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Research Article

Transient Expression of Plant-Codon-Optimized *Cry2ab39* Gene by Agroinfiltration in *N. Benthamiana*

Ngoc Thu Le[1,2], Huyen Thi Tran[1], Vy Thai Trinh[1], Tra Thi Nguyen[1], Ha Hoang Chu[1,2], Phat Tien Do[1,2*], Thanh Minh Thi Le[1], Ngoc Bich Pham[1,2*]

[1]Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

[2]Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

[*]**Correspondence to: Phat Tien Do, PhD, Head,** Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cay Giay, Hanoi 13111, Vietnam; Email: dtphat@ibt.ac.vn
Ngoc Bich Pham, Vietnam Academy of Science and Technology, 8 Hoang Quoc Viet, Cay Giay, Hanoi 13111, Vietnam; Email: pbngoc77@gmail.com

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Abstract

Objective: This study was performed to modify the coding sequence of a novel *Cry2Ab39* gene derived from *B. thuringiensis serovar canadensis* strain SP142 in Vietnam and investigate the expression of this codon-optimized gene in *Nicotiana benthamiana* (*N. benthamiana*) leaves.

Methods: The *Cry2Ab39* gene sequence (Genebank No. MN319700.1) was modified based on codon bias of *N. benthamiana*. A sequence coding the legumin B4 signal peptide and a His-tag coding sequence followed by endoplasmic reticulum (ER) retention signal KDEL were added to the 5′ and 3′ ends of the codon optimized *Cry2Ab39* gene, respectively. The binary vector pFGC5941 harboring optimized *Cry2Ab39* under the control of either CaMV 35S or Arabidopsis rbcS1A promoters was constructed and used for transient gene expression in *N. benthamiana* via agroinfiltration.

Results: The native *Cry2Ab39* gene were optimized based on codon usage of *N. benthamiana* along with a preference of G/C-containing codons to increase the overall G-C content and the potential mRNA stability sequences. A significantly higher *Cry2Ab39* protein quantity was produced from the construct driven by the Arabidopsis rbcS1A promoter as compared to the CaMV 35S promoter. In addition, the highest recombinant protein was accumulated in tobacco leaves at 6 days-post-infiltration (dpi) with bacterial density OD_{600} of 0.5. The His-tagged *Cry2Ab39* was successfully purified by affinity chromatography using Ni-NTA columns.

Conclusion: The recombinant *Cry2Ab39* protein was successfully produced and purified from *N. benthamiana* leaves in appropriate amounts using suitable promoters and optimal transformation parameters. These results suggest high production of codon-optimized *Cry2Ab39* gene in the plant system and show its potential for further applications in creating insect resistant crops.

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Keywords: Agroinfiltration, codon optimization, *Cry2Ab39* gene, recombinant protein, tobacco

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1 INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive, endospore-forming soil bacterium and can be isolated from diverse habitats, including soil, water, plants, insects, stored-product dust[1-3]. Its parasporal inclusion bodies are well-known as insecticidal crystal proteins (or δ-endotoxins), including crystal (Cry) and cytolytic toxins at the onset of sporulation and non-crystalline toxins (Vip and Sip proteins) during the vegetative growth phase[4,5]. These proteins are toxic against a number of insect species from different orders such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Mallopphaga, and other organisms, i.e., Nematodes[6,7]. Among Bt insecticidal proteins, Cry toxins were most widely employed in insect management either as biopesticidesor pest-resistant transgenic plants through genetic engineering[8]. Genes Cry coding Cry proteins mostly locate in large plasmids in Bt bacteria[9]. Up to now, 75 different Cry toxin groups (Cry1 to Cry75) have been found with more than 800 members[10].

