A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Improved insecticidal activities of Novel *Bacillus thuringiensis Cry1*-type genes

향상된 살충활성을 가지는

신규 ***Bacillus thuringiensis Cry1*-type** 유전자의 제작 및 특성 파악

By

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UNDER THE DIRECTION OF ADVISER YEON HO JE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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APPROVED AS A QUALIFIED THESIS OF JONG-HOON KIM
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMBERS

CHAIRMAN **Young-Joon Ahn** _____

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ABSTRACT

Bacillus thuringiensis (Bt) is a well-known, gram positive soil bacterium, capable of forming spores and numerous types of crystal proteins, which kill a variety of host insects and nematode pests. On the other hand, these proteins do not harm humans or non-target organisms, and are also environmentally safe. Thus, these Cry proteins have been widely used as effective biological control agents. Recently, Bt *cry* genes encoding insecticidal Cry proteins have been widely applied for the construction of transgenic crops that are

resistant to insect pests. This study aimed to construct novel mutant *Cry1Ac* genes for genetically modified crops with enhanced insecticidal activities.

To achieve this objective, modified *Cry1Ac* genes that were converted to plant-preferring codon usage, were used as templates, and multi site-directed mutagenesis was conducted using a total of 21 primers randomly. As a result, 10 mutant *cry1Ac* genes were synthesized and converted at 24 amino acid residues, located on domain I (8 residues) and domain II (16 residues). These mutant genes were expressed as a fusion protein with polyhedrin using the baculovirus expression system. The expressed proteins were occluded into polyhedra and stably activated to 65 kDa by trypsin. Among these, Mut-N16, constructed from previous studies, showed high levels of insecticidal activities against larvae of *Plutella xylostella*, *Spodoptera exigua*, and *Ostrinia furnacalis*. To confirm the suitability of Mut-N16 protein for introduction into transgenic crops, quantitative bioassays were performed with Cry1Ab protein of SYN-EV176-9. LD_{50} values of Mut-N16 protein were about 6 times lower against *P. xylostella* and 1.4 times lower against *O. furnacalis* than that of Cry1Ab. In the bioassay against *S. exigua*, the LD_{50} value of Mut-N16 protein was 234.28 ng / larva, while Cry1Ab showed very low insecticidal activity.

Mut-N16, which showed the highest insecticidal activity, is expected to be a desirable *cry* gene for introduction into transgenic crops. This study could provide useful means to construct mutant *cry* genes with improved insecticidal activities and expand host spectra for transgenic crops.

Key words: *Bacillus thuringiensis*, mutant *Cry1Ac* gene, multi site-directed mutagenesis, transgenic crop, baculovirus expression system

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INTRODUCTION

Bacillus thuringiensis (Bt) is a well-known, gram positive soil bacterium, capable of forming spores and numerous types of crystal proteins. During the sporulation phase, proteinaceous parasporal inclusions are produced and these parasporal inclusions contain various types of insecticidal crystal protein (ICP). Insecticidal activities of Bt thus relies on crystal protein (Bravo et al., 2007; Choi et al., 2007). These crystal proteins are activated by the proteases of the insect midgut. The activated forms have a high affinity to specific receptors located at the apical brush border of the microvilli of the midgut epithelium of susceptible insects. The binding of the receptor and the protein is an essential factor defining specificity. This process results in the formation of pores within the digestive system that ultimately kills the host insect. On the other hand, these proteins do not harm humans or non-target organisms, and are also environmentally safe. They therefore have been continuously developed, and widely used as effective biological control agents (de Maagd et al., 2001; Herrero et al., 2004; Schnepf et al., 1998; Wang et al., 2008; Whalon and Wingerd, 2003).

Depending on the strain, crystal proteins produced by Bt vary in quantity and type. These diverse crystal proteins have a defined host spectrum of insecticidal activity, usually restricted to a few species within specific orders including Lepidotera, Diptera, Coleoptera and Hymenoptera (Johnson et al., 1998; Kim et al., 2008). Moreover, several crystal proteins have susceptible activities against non-insect species such as nematodes, mites and protozoa (Marroquin et al., 2000). Since the first cloning of the *Cry1Aa* gene

from Bt subsp. *kurstaki* strain HD1 (Schnepf and Whiteley, 1981), various *cry* genes were reported (Lecadet et al., 1999; Li-Ming et al., 2008). The objective of finding novel Cry proteins focuses on three main purposes: 1) the search for a new range of activities, 2) the search for higher levels of toxicity, and 3) the search for alternative toxins in case of resistance development (Noguera and Ibarra, 2010).

Even if Bt products have momentous advantages as biological insecticides, each type of Bt crystal protein has a very narrow host spectrum, as well as the fact that the target insects may develop resistance. In order to alleviate these problems, many genetic engineering studies have been conducted using Bt strains and *cry* genes for enhancement of insecticidal activity, faster effects and delay of resistance development (Aronson et al., 1995; Kalman et al., 1995). The prospects of genetic engineering of Bt are as follows: 1) expression of a different *cry* gene in natural Bt by transformation or conjugation, 2) expression of cry genes in alternative hosts, and 3) transgenic insecticidal plants transformed with *cry* genes (Gao et al., 2004; Khasdan et al., 2003; Murphy and Stevens, 1992).

Recently, studies that introduce *cry* genes into crops to create pest resistance have made much progress, and the total area of land planted with Bt crops has increased substantially (James, 2014; Tabashnik et al., 2003). Most Bt crops however express cry1-type proteins which are specific to a particular lepidopteran host because of the difficulty in converting DNA nucleotide sequences to crop-preferring codon usage, and recent discoveries have shown that these target species have developed resistance against the introduced cry proteins (Romeis et al., 2006; Tabashnik et al., 2003). The purpose of this study is to overcome these problems by synthesizing novel Bt *cry* genes with

improved insecticidal activities and host range that can be substituted.

In this study, mutant *Cry1Ac* genes with enhanced insecticidal activity were synthesized for transgenic crops. For the construction of various mutant *Cry1Ac* genes, multi site-directed mutagenesis was performed using a modified 1.8 kb *Cry1Ac* gene (*mod-Cry1Ac*). These mutant *Cry1Ac* genes were expressed as a fusion protein with polyhedrin using the baculovirus expression system and their insecticidal activities were investigated against larvae of several lepidopteran pests. Furthermore, a comparison quantification bioassay was performed to confirm a suitable *Cry* gene candidate. Based on the result of this bioassay, the most appropriate novel *Cry* gene was selected for the construction of genetically modified crops.

LITERATURE REVIEW

1. Basic biological characteristic of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) was first discovered by Shigetane Ishiwata as the causative agent of the "sotto" disease responsible for the silkworm deaths in 1901 (Federici et al., 2010). The bacteria was given its name, Bacillus thuringiensis, by the German scientist Berliner in 1911, who discovered the insecticidal activities of Bt against the flour moth, *Ephestia kuehniella*, found in the town of Thuringia, Germany. The first application of Bt as an insecticidal agent, commonly in the form of liquid sprays, occurred in the late 1920s after Berliner confirmed the existence of Bt parasporal inclusions (Lemaux, 2008). The bacteria was given its name, Bacillus thuringiensis, by the German scientist Berliner in 1911, who discovered the insecticidal activities of Bt against the flour moth, *Ephestia kuehniella*, found in the town of Thuringia, Germany. The first application of Bt as an insecticidal agent, commonly in the form of liquid sprays, occurred in the late 1920s after Berliner confirmed the existence of Bt parasporal inclusions (Lemaux, 2008). Bt was considered to be toxic only against Lepidoptera until other subspecies lethal to dipterans, coleopterans, hymenopterans and nematodes were discovered in the 1970s. In 1981, Schnepf and Whiteley cloned and expressed the first *cry* gene, which encodes the insecticidal crystal proteins. More than 60, 000 Bt have been acquired by numerous industries in order to obtain novel *cry* genes, and over 371 *cry* genes have been reported (Choi et al., 2007; Li-Ming et al., 2008).

Bt is a gram positive bacteria closely related to B. cereus, which is the causative agent

of human gastroenteritis. Bt however is ineffective against mammals, and are known to produce one or more proteinaceous inclusions during sporulation as a phenotypic difference (Kolstø et al., 1990). These inclusions include endotoxins, exotoxins, haemolysins and enterotoxins. Among these proteins, the delta-endotoxins (Cry protein) show high toxicity to a wide range of significant agricultural and health-related insect pests as well as high host specificity. Due to the aforementioned characteristics, Bt have been often valued as effective biological control agents for more than 40 years, consisting of more than 90% of commercially available biopesticides (Harris, 1991; Raddadi et al., 2009; Van Rie, 2000). Bt by themselves as insecticides however, are limited by their narrow host range, a short shelf life, and the development of resistance by their target insects. In order to overcome these disadvantages, Bt strains have been subjected to genetic modifications which improve their effectiveness against their targets, as well as increasing host spectra (Lecadet et al., 1992; Wang et al., 2008).

Classification of Bt strains into serotypes were conducted according to their H-flagellar antigenic determinants (De Barjac and Frachon, 1990; De Barjac et al., 1981). Sixty nine serotypes and 13 sub-antigenic groups which gives rise to 82 different serovars have been identified to date, and were sorted into subspecies accordingly (Lecadet et al., 1999). Serotyping is one of the most frequently used classification techniques used throughout the world. It is however, limited by the fact that it considers only one aspect of the species, which means that it is not a reliable predictor of insecticidal activity.

