amplification of the terminator (*Ter*) gene from the pUCIDT vector using the Fter/Rter primer pair, and restriction digestion of the purified *Ter* fragment with *Pst*I and *Hin*dIII.
L: Standard DNA ladder; Lane 1: Positive control; Lanes 2–3: PCR products amplified from pUCIDT using Fter/Rter primers; Lane 4: Purified Ter fragment digested with PstI and *Hin*dIII.

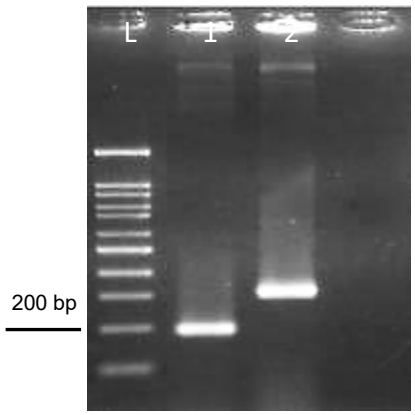



**Figure 12** Agarose gel electrophoresis of PCR-based screening of cell lines harboring the recombinant vector pUDBt-Ter using the Fter/Rter primer pair.
L: Standard DNA ladder; Lanes 1–2: PCR amplification of the Ter gene from putative recombinant clones; Lane 3: Positive control.

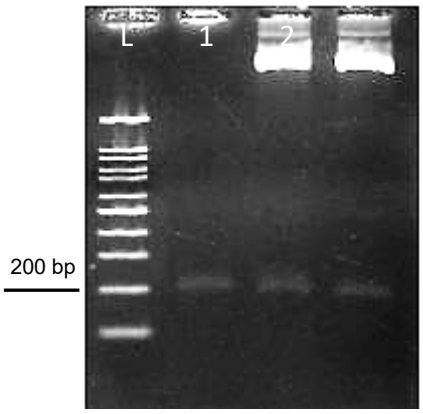



**Figure 13** Agarose gel electrophoresis of restriction digestion of the terminator gene fragment from the pUCIDT vector using *Hin*dIII and *Pst*I. L: Standard DNA ladder; Lane 1: Positive control; Lanes 2–3: Terminator gene fragments digested with *Hin*dIII and *Pst*I.

**Creating expression pUCBt$^{Ac}$ vector conferring target Cry1Ac gene and transforming into *Pseudomonas fluorescens***

The final assembly of the recombinant vector pUDBtAc (approximately 5920 bp in size) involved insertion of the *Kan-Tac* fragment into pUDBt-*Ter*. PCR screening using primers Fkd/Rkd confirmed the presence of the *kan* gene (1219 bp) in *E. coli* DH5α transformants (lane 4), matching the positive control (Figure 14). Restriction digestion of pUDBtAc with *EcoRI* yielded the expected fragments of ~1219 bp (*kan*) and ~4700 bp (vector backbone). Digestion with *BamHI/HindIII* produced fragments of ~2010 bp (*cry1Ac* + *Ter*) and ~3900 bp. Combined *EcoRI/HindIII* digestion generated three fragments (~1219 bp, ~2010 bp, and ~2700 bp), corresponding to the *kan*, *cry1Ac* + *Ter*, and remaining vector regions, respectively. The observed digestion patterns (Figure 15, lanes 1–4) matched the predicted fragment sizes, confirming the successful construction of the pUDBtAc expression vector harboring the *cry1Ac* gene.

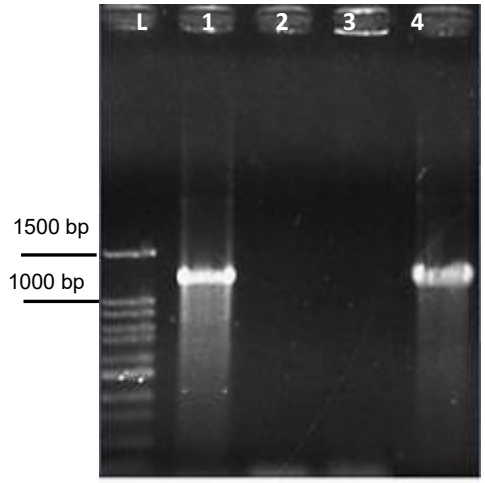



**Figure 14** Agarose gel electrophoresis of PCR screening of the kanamycin resistance gene (kan) in putative positive colonies harboring the recombinant vector pUDBtAc using the primer pair Fkd/Rkd.
L: Standard DNA ladder; Lane 1: Positive control (pJet-TK vector); Lanes 2–4: PCR products from putative positive colonies.