The rapid discoveries of Bt genes in conjunction with advances in transgenic technology have promoted the generation of Bt crops resistant to pests on a global scale[11,12]. The genes of cry1-type were the most popular source for production of the first-generation Bt crops. However, due to long-term use and large-scale cultivation, target insects were escaped from these toxins due to the evolution of resistance[13-15]. Therefore, Cry2A toxins currently are alternative candidates for production of second-generation transgenic plants to control insect pests instead of Cry1-type toxins. The Cry2A toxin family (65-70kDa proteins) formed cuboidal crystals in *B. thuringiensis* strains and actively targeted lepidopteran as well as dipteran insects[16]. One important note is that Cry2A proteins were found to be lethal to some lepidopteran pests, which were unaffected by Cry1 toxins[17]. Furthermore, with different receptors on brush border membrane vesicles, those toxins exhibited the low level of cross-resistance in Cry1-resisitant insects[18,19]. In addition, due to their smaller molecular size than Cry1 genes, the genes encoding Cry2A toxins are thought to have greater advantages for transformation as well as expression in plants[20]. In this regard, research for new Cry2A-type toxins with high insecticidal activity and wide spectrum of toxicity seems an attractive approach not only for the development of transgenic plants to control crop pests but also the management of insect resistance evolution.

The challenge in development of Bt transgenic crops is the efficient and stable expression and translation of insecticidal genes and toxic protein in plant cells[21,22]. Initial attempts to introduce native Bt genes into transgenic plants resulted in extremely low levels of mRNAs and the corresponding proteins which were insufficient for protection of the crops from target insects[23]. Codon optimization of bacterial genes based on codon usage of host plants was proved as one of the efficient ways to solve this issue. Synthetic Bt genes with modification in coding sequences were found to be expressed with the high efficiency in host plants and conferred insect resistance of the hosts[22,24-26].

In our previous work, the novel gene *Cry2Ab39* derived from *B. thuringiensiss* erovar *canadensis* strain SP142 isolated in Vietnam was characterized and its toxicity was evaluated (data not published yet). In this study, the *Cry2Ab39* codon sequence was optimized for transient expression in *N. benthanmia* via agroinfiltration method. Different transformation parameters as well as promoters were evaluated to improve expression and accumulation of the *Cry2Ab39* protein in tobacco leaf tissue. The recombinant *Cry2Ab39* was successfully purified using affinity chromatography via Ni-NTA columns. Our results provide strategies for expression and production of bacterial proteins in plant systems. In addition, the codon-optimized *Cry2Ab39* gene is potential for further researches to create insect resistant crops.

2 MATERIALS AND METHODS

2.1 Plasmids, Bacteria and Plant Materials

The vector pBT/At-rbcs1Aprocarrying Arabidopsis rbcs1A promoter (provided by Plant Cell Biotechnology Lab., Institute of Biotechnology, VAST, Vietnam) and the binary vector pFGC5941 (Addgene, USA, #44182) were used to construct the plant expression vectors. pBI121/35S_Hc-Pro PVY, a pBI121-based expression vector for the production of helper component-proteinase (HcPro) of Potato virus Y (a gift from Applied DNA Technology Lab., Institute of Biotechnology, VAST, Vietnam), 4-week hydroponic *N. benthamiana* plants and the *Agrobacterium tumefaciens* strain AGL1 (purchasedfrom Nova Lifetech, Hongkong) was used for transientexpression experiments.

2.2 Codon Modification and Gene Synthesis

Based on the codon usage database for *N. benthamiana* (https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4100)[27], rare codons (below 20%) in coding sequence of the wild-type *Cry2Ab39* gene (Genebank No. MN319700.1) were eliminated and edited to expected codons without changing the amino acid sequence. The web-based tool Seqool (http://www.biossc.de/seqool/index.html) was used to find AT-rich regions, potential plant polyadenylation

signal sequences as well as sequence motifs known as triggers of RNA instability in plants[28]. These sequences were then manually substituted by degenerate codons. In addition, the mRNA secondary structure was analyzed using NUPACK66 software (http://www.nupack.org) to eliminate the stable loop structure formation. In order to translocate this insecticidal protein to the endoplasmic reticulum (ER), the legumin B4 signal peptide and ER retention signal (KDEL) were fused to the N and C termini of the optimized *Cry2Ab39* gene, respectively. The His-tag coding sequence and *Nco*I and *Sma*I cloning sites were added to C-terminal region and the 5′ and 3′ ends of the modified *Cry2Ab39* gene, respectively to facilitate protein purification procedure. The codon-optimized *Cry2Ab39* was synthesized by GenScript (Hong Kong) and cloned into pUC57 cloning vector.