2. Bt Toxins.

Bacillus thuringiensis (Bt) crystalline inclusions containing insecticidal

delta-endotoxin proteins are formed during sporulation. When these crystalline inclusions are ingested by the host, they dissolve due to the alkaline conditions of the midgut and one or more of the delta-endotoxins are activated by proteolytic enzymes depending on the strain. Depending on growth conditions and the type of toxin present in the crystal, the shape of the crystal may vary, with a variety of crystal morphologies sometimes occurring concurrently. Most natural isolates are bipyramidal or circular in shape (Bernhard et al., 1997; Martin and Travers, 1989).

A majority of delta-endotoxins are encoded by the *cry* genes, whose gene products consist of approximately 20~30% of all bacterial proteins that are expressed (Boucias and Pendland, 2012). These *cry* genes were initially organized into four different types (Höfte and Whiteley, 1989) based on amino acid sequence homology and host specificity. Expression of type 1 genes produce proteins that are approximately 130 kDa in size, which are specific to lepidopterans. Type II genes encode proteins that are 70 kDa in size, and are specific to both lepidopterans and dipterans. Type III on the other hand, produce proteins that are specific to coleopterans. Type IV gene products are specific to dipterans. Type V genes that encode proteins specific to lepidopterans and coleopterans were included (Tailor et al., 1992). The current classification system groups the genes based only on amino acid sequence homology. Each protoxin is given a name that begins with the mnemonic Cry, and includes four hierarchical ranks consisting of numbers. The four numerical ranks of genes are given depending on their place within a phylogenetic tree. Proteins that have less than 45% sequence identity differ in primary rank, and 78% and 95% sequence identity define the boundaries of secondary and tertiary ranks. All known Cry proteins have been grouped and distributed into classes only by the similarity in

amino acid sequences. Several classes such as Cry 6 and Cry 15, show little similarity with most other Cry proteins. This new classification system has replaced the former nomenclature using roman numerals.

Each Cry protein has its own unique host spectrum. Cry 1, Cry 2 and Cry 9 proteins have the strongest effect on lepidopterans while Cry 2, Cry 4, Cry 10, Cry 11, Cry 16, Cry 17, Cry 24, Cry 25, Cry 27, Cry 29, Cry 30, Cry 32, Cry 39 and Cry 40 have the highest toxicity against Dipterans. In the case of coleopterans, Cry 3, Cry 7, Cry 8, Cry 18, Cry 34, Cry 35 and Cry 43 are most toxic. Some types were found to show activities against more than one insect order. Cry 1I for example, is effective against both lepidopterans and coleopterans. Another protein, Cry1B, has insecticidal activities against lepidopterans, coleopterans and dipterans. Many of these proteins are used as efficient control agents but target insects have developed resistance to a few of them (Tan et al., 2009).

3. Structure of Cry protein

Cry proteins consist of three major groups which are phylogenetically unrelated. These are the three domain Cry toxins, mosquitocidal-like Cry toxins and binary-like Cry toxins. Each group may operate under different modes of action. Out of the three, the three domain Cry proteins represent the largest group. The N-terminal domain I is a bundle comprised of seven alpha-helices, may help the insertion of the toxin into the cell membrane, causing pore formation. Domain II consists of a beta-prism of three anti-parallel beta-sheets surrounding a hydrophobic core, and has been found to play a role in the interaction of the toxin protein and its binding sites in the midgut epithelium.

This suggests that domain II is an important factor in the insect specificity of the toxin. The interaction of domain II with the cellular binding site occurs through three main loops in its structure. Mutations of several residues within these loops leads to a decreased binding affinity of the toxins, and a decrease in toxicity as a result (Schnepf et al., 1998).

Domain III is a beta-sandwich, consisting of two antiparallel beta-sheets. This domain has also been revealed to take part in receptor recognition, and thus specificity (De Maagd et al., 1996; Lee et al., 1995). Upon binding, domain I or at least a part of domain I is inserted into the cell membrane as an oligomer, forming an aqueous pore with other toxins. In this regard, domain I is similar to other pore-forming or membrane-translocation domains of bacterial toxins. An umbrella-like model shows that alpha-helices 4 and 5 of the oligomer make up the pore, and the rest of the protein spreads over the surface of the membrane (Gazit et al., 1998).

4. Mode of action of Cry protein

Two models have been used to explain the mode of action of Cry proteins: the pore-forming model and the signal transduction model.

Lepidopteran larvae were mainly used to study and identify the modes of action of Cry proteins. The major function of Cry proteins is to facilitate bacterial infiltration into the hemocoel by disrupting the midgut epithelium. Once the larva ingests the crystal proteins, the alkaline pH of the midgut induce solubilization of inactive protoxins. The protoxins are then subjected to proteolysis by host proteases, and are activated as a result. Activated toxins are resistant to further proteolysis, and pass through the peritrophic matrix. Once

they have traversed the peritrophic membrane, the toxins bind with the brush border membrane of midget epithelia. The binding of toxins to the brush border initiates the formation of pores, which eventually leads to the death of the insect (Bietlot et al., 1989; Bravo et al., 1992; Herrero et al., 2004). Cadherin has been recently recognized as a functional receptor in *An. gambiae*, a dipteran. Binding of cadherin and cry proteins induce further proteolytic cleavages of the N-terminal end of the proteins by proteases, which results in the elimination of helix alpha-1 of domain I. The oligomeric forms of the toxins formed by this cleavage have enhanced binding affinities to the secondary receptors, which are glycosylphosphatidyl-inositol (GPI)-anchored proteins such as aminopeptidase N (APN) and alkaline phosphatase (ALP). These oligomers insert into the sites of membrane microdomains where GPI-anchored receptors are localized. This causes the formation of pores in the apical membrane of midgut epithelial cells, which lead to osmotic shock and bursting of the cells (Bravo et al., 2004; Soberon et al., 2009).

The signaling pathway model is an alternative mechanism which considers the activation of stimulatory guanine nucleotide-binding proteins (G proteins) and adenylyl cyclases (AC), the increase in cyclic adenosine monophosphate (cAMP) levels that follow, and the activation of protein kinase A (PKA) (García-Robles et al., 2012). This model proposes that Cry proteins achieve their toxic effects through the activation of Magnesium dependent signal cascade pathways that is induced by the interaction of monomeric three domain Cry toxins (3D-Cry) with cadherin, the primary receptor. The interaction of the toxin and cadherin stimulates a G protein which then activates an AC. The activated AC then causes an increase in intracellular cAMP levels, activating PKA as a result. PKA then turns on an intracellular pathway leading to cell death. The model

therefore suggests that cell death occurs without the pore forming oligomeric structures of Cry toxins or other receptors such as GPI-anchored proteins (Zhang et al., 2006).

5. Transgenic crops expressing Bt Cry protein

Several *Bacillus thuringiensis* (Bt) *cry* genes have been introduced into a variety of crops as protection against pests, starting first with tobacco and now with major crops such as cotton, maize, potato, rice, broccoli, lettuce, walnut, apple, alfalfa and soybean (Barton, 1987). For example, transgenic Bt maize is very effective against the European corn borer, *Ostrinia nubilalis*. Transgenic maize is also highly effective against the spotted stem borer, *Chilo partellus* and the maize stalk borer, *Busseola fusca* (Koziel et al., 1993; Van Rensburg, 1999). Even without considering these examples, Bt transgenic crops have been proven to be effective against many other pest species, depending on the type of *cry* genes introduced. Transgenic plants with Bt *cry* genes have been planted for cultivation mainly in the United States, Brazil, Argentina, India, Canada and China. A record of 181.5 million hectares of transgenic crops were cultivated globally. Since 1996, the growth of transgenic plant hectarage continued after eighteen consecutive years of increase, with 12 out of 18 years having double-digit growth rates. The spread of transgenic crops is becoming more rapid than any other agricultural technology in history, which suggests that farmers perceive significant advantages in using them (James, 2014; Raney, 2006).

However, one problem was that native *cry* genes were expressed poorly in plants. This suggested that in order to produce transgenic plants that are effective against economically significant pests, a large improvement of *cry* gene expression in plant

systems was necessary. The inadequate expression of protoxin genes from plant nuclei has required the use of truncated versions of the genes, which only code for the active toxin fragments (Gleave et al., 1998). Bt DNA, which is A-T rich, contains several sequences that may produce signals deleterious to gene expression in plants such as splice sites, poly(A) addition sites, ATTTA sequences, mRNA degradation signals and transcription termination sites as well as rare codons (Liu et al., 2004). When synonymous codons are used to modify Bt sequences such that the deleterious signals are reduced or eliminated, and a more plant like codon bias is induced as a result, expression levels of *cry* genes have been shown to improve drastically (Perlak et al., 1993). In several instances, smaller changes in the coding regions have also resulted in dramatic increases in expression levels (Perlak et al., 1991; Van Der Salm et al., 1994). Modified *cry* genes have led to the successful development of commercially used insect resistant transgenic plants.

A recent issue is the rise of insect pest resistance to transgenic crops which encoding *cry* genes. One of the major mechanisms of toxin resistance is the reduction in toxin binding to midgut epithelia through mutations of Cry toxin receptors such as cadherin, ALP or APN. The most commonly occurring phenotype of resistance, also known as Mode 1 of Resistance, is characterized by the reduction of Cry1A toxin binding, including cross resistance of Cry1Aa, Cry1Ab and Cry1Ac. In some lepidopterans, the mode 1 of resistance is related to mutations in the cadherin gene (Bravo et al., 2011; Gahan et al., 2001). Many studies using genetic methods such as mutagenesis and imposition of high-performance genes via gene fusion were conducted in order to delay the development of insect resistance.