**Induction of Cry1Ac protein expression under shaking culture conditions and testing of protein after expression by SDS-PAGE and DAS-ELISA**

The presence of the pUDBtAc vector in *E. coli* BL21-DE3 and *Pseudomonas fluorescens* was verified by PCR using Fkd/Rkd primers targeting the kanamycin resistance gene (kan, 1219 bp) (Figure 16). Amplification confirmed successful transformation in *P. fluorescens* (sample 2) and *E. coli* (samples 3 and 4). Additionally, PCR with Fbt1/Rbt1 primers confirmed the presence of the *cry1Ac* gene in both host strains (Figure 17). Further validation was performed by *Bam*HI/*Hin*dIII restriction digestion of plasmids from colonies 2, 3, and 4, yielding a 2010 bp fragment corresponding to the *cry1Ac* gene (1848 bp) and the terminator sequence (~160 bp) (Figure 18). These results confirm the successful introduction of the pUDBtAc vector into both *P. fluorescens* and *E. coli* BL21-DE3.

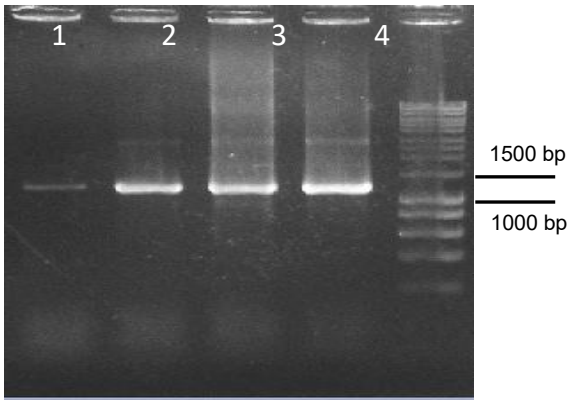



**Figure 16** Agarose gel electrophoresis of PCR screening for the kanamycin resistance gene in *E. coli* BL21-DE3 and *Pseudomonas fluorescens* transformants carrying the pUDBtAc vector using the Fkd/Rkd primer pair.
L: Standard DNA ladder; Lanes 1–2: PCR products from *P. fluorescens* colonies; Lanes 3–4: PCR products from *E. coli* BL21-DE3 colonies.

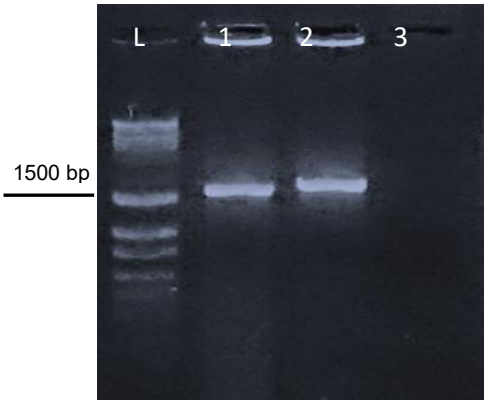



**Figure 17** Agarose gel electrophoresis of PCR screening for the *cry1Ac* gene in *E. coli* BL21-DE3 and *Pseudomonas fluorescens* transformants harboring the pUDBtAc vector using the Fbt1/Rbt1 primer pair. L: Standard DNA ladder; Lane 1: PCR product from *P. fluorescens*; Lane 2: PCR product from *E. coli* BL21-DE3; Lane 3: Negative control.

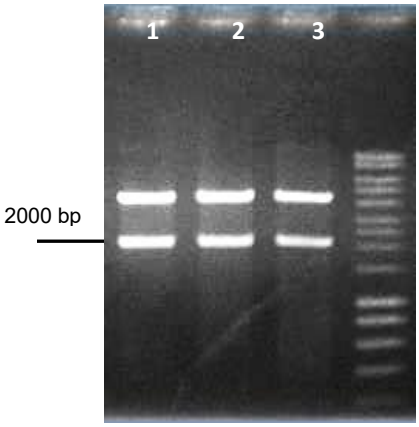



**Figure 18** Agarose gel electrophoresis of *Bam*HI/*Hin*dIII digestion of the pUDBtAc vector isolated from *E. coli* BL21-DE3 and *Pseudomonas fluorescens*. L: Standard DNA ladder; Lanes 1–3: Digestion products showing two fragments of approximately 3900 bp and 2000 bp.