2.3 Vector Construction

The full-length *Cry2Ab39* gene was obtained from cloning vector pUC57/*Cry2Ab39* using *Nco*I and *Xma*I (Thermo Scientific, USA), and then mobilized into the binary vector pFGC5941 backbone to generate pFGC5941/35S_*Cry2Ab39*. The 35S promoter of the pFGC5941/35S_*Cry2Ab39* vector was replaced with the At-rbcS1A promoter obtained by digesting pBT/At-rbcS1Spro using *Eco*RI and *Xho*I (Thermo Scientific, USA) to produce the second vector, pFGC5941/At-rbcS1A_*Cry2Ab39*. The two plasmids were introduced individually into *Agrobacterium tumefaciens* strain AGL1 using freeze-thaw method[29].

2.4 Agroinfiltration

The expression of the recombinant protein in plant by agroinfiltration was conducted as described by Ho et al.[30] with optimization. Briefly, a single colony of AGL1 straincarrying *Cry2Ab39* or Hc-Pro (an RNA silencing suppressor that prevents degradation of T-DNA transcripts[31,32]) expression constructs were cultured in 200mL of LB medium containing appropriate antibiotics in the 200rpm shaker (MaxQ 6000, Thermo Fisher, USA) at 28°C for 14-16h to reach optical density at 600nm (OD_{600}) of 1.8 (measured using Beckman Coulter DU800 spectrophotometer, USA). Bacterial cells were harvested and resuspended in the infiltration buffer (10mM 2-(N-morpholino) ethanesulfonic acid, 10mM $MgSO_4$, pH 5.6) to get the final OD_{600} of 0.5. Equal volumes of two bacterial suspensions were combined and used for infiltration.Whole plants of *N. benthamiana* were placed upside down in the bacterial mixture inside a desiccator under the vacuum of 40Torr for 2min. After infiltration, the plants were returned to the greenhouse at 21°C with 16/8h light and dark photoperiod. Leaf samples were collected at 5 days-post-infiltration (dpi) and stored at -80°C for further analysis. Different factors including gene constructs (pFGC5941/35S_*Cry2Ab39*, pFGC5941/At-rbcS1A_*Cry2Ab39*), *Agrobacterium* density (OD_{600}=0.1, 0.3, 0.5, 0.8) and leaf harvesting time (1, 2, 3, 4, 5, 6, 7dpi) were evaluated to reveal the optimal conditions for production of recombinant *Cry2Ab* protein in tobacco leaves. The expression of the target protein was assessed through Western blot analysis, and the optimal conditions were employed in large-scale to produce *Cry2Ab39* for the subsequent purification experiments.

2.5 SDS-PAGE and Western Blot

Leaf samples were ground in liquid nitrogen and homogenized in PBS buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.4) at a 1:3 (w/v) ratio. The raw extracts were centrifuged at 13,000rpm (Sorvall legend micro21 microcentrifuge, Thermo Scientific, USA), 4°C for 30min to remove cell mass. The total soluble protein contents were determined by Bradford assay[33] with bovine serum albumin (Sigma-Aldrich) as a standard. The extracted proteins were then diluted to the final concentration of 3μg/μL. About 30μg total protein of each sample was subjected to glycine SDS-PAGE according to the description of Laemmli[34]. The SDS-PAGE gel was stained for 30min with 0.2% (w/v) Coomassie Brilliant Blue R-250 (Merck, Germany) in a mixture of methanol/acetic acid (40:10, v/v). Western blot procedure was carried out using monoclonal anti-6X His tag antibody following the protocol described by Pham et al. (2019)[31].

2.6 Protein Purification

Cry2Ab39 protein expressed in *N. benthamiana* was purified using immobilized metal ion affinity chromatography (IMAC) which is designed for purification of 6xHis-tagged recombinant proteins. One hundred grams of tobacco leaves were ground in liquid nitrogen and blended in 300mL of binding buffer (20mM NaH_2PO_4, 0.5M NaCl, 20mM imidazole, pH 8.0). The crude extract was centrifuged twice at 13,000rpm, 4°C for 30min to clarify the cell lysate. The supernatant was subsequently incubated with 10 mL of Ni-NTA resin (Cube Biotech, Germany) equilibrated in binding buffer and mixed gently at 4°C for an hour to allow affinity binding with *Cry2Ab*. The protein/resin complex was applied to a Econo-Pac chromatographycolumn (Bio-Rad 7321010) and extensively washed with a washing buffer (20mM NaH_2PO_4, 0.5M NaCl, 40mM imidazole, pH 8.0) to remove the unbound nonspecific proteins. Recombinant protein *Cry2Ab39* was finally eluted from the column using an elution buffer (20mM NaH_2PO_4, 0.5M NaCl, 500mM imidazole, pH 8.0). One-milliliter fractions were collected separately in microcentrifuge tubes and SDS-PAGE assay was carried out to analyze the purity of protein samples. The fractions containing purified protein were then pooled for dialysis against PBS to remove the imidazole and finally concentrated using the 3K MWCO protein concentrator column (Thermo Scientific, USA). Protein concentration was determined using the Bradford assay[33].