MATERIALS AND METHODS

1. Construction of a transfer vector including mutant *Cry1Ac* gene.

A. Cloning of pOB-*Mod-Cry1Ac* transfer vector

The *mod-Cry1Ac* gene, which was constructed according to a crop-preferring codon usage, was kindly provided by Dr. Beom-Seok Park (National Institute of Agricultural Biotechnology, RDA, Korea). For the mutation of *mod-Cry1Ac*, the alignment of the amino acid sequence of the Mod-Cry1Ac and other Cry1-type proteins was performed using MegAlign (DNASTAR Inc., USA). For the mutagenesis of *mod-Cry1Ac* gene, *mod-Cry1Ac* gene was amplified using specific primers, Mod1Ac-ATG-F containing Xho I at 5'-end (5'-AAACTCGAGATGGACAACAACCCAAAC-3') and Mod1Ac-TAA-R containing EcoRⅠ at 5'-end (5'-TTTGAATTCTTAAAGATTGTACTCAGCCTC-3'). The PCR-amplified *mod-Cry1Ac* gene fragment was digested with XhoI and EcoRI, and inserted into pOBI vector digested with the same restriction endonucleases to obtain the pOB-*Mod-Cry1Ac* (Fig. 1).

B. Cloning of pOBI-*Cry1Ab* transfer vector

In order to construct the pOBI-*Cry1Ab* transfer vector, total genomic DNA was isolated from SYN-EV176-9 (Syngenta seeds. Switzerland) powder using the DNeasy® Plant Mini Kit (QIAZEN, GERMANY), following the manufacturer's instructions. Using total genomic DNA of SYN-EV176-9 as a template, *Cry1Ab* gene was amplified using specific primers, Bt176Cry–XhoIF containing Xho I restriction endonuclease site at the

5’-end (5’-CGCCTCGAGATGGACAACAACCC-3’) and Bt176Cry-EcoRIR containing EcoR I restriction site at the 5’-end (5’-CGCGAATTCCTACACCTGATCGATGTG-3’). The amplified *Cry1Ab* gene digested by XhoI and EcoRI was introduced to a pOBI vector digested by the same restriction endonucleases to obtain the pOBI-*Cry1Ab* (Fig. 2).

C. Multi site-directed mutagenesis

The primer sequences were based on the codon usage in pooled sequences of Chinese cabbage genes (Table 1) and 21 primers for mutagenesis were designed to change 24 amino acid sequences located on domain I and domain II (Table 2). All of the primers used for simultaneous mutagenesis were annealed to the same strand of the template plasmid. Primers were designed to be between 25 and 45 bases in length with a melting temperature (Tm) over 75°C, which was calculated using the QuickChange® Tm calculator (available online at http://www.stratagene.com).

The *mod-cry1Ac* gene was mutated using the QuickChange® multi site-directed mutagenesis method (Stratagene, USA) (Fig. 3). Template DNA was replicated with 3 to 5 mutagenic primers using enzyme blend including QuickChange® DNA polymerase (Stratagene, USA) according to the following cycle parameters; step 1, 95°C, 1min; step 2, 95°C, 1min; step 3, 55°C, 1min; step 4, 65°C, 17min (every successive cycle repeats steps 2 to 4, 34 times); step 5, 4°C, unlimited. All DNA amplifications were performed with the DNA Thermal Cycler (BIO-RAD, USA). The disintegration of methylated or hemimethylated template DNA was performed with DpnI (New England Biolab, USA). Mutated single stranded-DNA was transformed into XL-10 Gold Ultracompetent cells

(Stratagene, USA). Each mutant was confirmed by DNA sequencing analysis using specific primers, Mod-cry1Ac-F sequence with 5'-ACCGACTACGCTGTTCG-3' and Mod-cry1Ac-R sequence with 5'-AATGTTGTTGCCAGAGC-3'.

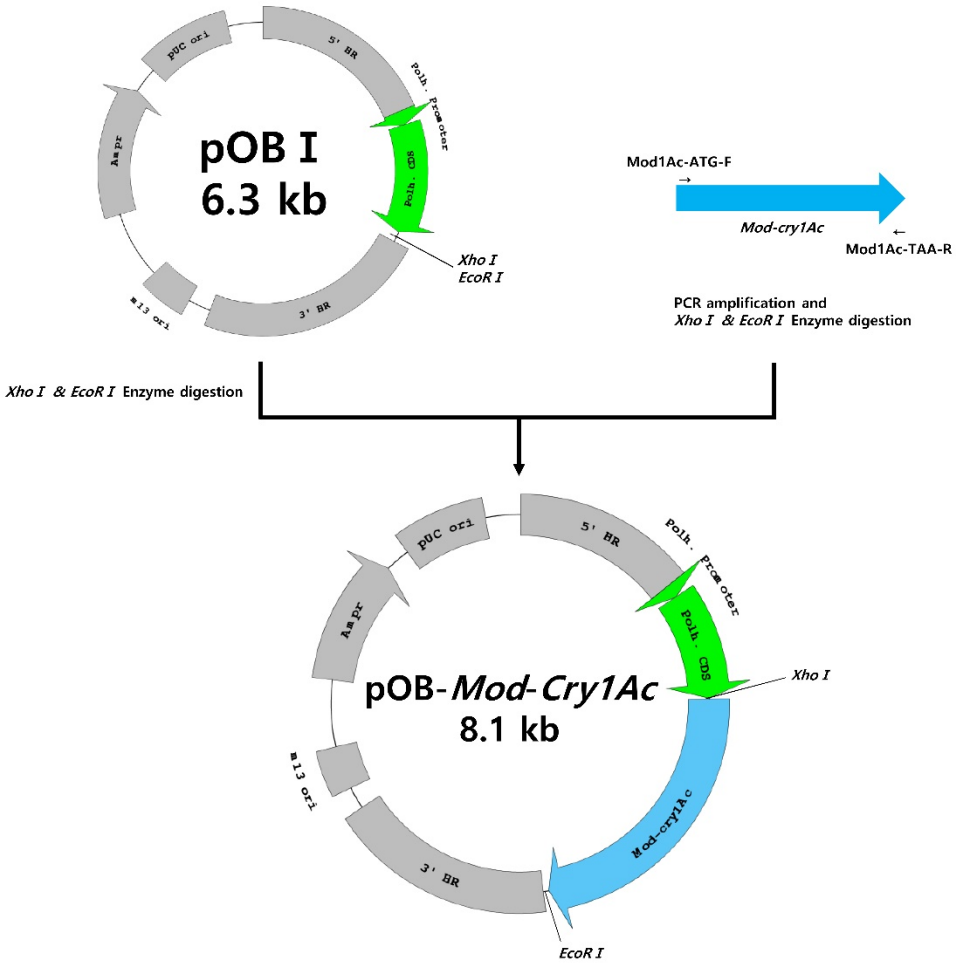

Figure 1. Construction map of transfer vector, pOB Ⅰ-*Mod-Cry1Ac*, expressing *Mod-Cry1Ac* gene. Modified *Cry1Ac* gene was inserted into pOBI vector to obtain the pOB-*Mod-Cry1Ac* transfer vector.

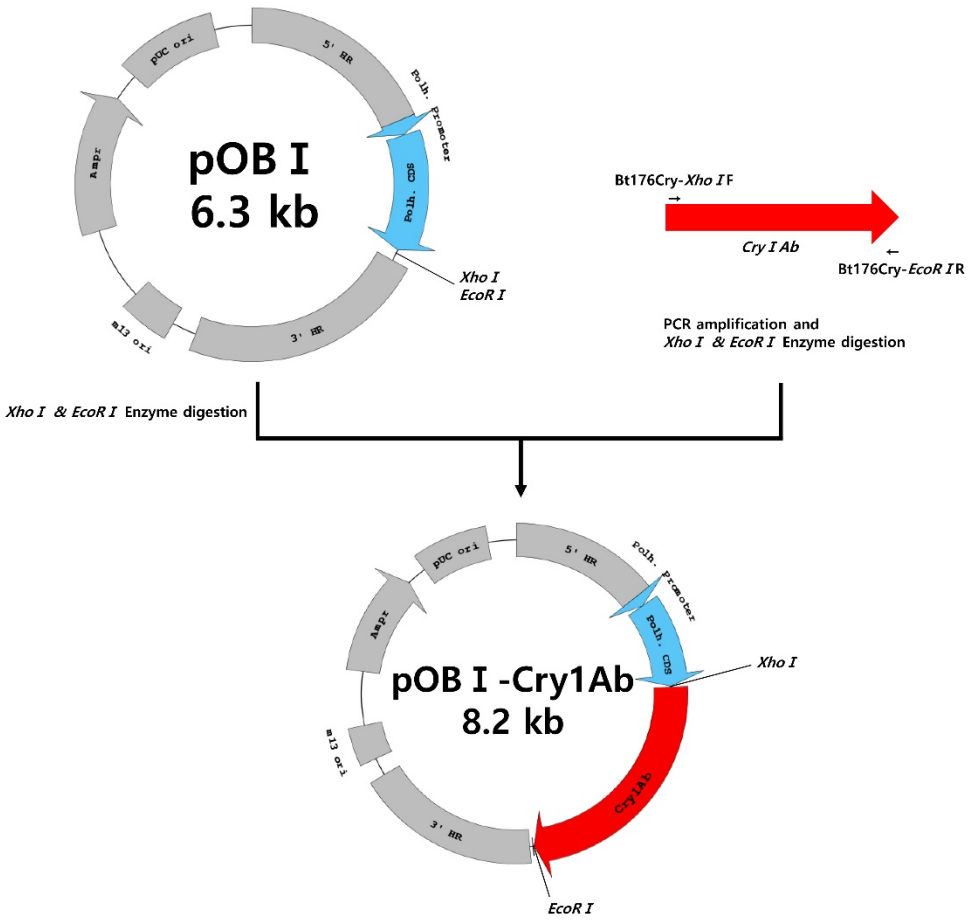

Figure 2. Construction map of transfer vector, pOB Ⅰ-Cry1Ab, expressing Cry1Ab gene of SYN-EV176-9. Cry1Ab gene of SYN-EV176-9 was inserted into pOBI vector to obtain the pOB-Mod-Cry1Ab transfer vector**.**

Table 1. Codon usage in pooled sequences of Chinese cabbage genes.