**Examination of Bt protein expression by SDS-PAGE in *Pseudomonas fluorescens***

According to the design, the target protein has an expected molecular weight of approximately 65 kDa. In practice, the expression results of the Cry1Ac protein (Figure 19) showed protein bands in lanes 1 to 6 with molecular weights ranging from 60 to 70 kDa, as compared to the protein marker. This result is consistent with the initial theoretical estimation. The toxin encoded by the *cry1Ac* gene expressed in this study also yielded similar results to those reported by Peng et al., 2003.

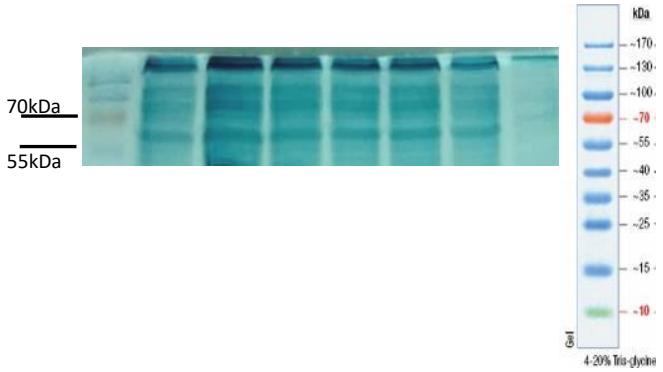
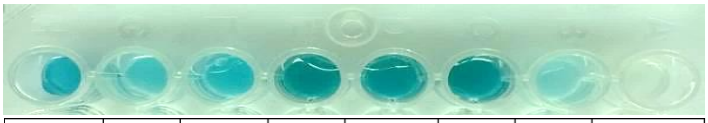



**Figure 19** SDS-PAGE analysis of Bt-Cry1Ac protein expression in *Pseudomonas fluorescens* strains. L: Protein molecular weight marker; Lanes 1–6: *P. fluorescens* strains harboring the Bt-Cry1Ac expression vector; Lane 7: Negative control strain of *P. fluorescens*.

**Testing the expression of Bt protein using DAS-ELISA method**

The recombinant Cry1Ac protein was identified by a colorimetric ELISA, in which a specific reaction between Cry1Ac and an enzyme-conjugated anti-Cry1Ac antibody produced a distinct blue color (Figure 20). $OD_{650}$ values were significantly higher than those of negative controls (samples A–H), confirming successful expression of Cry1Ac in *Pseudomonas fluorescens*. Expression was regulated by a *tac* promoter induced by 3-indolylacetic acid (IAA) (**John et al., 1996**). In the absence of IAA, a repressor binds the operator, blocking transcription. IAA binds the repressor, allowing transcription initiation and subsequent Bt protein production (**Allan et al., 1998**). Protein expression was positively correlated with IAA concentration, peaking at 200–400 µM, consistent with previous findings (**Xin et al., 2014**). Expression declined at concentrations above 500 µM, likely due to competitive inhibition. These results confirm that the recombinant protein is Cry1Ac, with optimal induction achieved at 200–400 µM IAA.



| Positive samples | IAA 50 µM | IAA 100 µM | IAA 200 µM | IAA 300 µM | IAA 400 µM | IAA 500 µM | Water |
|---|---|---|---|---|---|---|---|
| H | G | F | E | D | C | B | A |
| 1.856 | 0.935 | 1.676 | 2.568 | 2.573 | 2.487 | 0.865 | 0.0521 |

**Figure 20** Evaluation of Bt protein expression in *Pseudomonas fluorescens* using ELISA and OD measurement methods.
Well A: Distilled water (negative control); Wells B–G: *P. fluorescens* strains carrying the IAA-inducible Bt gene, induced with 50 µM (Well B), 100 µM (C), 200 µM (D), 300 µM (E), 400 µM (F), and 500 µM (G) of IAA; Well H: Positive control – *E. coli* DH5α strain harboring the inducible Bt gene.