3 RESULTS

3.1 *Cry2ab39* Codon Optimization and Synthesis

The proper expression of insecticidal genes in plant cells is a critical factor required for the insect resistance of transgenic

crops[35]. Thus, in this study, the native *Cry2Ab39* gene sequence was optimized for expression in *N. benthamiana* with a preference of G/C-containing codons to increase the overall G-C content. Furthermore, ATTTA motifs as messenger destabilization elements and potential poly A signal sites (AATATT, AATTAA, AATAAA and AATAAT) as well as predicted potential splice sites were eliminated. In addition, the RNA secondary structure was removed and RNA instability motifs were avoided. As a result, the optimized sequence differed from the original one by 24.15% at the nucleotide level, but no change in the amino acid sequence. In addition, the GC content of the codon-optimized sequence increased to 45.1%. Importantly, the sequences conferring potential mRNA secondary structures as well as mRNA destabilization and splicing were eliminated (Table 1).

3.2 Plant Transient Vector Construction

To evaluate the influence of the constitutive CaMV 35S promoter and At-rbcS1A promoter on *Cry2Ab39* expression in tobacco, pFGC/35S_*Cry2Ab39* and pFGC/At-rbcS1A_*Cry2Ab39* were constructed (Figure 1A), respectively. The pFGC/35S_*Cry2Ab39* vector was created by subcloning of the *Cry2Ab39* gene (2037bp) digested from pUC57/*Cry2Ab39* into pFGC5941 at *Nco*I and *Xma*I sites (Figure 1B, lanes 1 and 2). Restriction analysis ofthe recombinant vector pFGC/35S_*Cry2Ab39* by *Nco*I-*Xma*I (Figure 1B, lane 3) exhibited the expected fragment of 2037bp corresponding to *Cry2Ab39* as well as the presence of 10-kbvector backbone, indicating the vector successful designed. The latter construct was built based on the former by replacing the 35S promoter with At-rbcs1A promoter. Both pFGC/35S_*Cry2Ab39* and pBT/At-rbcs1Apro were first treated by *EcoR*I-*Xho*I (Figure 1B, lanes 4 and 5). The fragments of At-rbcs1A promoter(1100bp) and digestedbackbone vector (10.6kb) were ligated to generate pFGC/At-rbcS1A_*Cry2Ab39* (Figure 1B, lane 6). Both constructed expression vectors were then introduced into *Agrobacterium* for plant transformation.

3.3 Transient Expression of *Cry2Ab39* in Nicotiana Benthamiana

3.3.1 Effects of Promoters

The efficiency of the constitutive CaMV 35S promoter and Arabidopsis rbcS1A promoter on transient expression of *Cry2Ab39* was evaluated using the tobacco agroinfiltration system. The total protein contents of crude extracts from infiltrated tobacco leaves of each construct were analyzed by Western blot using anti-6X His antibody (Figure 2A). The expected protein band with a size of nearly 75kDa representing the recombinant his-tagged *Cry2Ab39* was detected in leaf samples infiltrated with both designed constructs, but absent in the negative control. A significantly higher Cry protein content was observed from the pFGC/At-rbcS1A_*Cry2Ab39* construct as compared to pFGC/35S_*Cry2Ab39* based on protein band intensity. This result indicated that the At-rbcS1A promoter is more effective for transient expression of *Cry2Ab39* gene in *N. benthamiana* leaves.