Amino acid	Codon	Preference, %	Amino acid	Codon	Preference, %
ARG (R)	CGA	9.4	**ASP (D)**	GAC	41.1
	CGC	13.7		GAU	58.9
	CGG	6.0	**ILE (I)**	AUA	18.5
	CGU	20.5		AUC	45.7
	AGA	25.6		AUU	35.8
	AGG	24.8	**SER (S)**	UCA	19.4
PRO (P)	CCA	32.5		UCC	14.7
	CCC	13.3		UCG	11.6
	CCG	19.2		UCU	19.4
	CCU	34.9		AGC	19.4
LYS (K)	AAA	29.8		AGU	15.5
	AAG	70.2	**GLY (G)**	GGA	32.1
GLU (E)	GAA	45.3		GGC	12.3
	GAG	54.7		GGG	18.5
PHE (F)	UUC	55.0		GGU	37.0
	UUU	45.0	**GLN(G)**	CAA	48.6
LEU (L)	CUA	5.0		CAG	51.4
	CUC	23.5	**TYR (Y)**	UAC	63.0
	CUG	8.9		UAU	37.0
	CUU	24.0	**THR (T)**	ACA	24.6
	UUA	13.4		ACC	31.9
	UUG	25.1		ACG	17.4
ALA (A)	GCA	21.6		ACU	26.1
	GCC	16.7	**VAL (V)**	GUA	12.3
	GCG	14.2		GUC	23.5
	GCU	47.5		GUG	34.6
ASN (N)	AAC	60.2		GUU	29.6
	AAU	39.8	**HIS (H)**	CAC	37.2
MET (T)	GAC	100.0		CAU	62.8
TRP (W)	TRP	100.0	**TER**	UAA	33.3
CYS (C)	UGC	40.0		UAG	11.1
	UGU	60.0		UGA	55.5

Table 2. Nucleotide sequences of primers used in the multi site-directed mutagenesis.

	Primer	Primer sequence[a] (5′→ 3′)	Mutated residues
Domain I	E116A	GCAGAGAGCTTCAGAGCTTGGGAAGCCG	E116A
	E128V	CCCAGCTCTCCGCGTGGAAA	E128V
	G183E	GGGCAAAGATGGGAATTCGATGCTGCAA	G183E
	A187T	GGGGATTCGATGCTACCACCATCAATAGCCG	A187T
	V227I	CTGATTCTAGAGATTGGATCAGATACAACCAGTTCAGG	V227I
	A245S	CAGTTTTGGACATTGTGTCTCTCTTCCCGAAC	A245S
	P248S	ATTGTGGCTCTCTTCAGCAACTATGACTCCAGA	P248S
	R254T	CCCGAACTATGACTCCAGAACCTACCCTATCCGTAC	R254T
Domain II	S283M	GCTTCCGTGGTATGGCCCAGGGTATCG	S283M
	S283M&G286R	CCGTGGTATGGCCCAGAGGATCGAAAGATC	S283M, G286R
	G286R	CGTGGTTCTGCCCAGAGGATCGAAAGATCC	G286R
	A309V	GCATAACTATCTACACCGATGTGCACAGAGGATACTATTACTGGT	A309V
	M322T	CTGGACACCAGATCACCGCCTCTCCAGTTGG	M322T, F324S
	T334A	CCGGACCTGAGTTTGCTTTTCCTCTCTATGG	T334A
	Y338F	GTTTACCTTTCCTCTCTTCGGAAACGCTGGAAACGCCGCTCCA	Y338F, T340N, M341A
	I375P	CCCTTCAATATCGGTCCTAACAACCAGCAAC	I375P
	Q379E	GGTATCAACAACCAGGAACTTTCCGTTCTTGACGGAAC	Q379E
	S381F	GGTATCAACAACCAGCAACTTTTCGTTCTTGACGGAAC	S381F
	E412V	CGTTGATTCCTTGGACGTGATCCCACCACAG	E412V
	N417D	GATCCCACCACAGGATAACAGCGTGCCACCCAGGC	N417D, N419S
	Q424A	GTGCCACCCAGGGCTGGATTCTCCCAC	Q424A

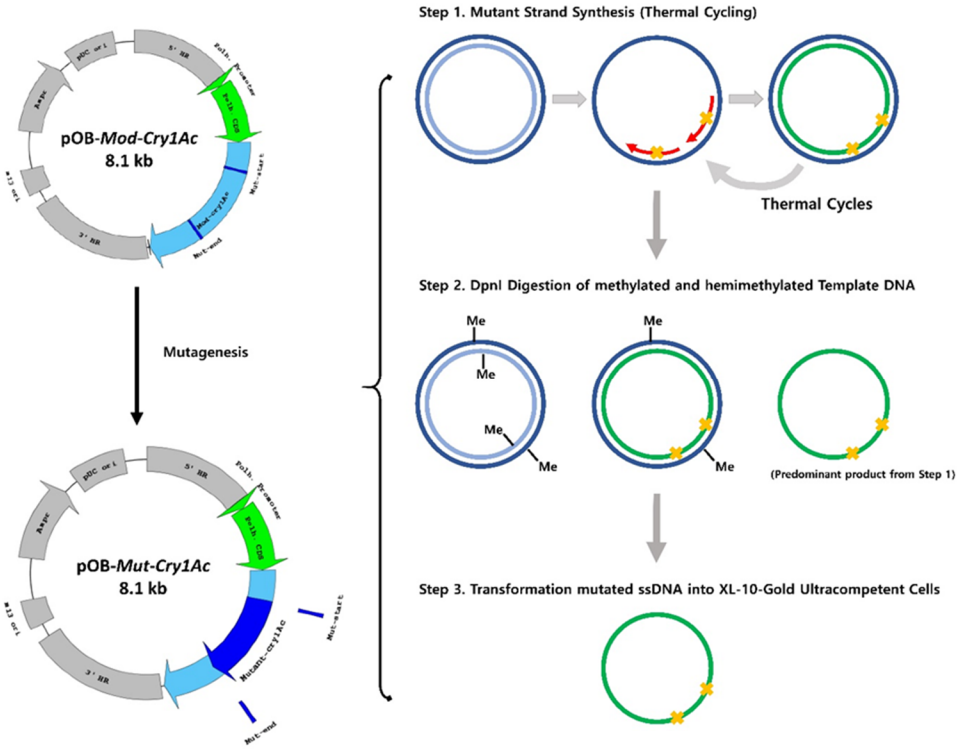

Figure 3. Model of multi site-directed mutagenesis using pOB-*Mod-Cry1Ac*.

2. Expression of mutant *Cry1Ac* genes using Baculovirus expression system

A. Insect cell lines and baculoviruses

Spodoptera frugiferda cells (Sf9 cells) were maintained on TC-100 medium (WelGENE, Korea) supplemented with 10% heat-inactivated (55℃, 30 min) fetal bovine serum (WelGENE, Japan) at 27℃. All insect cell lines were cultured at 28℃ and sub-cultured for 5 days.

Wild-type *Autographa californica* nucleopolyhedrovirus (AcMNPV) and all of recombinant AcMNPVs used in this study were propagated in Sf9 cells maintained in TC-100 medium. A wild-type AcMNPV was used as a control virus.

B. Construction of recombinant baculovirus expressing recombinant protein

Mutant Cry1Ac proteins were expressed using the baculovirus expression system with a defective viral genome, bApGOZA (Je et al., 2003). The cell culture dish (60-mm diameter) seeded with 5-7 × 10^5 Sf9 cells was incubated at 27℃ for 30 min to let the cells attach. The bApGOZA DNA (500ng) and each transfer vector DNA (2 ug) were cotransfected into Sf9 cells using of Cellfectin II™ reagent (Invitrogen Co., USA) according to the manufacturer's instruction. The bApGOZA DNA, transfer vector DNA and 100 ul of the serum-free TC-100 medium were mixed in a polystyrene tube. 20 ul of Cellfectin II™ reagent and 100 ul of the serum-free TC-100 medium were mixed in another polystyrene tube. The two solutions were gently mixed and the mixture was incubated at room temperature for 30 min. The attached cells were washed with 3 ml

serum-free TC-100 medium and refreshed twice with 2 ml of the same medium. After 30 min, the Cellfectin II-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubation at 27℃ for 5 h, 3ml of the TC-100 medium containing 30 ul antibiotics and 10% FBS were added to each dish and incubation at 27℃ continued. At 5 days post-transfection (p.i.), the supernatant containing recombinant virus was harvested and used as inoculum for the proliferation of recombinant virus.

C. SDS-PAGE

To analyze fusion protein expression, Sf9 cells infected with the recombinant viruses were lysed with cell-lysis buffer (50 mM Tris-HCl, pH 8.0; 0.4% SDS; 10 mM EDTA; 5% 2-Mercaptoethanol), sonicated (Duty cycle, 30; Output Control, 3) for 120 s and centrifuged at 15,000 rpm for 10 min. The resulting pellet was washed with 0.5% SDS and with 0.1% Tween 20 solution, and then analyzed in 12% SDS-PAGE. To digest mutant proteins as active forms, the expressed fusion proteins were treated with alkaline lysis buffer (0.1 M Na_2CO_3, 0.01 M EDTA, 0.17 M NaCl, pH 10.5) at 37°C for 1 h, and treated with trypsin at 37°C for 2 h. Wild type AcMNPV was used as a negative control and mod-Cry1Ac was used as a positive control. SDS-PAGE samples were mixed with an equal volume of 2 × SDS-PAGE sample buffer (Sigma, USA). After boiling at 100℃ for 10 min, each sample was loaded into the 12% Mini-PROTEAN® TGX™ gels (BIO-RAD, USA), and the gel was stained with Coomassie brilliant blue for 1 hour and then destained with destain buffer (Methanol : Glacial acetic acid : Distilled water = 1 : 1 : 8).