**Analysis of insecticidal activity at laboratory scale**

The undiluted recombinant protein induced the highest larval mortality (44.44%) and insecticidal efficacy (43.82%) as shown in Table 1, with both parameters decreasing proportionally with dilution. A positive correlation was observed between protein concentration and insecticidal activity. At a 1:1000 dilution, mortality and efficacy were minimal and comparable to negative controls (*Pseudomonas fluorescens* without the *Bt* gene and distilled water). In comparison, the commercial product Vi-BT achieved 94.44% mortality and 94.38% efficacy, while BICILUS reached 51.11% and 50.56%, respectively. Based on manufacturer-recommended dilutions (25 g/8 L), Vi-BT had an estimated concentration 1.78 times higher than BICILUS, corresponding to 1.85-fold and 1.87-fold increases in mortality and efficacy. These findings confirm the dose-dependent insecticidal effect of *Bt*-Cry1Ac protein. Although the recombinant formulation showed lower efficacy than commercial products, it maintained significant bioactivity even at high dilutions, demonstrating its potential as an effective bioinsecticide.

**Table 1** Testing results of insecticidal efficacy of Bt protein

| Experiments | Bt concentration | The death rate of worm (%) after 1 weeks | Insecticidal efficacy (%) |
|---|---|---|---|
| $X_1$ | Negative control | $2.22^{g} \pm 1.11$ | 1.12 |
| $X_2$ | 1 | $44.44^{c} \pm 1.11$ | 43.82 |
| $X_3$ | 1:10 | $33.33^{d} \pm 1.93$ | 32.59 |
| $X_4$ | 1:100 | $26.67^{e} \pm 1.93$ | 25.84 |
| $X_5$ | 1:1000 | $18.89^{f} \pm 1.11$ | 17.98 |
| $X_6$ | Vi-BT | $94.44^{a} \pm 1.11$ | 94.38 |
| $X_7$ | Bicilus | $51.11^{b} \pm 2.22$ | 50.56 |
| $X_8$ | Distilled water | $1.11^{g} \pm 1.11$ | 0.00 |
| CV(%) | | 7.25 | |
| LSD 0.05 | | 4.32 | |

The letters a, b, c, d, e, f, g in the same colume indicate statistically significant differences with $p < 0.05$ (Turkey's test)

The insecticidal activity of Cry1Ac, particularly against Lepidopteran species, has been well-documented (**Hernández-Rodríguez et al., 2013**). Expression of *Bacillus thuringiensis* (Bt) δ-endotoxins in *Pseudomonas fluorescens* has been demonstrated in multiple studies (**Peng et al., 2003; Gao et al., 2004; Alberghini et al., 2005**), and commercial formulations utilizing this system (e.g., CellCap) have been available since 1991 (**Panetta, 1999**). A key advantage of this heterologous expression platform is the encapsulation of Bt toxins within non-viable *P. fluorescens* cells, protecting the protein from rapid environmental degradation and extending its shelf life. While Bt spores and crystal proteins typically lose efficacy within days post-application, encapsulated toxins retain insecticidal activity even after UV exposure. This stability reduces the need for repeated applications and minimizes environmental spore accumulation. Importantly, the fixation process ensures the absence of viable *P. fluorescens* cells during field use (**Panetta, 1993**). Additionally, co-expression of insecticidal proteins from *Pseudomonas* spp. with Bt toxins in *Escherichia coli* has shown enhanced activity against coleopteran pests, highlighting the potential of *Pseudomonas*-derived proteins as promising biopesticidal agents (**Barbosa Rodrigues et al., 2023**).

## CONCLUSION

The expression vector pUDBtAc, carrying the gene encoding the Cry1Ac protein—comprising only the toxic core fragment (65 kDa) from *Bacillus thuringiensis* (Bt)—was successfully constructed using the tac promoter sequence for expression and a kanamycin resistance gene as a selectable marker. The recombinant *cry1Ac* protein derived from *Bacillus thuringiensis* was also successfully expressed in *Pseudomonas fluorescens* under induction with indole-3-acetic acid (IAA) at concentrations ranging from 200 µM to 400 µM. The recombinant Cry1Ac protein obtained exhibited specific reactivity with anti-Cry1Ac antibodies. Furthermore, the recombinant protein demonstrated high larvicidal activity against *Spodoptera litura* caterpillars under laboratory conditions. Based on the successful design and expression of this system, other Cry proteins can be expressed by substituting the target *cry* genes into the constructed vector.

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