3.3.2 Effect of Bacterial Density

To find an optimal bacterial density for *Cry2Ab39* transient expression in *N. benthamiana* leaves, the suspension of *Agrobacterium* AGL1 strain harboring pFGC/At-rbcS1A_*Cry2Ab39* was prepared at final densities (OD_{600}) of 0.1 to 0.8. Samples were collected at 5dpi and recombinant protein levels were assessed by Western blot. According to data on the Figure 2B, the highest efficiency of *Cry2Ab39* production was achieved at OD_{600}=0.5. However, the *Cry2Ab39* protein was not detectable from the treatment of OD_{600}=0.1. This result indicated that the bacterial suspension at OD_{600}=0.5 was suitable for transient expression of *Cry2Ab39* in tobacco via agroinfiltration.

3.3.3 Effect of Harvest Time

Cell suspension of *A. tumefaciens* strain AGL1 carrying pFGC/At-rbcS1A_*Cry2Ab39* adjusted to OD_{600}=0.5 was used for tobacco leaf infiltration. Recombinant protein was extracted from leave samples collected at 1, 2, 3, 4, 5, 6, 7 dpi. *Cry2Ab39* concentration was semi-quantified by Western blot (Figure 2C). Densitometry analysis of the protein bands showed that the expression of recombinant *Cry2Ab39* were detectable from 2dpi. The protein concentrations increased gradually and reached the highest level at 6dpi, then dramatically decreased. Therefore, 6dpi was selected for sample harvesting to obtain the highest desired protein content.

3.4 Purification of Recombinant *Cry2Ab39*

In this work, we used IMAC Ni-NTA under native conditions to purify His-tagged recombinant *Cry2Ab39* from crude extract of tobacco leaves. The SDS-PAGE and Western blot analysis of different fractions collected during the whole process presented in Figure 3 demonstrated the successful purification of the target protein in expected size (75kDa). The non-specific binding of contaminant proteins rich in histidine was reduced by adding low concentration of imidazole to binding and wash buffers. The purified recombinant protein was obtained with the purity greater than 90%, and purification yield was estimated about 1.5g protein per kg fresh weight of leaves.Therefore, using this method, *Cry2Ab39* protein was purified from tobacco leave extract with the high purity and concentration.

4 DISCUSSION

4.1 Plant Codon Optimization of *Cry2ab39* Gene Sequence

In many studies, bacterial Cry genes were poorly expressed in plants and could not help plants to protect themselves from relevant pests[23]. A major reason for the low-level expression of bacterial Cry genes in plant cells is that their mRNA transcripts are extremely unstable and degraded rapidly. The causes of the mRNA instability were

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Table 1. Comparison of the Original and Optimized *Cry2Ab39* Gene

Parameter	Original *Cry2Ab39*			Optimized *Cry2Ab39*		
	Base	No. of Bases	Percentage (%)	Base	No. of Bases	Percentage (%)
Base content	A	661	32.45	A	573	28.13
	T	659	32.35	T	626	30.73
	G	368	18.07	G	414	20.32
	C	349	17.13	C	424	20.81
GC%		35.2%			45.1%	
RNA instability motifs		76			0	
RNA secondary structure		12			0	