For the quantification of mutant Cry1Ac proteins occluded in the recombinant polyhedra, the activated proteins were subjected to 12% SDS-PAGE, and the amounts were determined by the 1D-gel analysis system (Kodak Co., USA).

3. Determining insecticidal activities of mutant Cry1Ac proteins

A. Screening bioassay of mutant Cry1Ac proteins

The insecticidal activities of recombinant polyhedra containing mutant Cry1Ac proteins were determined against the larvae of *Plutella xylostella*, *Spodoptera exigua*, and *Ostrinia furnacalis*. To evaluate their insecticidal activities against *P. xylostella* and *S. exigua*, recombinant polyhedra were treated on Chinese cabbage leaf discs (1.5 X 1.5 cm^2) and thirty larvae of *P. xylostella* (3rd-instar) or *S. exigua* (2nd-instar) were introduced to each leaf surface, respectively. For *P. xylostella*, each larva was treated with a concentration of 5 ng/larva and their mortality was scored at 3 days after inoculation. In the case of *S. exigua*, each larva was treated with a concentration of 300 ng/larva and their mortality was scored at 5 days after inoculation. To determine the insecticidal activity against *O. furnacalis*, the recombinant polyhedra were treated on a small slice of artificial diet (2.0 X 2.0 cm^2) and thirty neonates were laid on each slice. Each *O. furnacalis* larva was treated with a concentration of 50 ng/larva and their mortality was scored at 6 days after inoculation.

B. Quantitative bioassay

To compare the insecticidal activities of Mutant N16 Cry1Ac protein and Cry1Ab protein of SYN-EV176-9, a quantitative bioassay was performed. Cry1Ab proteins were expressed using the baculovirus expression system and then SDS-PAGE was performed. All processes use the same materials and methods described above. To determine the median lethal dose (LD_{50}) and median lethal time (LT_{50}), serial dilutions of the recombinant polyhedra were treated on Chinese cabbage leaf discs (1.5 X 1.5 cm^2) and thirty larvae of *P. xylostella* (3rd-instar) or *S. exigua* (2nd-instar) were introduced to each leaf surface, respectively. For *O. furnacalis*, serial dilutions of the recombinant polyhedra were treated on small slices of artificial diet (2.0 X 2.0 cm^2) and thirty neonates were laid on each slice. The mortality was checked at every 24 h interval for 2 days against *P. xylostella*, 5 days against *S. exigua*, and 6 days against *O. furnacalis*, respectively. The LD_{50} and LT_{50} values were calculated by a Probit analysis (Russell et al., 1977) using SPSS statistics 21 (IBM., USA). All assays were performed in triplicates at 25℃ in 60~70% humidity with a 16h : 8h light-dark cycle.

RESULTS

1. Construction of baculovirus transfer vector including mutant *Cry1Ac* genes.

A. Construction of pOBI-*Mod-Cry1Ac* transfer vector

In order to perform the mutagenesis of modified *Cry1Ac* gene (1857bp) and express mutant *Cry1Ac* genes using the baculovirus expression system, modified *Cry1Ac* gene was inserted into a pOBI vector (Yang-su Kim, thesis). pOBI-*Mod-Cry1Ac* transfer vector contains the pUC origin, ampicillin resistance gene and polyhedrin promoter gene, which can insert into bApGOZA and express recombinant proteins in Sf9 cells. Constructed transfer vector was confirmed by restriction endonuclease digestion patterns and DNA sequencing. The total size of pOBI-*Mod-Cry1Ac* was 8084 bp.

B. Multi site-directed mutagenesis

Through several repetitions of multi site-directed mutagenesis using 3~5 primers, a total of 10 different mutant *Cry1Ac* genes were obtained and named Mut-NX (the capital letter 'X' represents the clone number), respectively. The mutated regions of each clone were confirmed by DNA sequencing analysis. Each mutant had different mutagenic residues at 24 amino acid sequences (Fig. 4).

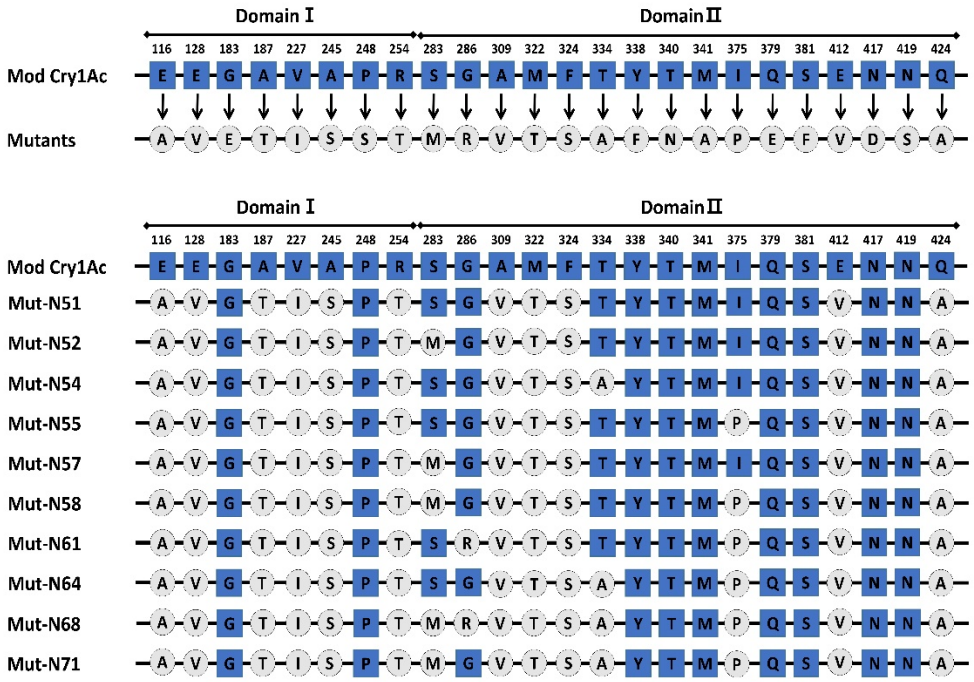

Figure 4. Mutated residues of 10 mutant Cry1Ac proteins. Ellipse represents mutated amino acid sequences and rectangle represents unchanged amino acid sequences.

2. Expression of mutant *Cry1Ac* genes using baculovirus expression system.

Ten mutant *Cry1Ac* genes were expressed using the baculovirus expression vector system. *Mod-Cry1Ac*, *Cry1Ac* and *Cry1C* gene were used as control. bApGOZA DNA was co-transfected with baculovirus transfer vectors into Sf9 cells (Fig. 5). The internal genome structure of the recombinant viruses harboring mutant *Cry1Ac* genes was confirmed by PCR analysis using specific primer sets (Fig. 6). Also, RT-PCR analysis showed that the polyhedrin-mutant *Cry1Ac* fusion genes were successfully transcribed in Sf9 cells infected with the recombinant viruses (Fig. 7). The supernatant containing budded viruses was collected at 7 days post infection (p.i.), re-infected into a monolayer of Sf9 cells, and infected cells were harvested at 7 days p.i. (Fig. 8).

In the SDS-PAGE analysis of the recombinant viruses, results showed fusion protein bands of Mutant Cry1Ac approximately 95kDa in size, while the wild-type *Autographa californica* nucleopolyhedrovirus (AcMNPV) yielded a polyhedron protein band about 30kDa in size (Fig. 9A). To confirm whether the recombinant polyhedra would be cleaved into the active toxin by proteolytic enzymes, fusion proteins were treated with trypsin, one of the major proteolytic enzymes present in the insect midgut. The activated mutant Cry1Ac proteins were approximately 65kDa in size as activated forms (Fig. 9B). For the bioassay, activated Mutant Cry1Ac proteins were quantitatively measured by a 1D-gel analysis system (Kodak Co., USA) using Bovine Serum Albumin (BSA) as the standard (Table 3).

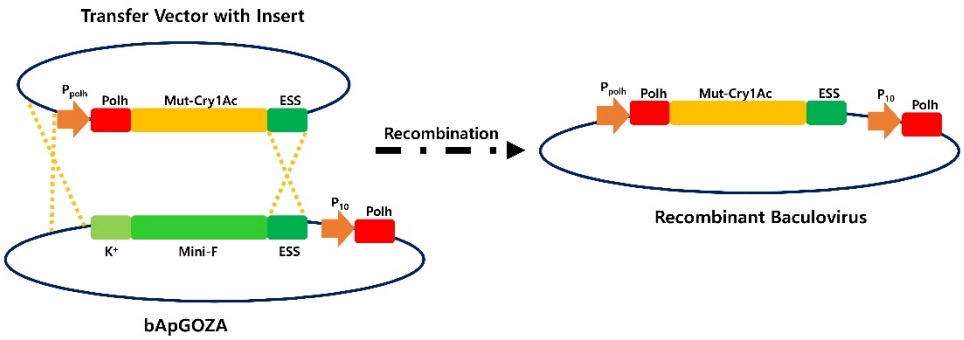

Figure 5. Schematic model of the construction of the recombinant baculoviruses expressing *Mut-Cry1Ac* genes.