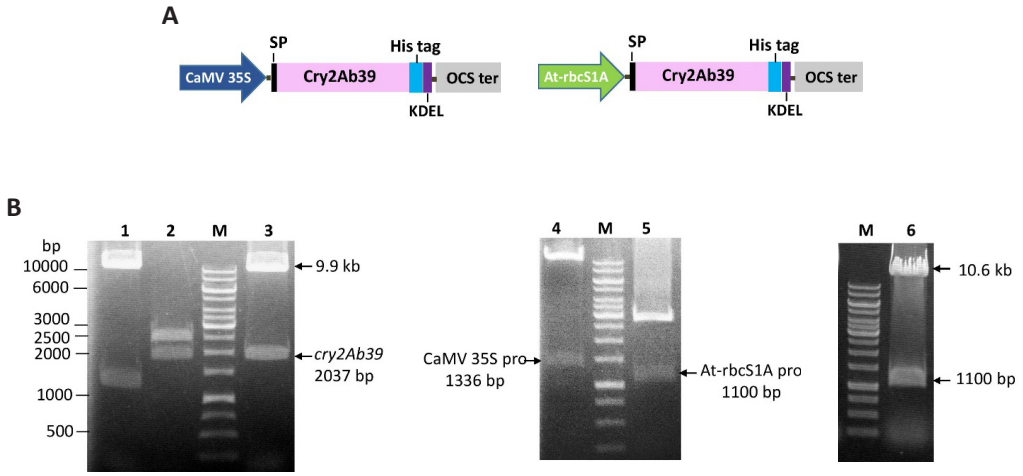

Figure 1. Plant transformation vector construction for *Agrobacterium* infiltration. A: *Cry2Ab29* expression cassettes regulated by either CaMV 35S promoter or At-rbcS1A promoter. SP: Le B4 signal peptide. KDEL: ER retention signal. OCS ter: octopine synthase terminator. His tag: six histidine (6xHis) residues; B: Clonning procedure to mobilize *Cry2Ab39* gene into pFGC5941 expression vector. Lanes 1 and 2: digestion of pFGC5941 and pUC57/*Cry2Ab39* using *Nco*I and *Xma*I, respectively. Lanes 4 and 5: digestion of pFGC/35S_*Cry2Ab39* and pBT/At-rbcS1A using *Eco*RI and *Xho*I, respectively. Lanes 3 and 6: Confirmation of recombinant vectors pFGC/35S_*Cry2Ab39* and pFGC/At-rbcS1A_*Cry2Ab39* by treatment with *Nco*I-*Xma*I and *Eco*RI-*Xho*I, respectively. M: DNA ladder 1kb (Thermo Scientific, USA).

a distinct difference in the DNA composition between bacterial Cry genes and plant genes. The A-T percentage of wild-type Cry genes is much higher than in a typical plant gene. These A-T-rich regions in bacterial genes might resemble plant introns or potential plant polyadenylation signal sequences and therefore cause premature termination of translation and subsequent degradation of the target mRNA[24,35-37]. In addition, different preferences in codon usage of plant and bacterial systems lead to ineffective translation[35,37]. In our study, five strategies of optimization were employed to enhance protein expression: (1) minimizing rare codons for tobacco by preferred codon substitution; (2) increasing the frequencies of GC-rich codons to upper the overall GC-content of the whole gene; (3) eliminating potential splice sites as well as polyA addition signal sequences; (4) avoiding RNA secondary structures and RNA instability motifs; (5) translocating the recombinant protein to the ER by addition of a signal peptide and ER retention signal sequence. The purification result showed that expression level of the insecticidal protein in leaf tissues was about 1.5μg/g, which is equal or higher than those of other previous reports[38,39], suggesting effective codon optimization.

4.2 Potential Promoter for *Cry2ab39* Gene Expression in Tobacco Leaves

Regulation of transgene transcription is one of the critical steps in recombinant protein production. Promoter is a key regulatory element responsible for direct control of the transcription initiation process and can explain up to 80% variance of corresponding protein level in host cells[40]. Thus, a suitable promoter is required for optimum gene expression[41]. In this work, effects of the two promoters, CaMV35S and Arabidopsis rbcS1A, on *Cry2Ab39* transient expression were investigated. The former is generally considered as a strong constitutive promoter and commonly used for high-level production of recombinant protein in plant cells[42], while the latter is a tissue-specific promoter for overexpression of transgenes in leaves and green fruits[43,44]. Recombinant protein quantification by Western blot showed that *Cry2Ab39* content in tobacco leaf tissuesunder the direction of At-rbcS1A promoter was significantly higher than that driven by CaMV35S promoter (Figure 2A), suggesting the superior effectiveness of At-rbcS1A. This is

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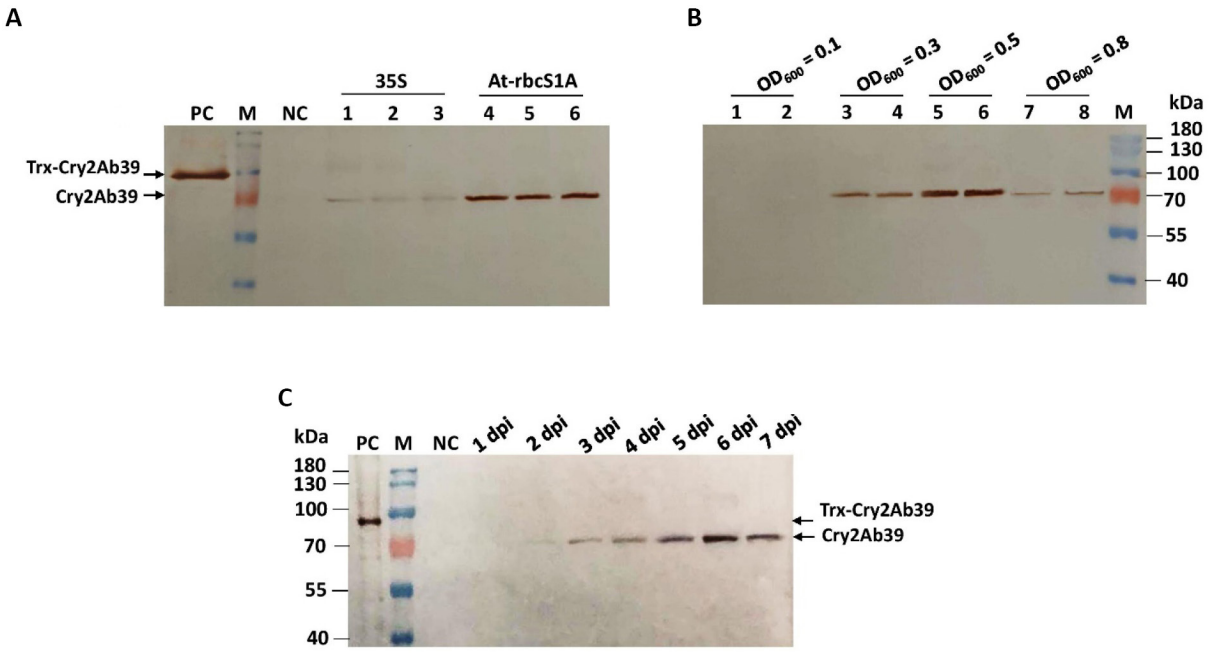

Figure 2. Optimization of transient expression of *Cry2Ab39* in *N. benthamiana*. A: Effect of different promoters (CaMV 35S and At-rbcS1A) on recombinant *Cry2Ab39* production in tobacco. The leaves (3 plants per construct) were infiltrated with *A. tumefaciens* strain AGL1 harboring either one of the two constructs at OD_{600}=0.5. Equal amounts of total soluble protein extracted from 5 dpi leaves were transferred to PVDF membrane and probed with anti-6xHis antibody; B: Optimization of bacterial concentrations for agroinfiltration. The leaves (2 plants per bacterial density) were infiltrated with *A. tumefaciens* strain AGL1 harboring pFGC/At-rbcS1A_*Cry2Ab39* at different concentrations (OD_{600}=0.1, 0.3, 0.5 and 0.8). Equal amounts of total soluble protein extracted from 5dpi leaves were transferred to PVDF membrane and probed with anti-6xHis antibody; C: Optimization of harvest time. Tobacco leaves were infiltrated with *A. tumefaciens* strain AGL1 harboring pFGC/At-rbcS1A_*Cry2Ab39* at OD_{600}=0.5. Equal amounts of total soluble protein extracted from leaves at 1, 2, 3, 4, 5, 6 and 7dpi were transferred to PVDF membrane and probed with anti-6xHis antibody. PC (positive control): Thioredoxin-fused *Cry2Ab39* (Trx-*Cry2Ab39*) expressed in E. coli BL21 (89kDa). NC (negative control): WT plants. M: Prestained Protein Ladder (10 to 180kDa,Thermo Scientific).

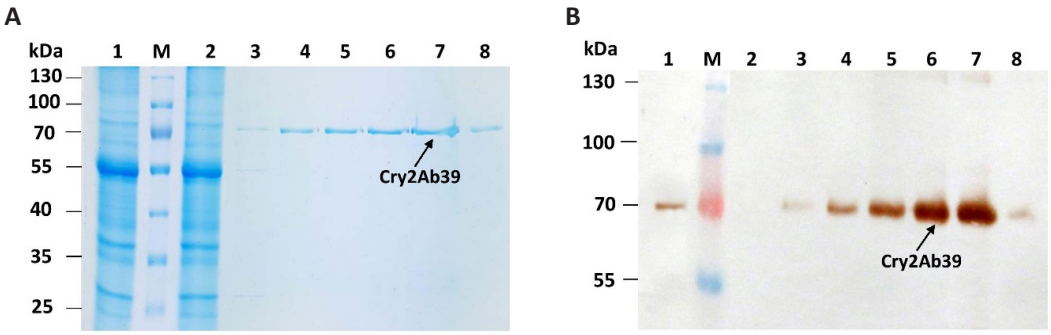

Figure 3. Purification of recombinant *Cry2Ab39*. A and B: SDS-PAGE (A) and Western blot (B) analysis of native purification of *Cry2Ab39*. Lane 1: raw extract of tobacco leaves. Lane 2: flow-through. Lanes 3 to 8: Fractions of eluted protein from Ni-NTA column. M: Prestained protein ladder (Thermo Scientific).