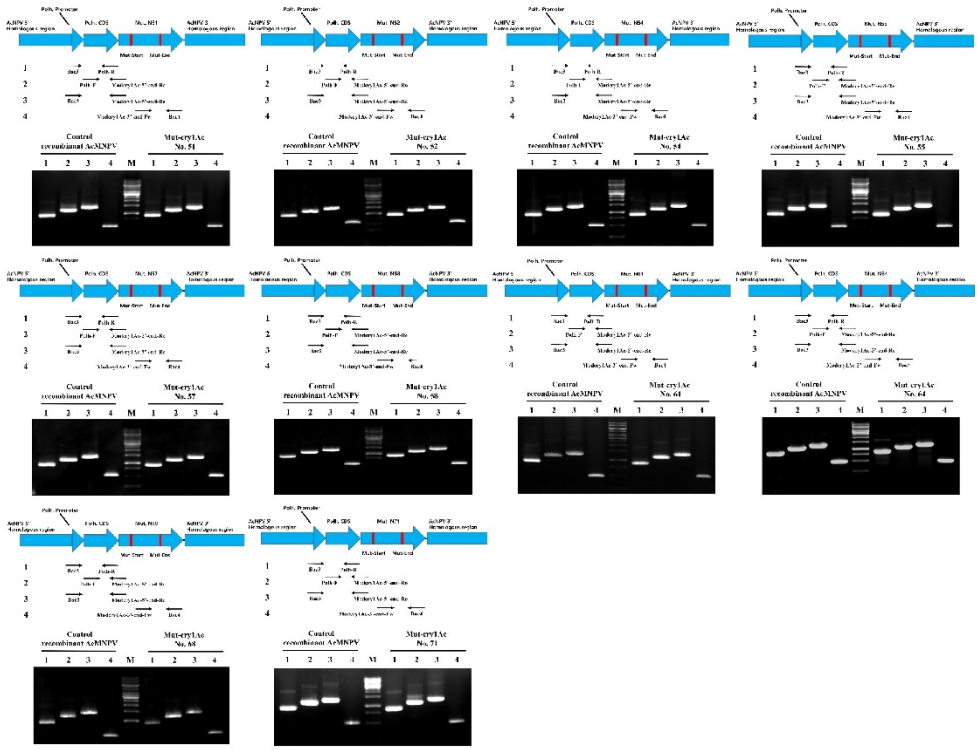

Figure 6. Verification of genome structure of the recombinant AcMNPVs expressing *Mut-Cry1Ac* genes by PCR using specific primer sets. Lane: M, 1kb ladder; 1, primers Bac3 and Polh-R; 2, primers Polh-F and Modcry1Ac-5'-end-Re; 3, primers Bac3 and Modcry1Ac-5'-end-Re; 4, primers Modcry1Ac-3'-end-Fw and Bac4.

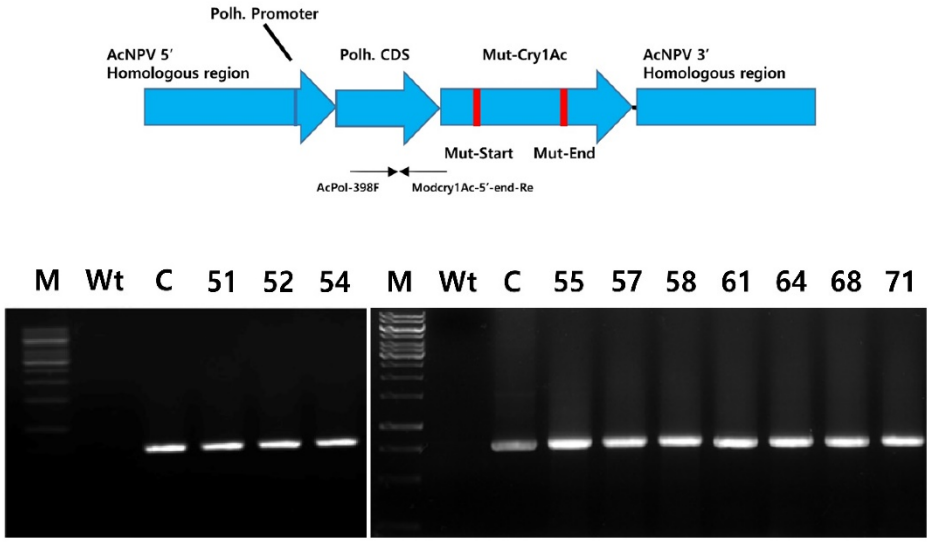

Figure 7. Verification of transcription of polyhedrin-*Mut-Cry1Ac* fusion genes from Sf9 cells infected with recombinant AcMNPVs expressing *Mut-Cry1Ac* genes. Lane: M, 1kb ladder; Wt, wild-type AcMNPV; C, recombinant AcMNPV expressing Mod-Cry1Ac, 16~71, recombinant AcMNPV expressing *Mut-Cry1Ac* 16~71 respectively.

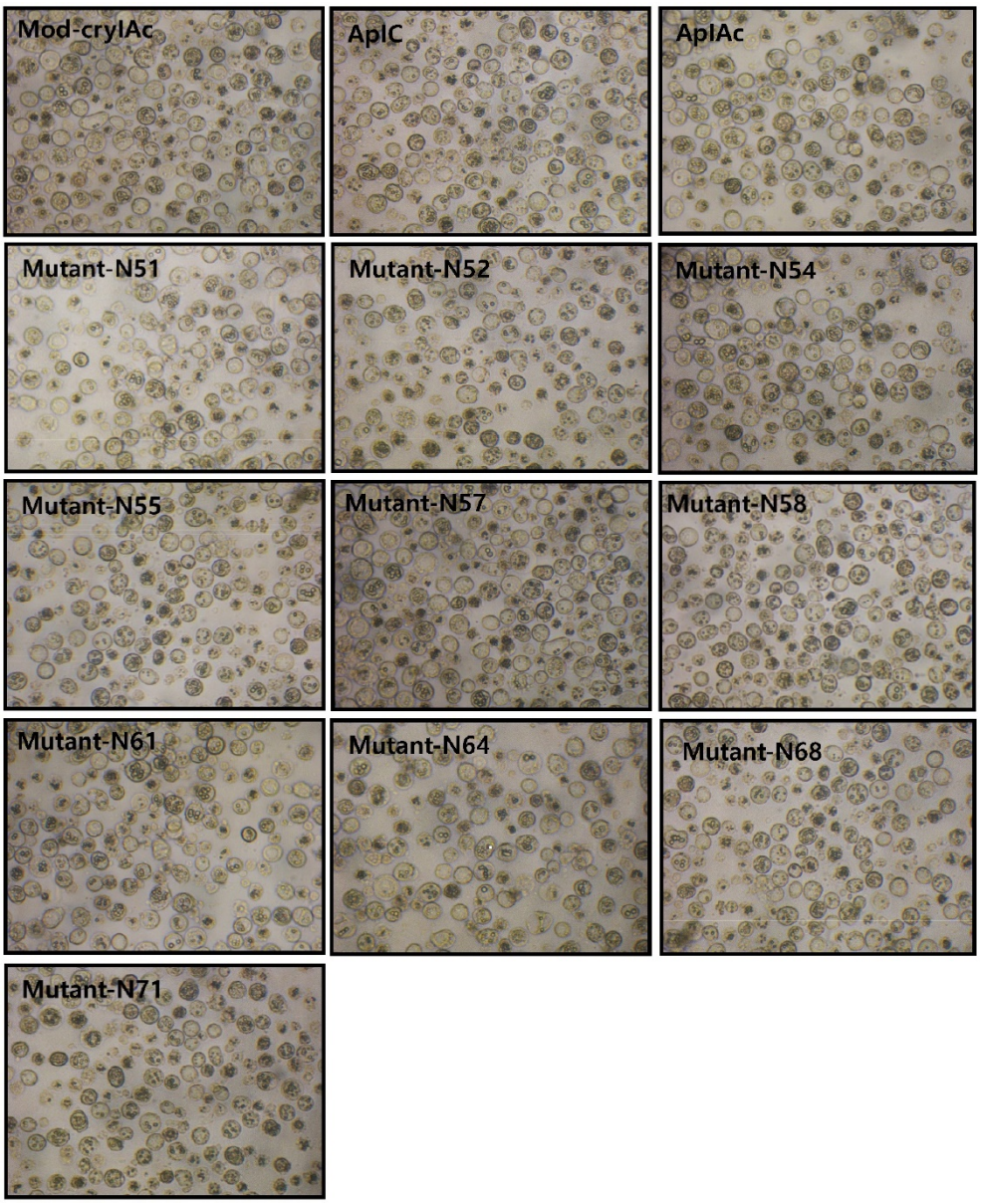

Figure 8. Phase-contrast microscopy of Sf9 cells infected with recombinant AcMNPVs expressing Mutant *Cry1Ac* genes (Magnification : X 1,000).