also proof of the fact that strong promoters are not always good choices for overexpression of recombinant proteins. In some situations, the utilization of a very strong promoter to drive the transgenes not only results in accumulation of target mRNAs in the cytoplasm, but also contributes to increase the amount of aberrant RNAs that might trigger post-transcriptional silencing[45,46], ultimately leading to loss of production yield. Furthermore, green-tissue-specific expression using rbcS promoters was proposed to be an efficient approach to improve transgene expression level in plant systems[47-49].

4.3 Optimal Conditions for *Cry2ab39* Protein Production in Tobacco Leaves

Agroinfiltration is a simple but effective tool to deliver heterologous genes into plant cells and rapid to product recombinant proteins[50]. Although this method has been used extensively on leaf tissue of *N. benthamiana*[51-54], depending on the target genes, several factors need to be examined to enhance *Agrobacterium* infection into leaf tissues and increase protein yields. In our study, cell density of bacterial inoculum and harvest time were found to be significant effect to the expression of *Cry2Ab39*. Bacterial

density for agroinfiltration was reported as one of the factors affecting transformation efficiency and subsequent transient expression of recombinant proteins[50,55,56]. Over diluted bacterial suspension may result in a low bacteria/target cell ratio, thereby decreasing transformation frequencies, whereas concentrated bacterial cultures may cause bacterial overgrowth which leads to excessive tissue damage[50,57]. Among tested OD_{600} of *Agrobacterium* suspension, the highest expression level of *Cry2Ab39* was observed at OD_{600}=0.5 (Figure 2B). *Agrobacterium* cell density OD_{600}=0.5 was also used for infiltration in many previous studies to obtain maximum transient expression efficiency of recombinant proteins in *N. benthamiana*[58-60]. A part from bacterial density, the leaf harvesting time after infection also affect the transient expression level of recombinant proteins[61]. Accordingly, agroinfiltrated leaves should be collected when they showed the least damage by *Agrobacterium* infiltration (i.e., chloroplasts loss such aschlorosis, necrosis, and wilting). The cellular damage can affect energy-generating capacity and therefore reduce protein production. As observed in our data (Figure 2C), 6dpi was the best time for obtaining recombinant protein *Cry2Ab39*. According to previous studies, the optimal harvest time for transient expression via agroinfiltration depended on the host plants and *Agrobacterium* strains used for transformation as well as individual introduced recombinant proteins[50,62].

5 CONCLUSION

In this study, anovel bacterial *Cry2Ab39* gene was codon optimized and constructed for expression in *N. benthamiana* leaves via *Agrobacterium* infiltration method. The recombinant *Cry2Ab39* protein was successfully produced and purified from this plant expression system using suitable promoters and optimal transformation parameters. Although the toxicity of the recombinant protein needs to be validated, the designed construct with the codon optimized *Cry2Ab39* gene is potential for further researches to create insect resistant crops.

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Conflicts of Interest

The authors declared no conflict of interest.

Author Contribution

Chu HH supervised the study. Pham NB and Le LMT designed the experiments and reviewed the entire manuscript. Le NT performed gene optimization, vector construction and protein analysis. Trinh VT, Nguyen TT and Tran HT conducted agroinfiltraion. Le NT wrote the main manuscript. Do PT provided proofreading and revising the manuscript.

Abbreviation List

Bt, Bacillus thuringiensis
Cry, Crystal
Cyt, Cytolytic
dpi, Days-post-infiltration
ER, Endoplasmic reticulum
HcPro, Helper component-proteinase
IMAC, immobilized metal ion affinity chromatography
KDEL, Endoplasmic reticulum retention signal
LeB4, Legumin B4
N. Benthamiana, *Nicotiana benthamiana*
OD_{600}, Optical density at 600nm
SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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