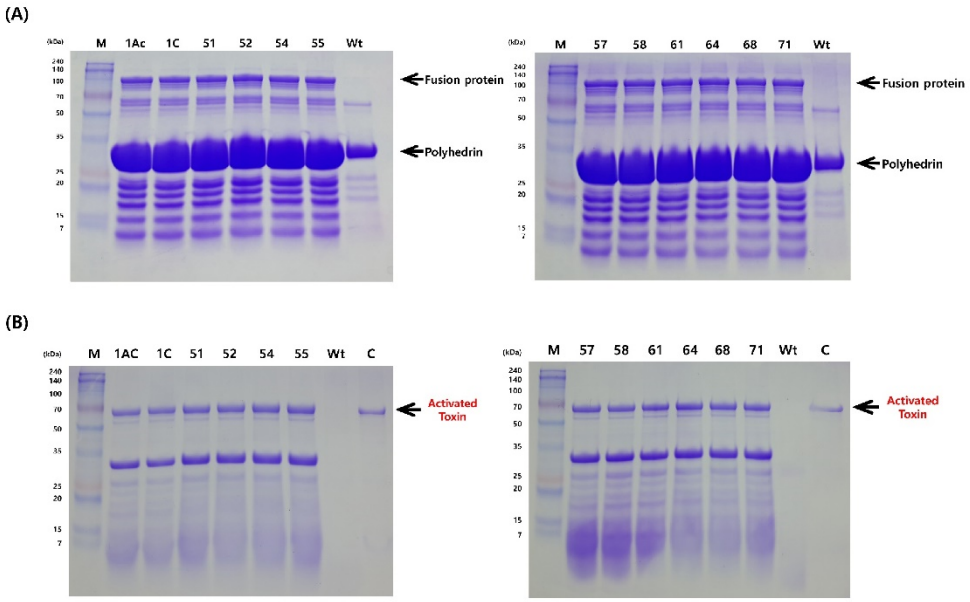

Figure 9. SDS-PAGE analysis of mutant Cry1Ac proteins fused with polyhedron (A) and activated by trypsin (B). Lanes: M, protein molecular weight marker; 1Ac, Cry1Ac; 1C, Cry1C; 51~71, Mut-N51~Mut-N71; Wt, wild-type AcMNPV; C, Mod-Cry1Ac.

Table 3. Quantification of activated forms of the Mutant Cry1Ac proteins which are occluded in the recombinant polyhedra.

Mutant No.	Polyhedra counting (PIBs / ml)	Bt Toxin (ng/1 X 10^6 PIBs)
1AC	1.8 X 10^9	37.9
1C	3.7 X 10^9	25.0
N51	1.94 X 10^8	893.9
N52	3.98 X 10^8	1097.0
N54	5.5 X 10^6	723.4
N55	1.47 X 10^7	581.8
N57	5.5 X 10^6	132.3
N58	9.5 X 10^7	1146.0
N61	1.3 X 10^9	758.6
N64	3.92 X 10^8	1024.0
N68	2.1 X 10^7	541.3
N71	3.1 X 10^7	421.6

3. Determining insecticidal activities of mutant Cry1Ac proteins

A. Insecticidal activities of mutant Cry1Ac proteins

In a previous study at our laboratory, the Mut-N16 C*ry* gene was synthesized and showed the highest insecticidal activities (Song-Eun Kim, thesis). To select desirable mutant *Cry1Ac* gene candidates, Mut-N16 *Cry1Ac* gene was also expressed through the baculovirus expression vector system and used in the following bioassay. To evaluate the insecticidal activity of mutant Cry1Ac proteins, bioassays were performed against *P. xylostella*, *S. exigua*, and *O. furnacalis*. Preliminary bioassays revealed that these mutant Cry1Ac proteins have similar or higher levels of insecticidal activities compared to those of Cry1Ac or Cry1C (Fig. 10). Among these mutant proteins, Mut-N16 showed the highest insecticidal activity against three lepidopteran pests. Based on the results, Mut-N16 was selected as the adequate candidate to be introduced into transgenic crops.

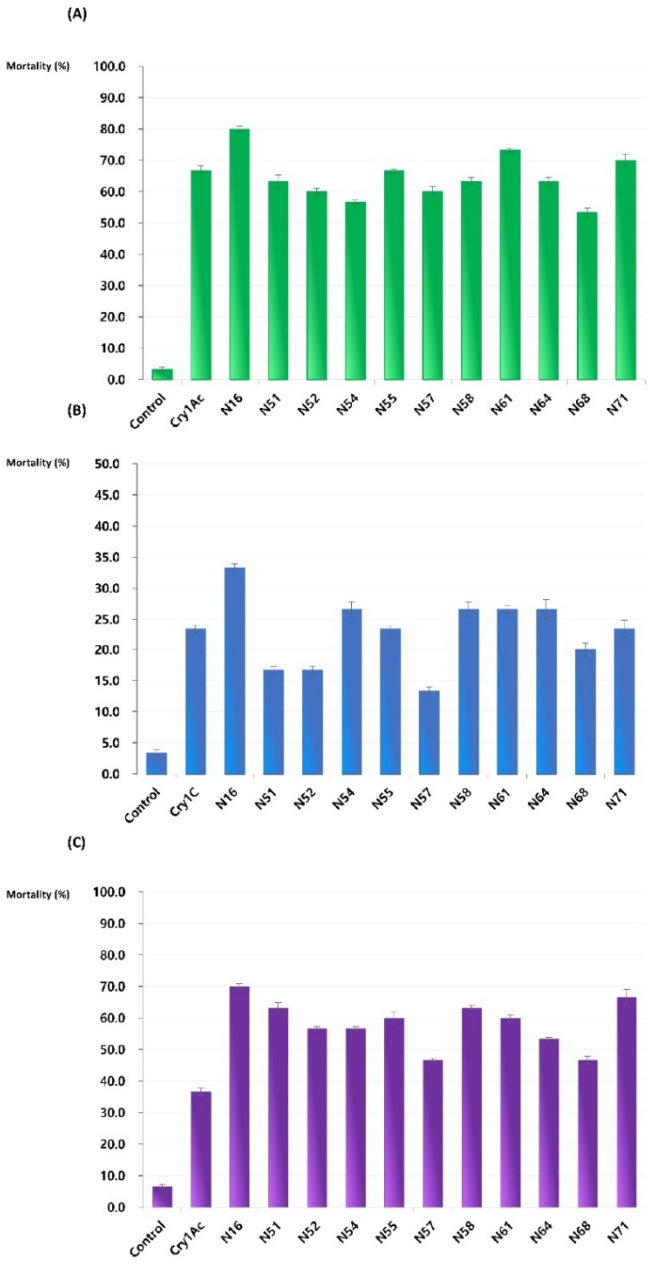

Figure 10. Insecticidal activities of Mutant Cry1Ac proteins against *P. xylostella* (A), *S. exigua* (B) and *O. furnacalis* (C). Third instar larvae of *P. xylostella* were fed with 5 ng/larva of activated mutant Cry1Ac proteins and their mortality was scored at 3 days after inoculation. Second instar larvae of *S. exigua* were fed with 500 ng/larva of activated mutant Cry1Ac proteins and their mortality was scored at 5 days after inoculation. Neonates of *O. furnacalis* were fed with 50 ng/larva of activated mutant Cry1Ac proteins and their mortality was scored 6 days after inoculation.

B. Insecticidal activity of Mutant-N16 protein

To confirm the suitability of Mut-N16 protein for use as introduced proteins, quantitative bioassays were performed with Cry1Ab protein of SYN-EV176-9. In order to express the *Cry1Ab* gene (1947bp) of SYN-EV176-9 using a baculovirus expression system, *Cry1Ab* gene was inserted into a pOBI vector. The constructed transfer vector was confirmed by DNA sequencing and the total size of pOBI-*Cry1Ab* was 8174 bp. *Cry1Ab* gene was expressed using the baculovirus expression vector system (Fig. 11A). In SDS-PAGE analysis of the recombinant polyhedra, recombinant viruses showed approximately 100kDa fusion protein bands of Cry1Ab and 70kDa activated forms of Cry1Ab protein respectively (Fig. 11B). For the bioassay, activated Cry1Ab protein was quantitatively measured by a 1D-gel analysis system.

The LD_{50} value of Mut-N16 protein was about 6 times lower against *P. xylostella* and 1.4 times lower against *O. furnacalis* than that of Cry1Ab. In the bioassay against *S. exigua*, the LD_{50} value of Mut-N16 protein was 234.28 ng/larva, while Cry1Ab showed very low insecticidal activity (Table 4). The LT_{50} value of Mut-N16 protein was about 1.7 days lower against *P. xylostella* and 1.2 days lower against *O. furnacalis* than that of Cry1Ab. In case of *S. exigua*, the LT_{50} value of Mut-N16 protein was 3.6 days (Table 5).

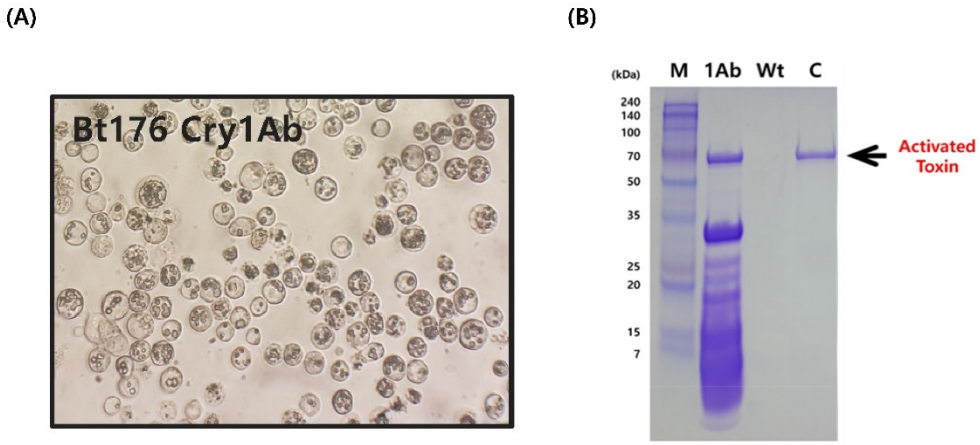

Figure 11. Phase-contrast microscopy of Sf9 cells infected with recombinant AcMNPVs expressing *Cry1Ab* genes (A). SDS-PAGE analysis of mutant Cry1Ab proteins fused with polyhedron and activated by trypsin (B). Lanes: M, protein molecular weight marker; 1Ab, Cry1Ab of SYN-EV176-9; Wt, wild-type AcMNPV; C, Mod-Cry1Ac.

Table 4. Median lethal dose (LD_{50}) of Mut-N16 and Cry1Ab against three lepidopteran species.

	P. xylostella[a]		*S. exigua*[b]		*O. furnacalis*[c]	
	LD_{50} (ng/larva)	95% Fiducial limits	LD_{50} (ng/larva)	95% Fiducial limits	LD_{50} (ng/larva)	95% Fiducial limits
Cry1Ab	**4.14**	**2.21-8.47**	**>10000.0**[d]	**ND**[e]	**19.95**	**3.56-35.30**
Mut-N16	**0.67**	**0.33-1.22**	**234.28**	**133.59-396.62**	**14.63**	**5.43-22.99**

[a]The mortality against third instar larvae of *P. xylostella* was scored at 2 days after inoculation.
[b]The mortality against third instar larvae of *S. exigua* was scored at 5 days after inoculation.
[c]The mortality against neonates of *O. furnacalis* was scored at 6 days after inoculation.
[d]The mortality was below 50% even at maximum dose tested.
[e]ND, not determined
LD_{50} was calculated by IBM SPSS Statistics 21.

Table 5. Median lethal time (LT_{50}) of Mut-N16 and Cry1Ab against three lepidopteran species.

	P. xylostella[a]		*S. exigua*[b]		*O. furnacalis*[c]	
	LT_{50} (Days)	95% Fiducial limits	LT_{50} (Days)	95% Fiducial limits	LD_{50} (Days)	95% Fiducial limits
Cry1Ab	**2.18**	**1.72-3.04**	**ND**[d]	**ND**[d]	**4.17**	**3.71-4.97**
Mut-N16	**1.25**	**0.57-1.68**	**3.60**	**2.92-4.87**	**3.48**	**2.74-3.87**

[a]Lethal time values were calculated at 10ng/larva.
[b]Lethal time values were calculated at 400ng/larva.
[c]Lethal time values were calculated at 100ng/larva.
[d]ND, not determined
LT_{50} was calculated by IBM SPSS Statistics 21.

DISCUSSION

Since the discovery of its insecticidal capabilities, *Bacillus thuringiensis* (Bt) has been widely used as a biological control agent. Also, there have been many studies regarding the introduction of Bt *cry* genes into crops to produce Bt transgenic crops, many of which are used in the field. However, it is not a simple technique to engineer Bt *cry* genes into crops, especially because Bt δ-endotoxin genes have an average A+T content of 64% as compared to that of about 44% and 55% in monocotyledon and dicotyledon exons, respectively. In addition, target insects have been developing resistance over the recent years (Entwistle et al., 1993; Gleave et al., 1998; Raymond et al., 2010; Tabashnik et al., 2003).

Previously, our laboratory has found that several mutated residues in domain I including E116A, E128V, G183E, A187T, V227I, A245S, P248S, and R254T improved insecticidal activity of the Cry1Ac against *S. exigua* (Xu et al., 2009). Also, mutations of S283M, G286R, A309V, M322T, F324S, T334A, Y338F, T340N, M341A, I375P, E412V, and Q424A in domain II improved insecticidal activity of the Cry1Ac against *O. furnacalis* (Kim et al., 2008). Therefore, in this study, we intended to construct novel Bt *cry1Ac* genes for genetically modified crops and select the most adequate *cry* gene for transgenic crops with enhanced insecticidal activities and broader host ranges against lepidopteran pests. To synthesize novel *Cry1Ac* mutant genes, mutagenesis experiments were performed repeatedly with 21 different primers used randomly. As a result, 10 different mutant *Cry1Ac* genes, randomly changed at 24 amino acid sequences, were

obtained. A total of 11 mutant *Cry1Ac* genes including the Mut-N16 *Cry1Ac* gene obtained in a previous study in our laboratory were expressed using the baculovirus expression system. As a result, all 11 mutant Cry1Ac proteins were stably activated as 65 kDa active toxins, respectively. By removing the first 28 amino acids from the amino-terminus (N-terminus) and the entire hydrophilic carboxy-terminus (C-terminal), the proteolytic enzyme activates the protoxin of a lepidopteron-specific crystal half to form a toxin of about 65kDa in molecular weight (Arvidson et al., 1989; Bietlot et al., 1989). In addition, insecticidal activities against larvae of several lepidopteran pests were investigated. These mutant Cry1Ac proteins showed higher or similar insecticidal activities in comparison to those of Cry1Ac and Cry1C. Among these, especially Mutant-N16 showed the highest insecticidal activity against three different lepidopteran pests.

Interestingly, these 11 mutant proteins had common mutated residues in domain II, namely A309V, M322T, F324S, E412V, and Q424A mutations. All mutant proteins showed much higher activities against *O. furnacalis* than Cry1Ac. Many studies have performed substitutions of amino acid residues at loop regions, and as a result, Cry protein structure was influenced. This in turn affected the mode of action of Cry proteins. The site-directed mutagenesis of domain II loop residue for example, produces mutant toxins that show increased insecticidal activities in some cases. Cry1Ab toxins with mutations in the domain II loop region resulted in higher insecticidal activity against *limantria dispar* (Lee et al., 2001; Lee et al., 2000). Modifications of different cry proteins in these amino acid regions have resulted in improved insecticidal activities against their corresponding target insect pests (Bravo et al., 2013). There are no related

reports with the 5 common mutated residues constructed in this study. However it is very hard to conclude which residue is responsible for the improvement of insecticidal activities. Thus, further studies on the role of each residue related to improved insecticidal activity against *O. furnacalis* are needed.

Bt maize expressing Cry1Ab protein were developed and commercialized to control major maize pests including the European corn borer, *Ostrinia nubilalis* Hübner (Felke et al., 2010; Rice and Ostlie, 1997). In this study, constructed novel Mut-N16 protein was assessed with commercialized SYN-EV176-9. SYN-EV176-9 produces a Cry1Ab protein with a N-terminal 648 amino acid sequence that is identical to the native Cry1Ab protein produced by Bt subsp. *kurstaki* strain HD1. As a result, SYN-EV176-9 showed excellent resistance to repeated heavy infestations of insects (Fearing et al., 1997; Koziel et al., 1993). To confirm the suitability of Mut-N16 protein for use as introduced protein, quantitative bioassays were performed with Cry1Ab protein of SYN-EV176-9. *Cry1Ab* gene of SYN-EV176-9 was expressed using the baculovirus expression system and comparison bioassays were performed with Mut-N16 protein against larvae of several lepidopteran pests. As a result, Mut-N16 protein showed higher insecticidal activities and broader host ranges than Cry1Ab protein of SYN-EV176-9.

In conclusion, 10 different mutant Cry1Ac proteins constructed in this study were considered to have the potential to be efficacious biological insecticides. Among these, Mut-N16 showed the highest insecticidal activity against lepidopteran pests, making it a desirable candidate to be introduced into transgenic crops. Moreover, this study can provide useful means of developing a high-throughput system for the screening of novel

recombinant Cry proteins with improved insecticidal activities and broader host spectra for transgenic crops.

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ABSTRACT IN KOREAN

향상된 살충활성을 가지는

신규 *Bacillus thuringiensis Cry1*-type 유전자의 제작 및 특성 파악

서울대학교

농생명공학부 곤충학전공

김 종 훈

초록

Bacillus thuringiensis (Bt)는 그람 양성 토양세균으로 포자 형성 시, 대상 해충에 대하여 높은 살충활성을 가지는 다양한 종류의 crystal 단백질을 생성한다. 반면, 인축에는 무해하며 환경에 안전하여 효과적인 생물학적 방제원으로 널리 사용 되고 있다. 또한, 최근에는 이 Cry 단백질을 encoding하는 Bt *Cry* 유전자를 작물에 도입함으로써 해충에 저항성을 가지는 형질전환 작물을 제작하는 연구가 진행되고 있다. 본 연구에서는 작물에 도입하기 위한 향상된

살충활성을 가지는 신규 Cry1Ac 유전자를 제작하는 것을 목적으로 한다.

이를 위해 식물이 선호하는 codon usage로 변환된 modified *Cry1Ac* 유전자를 template으로 하였으며, 총 21개의 primer를 이용하여 multi site-directed mutagenesis를 수행하였다. 그 결과, domain I의 8 residue 및 domain II의 16 residue에 위치하는 총 24개의 아미노산이 치환된 총 10종류의 Mutant *Cry1Ac* 유전자를 확보하였다. 이 Mutant 유전자들을 baculovirus expression system을 이용하여 다각체와 융합된 형태의 단백질을 발현하였으며, trypsin 처리 후 활성화시켜 SDS-PAGE를 수행한 결과, 65kDa으로 안정적으로 발현된 것을 확인하였다. 이들 중, 선행 연구에서 제작된 Mut-N16은 배추좀나방, 파밤나방 및 옥수수조명나방에 대해 가장 높은 살충활성을 보였다. 형질전환작물에 도입될 유전자로써의 적합성을 판단하기 위해, Mut-N16 단백질과 상용화된 SYN-EV176-9의 Cry1Ab 단백질의 정량 비교 생물검정을 수행하였다. 그 결과, 배추좀나방의 경우 Mut-N16의 LD_{50}값은 Cry1Ab에 비해 약 6배 낮았으며 옥수수조명나방에 대해서는 약 1.4배 낮았다. 파밤나방을 대상으로 한 생물검정에서 Cry1Ab는 살충성을 보이지 않았으나, Mut-N16의 LD_{50}값은 234.28 ng/larva의 값을 나타내었다.

가장 높은 살충활성을 보인 Mut-N16은 형질전환 작물에 도입될 *Cry* 유전자로써 적합할 것이라 기대된다. 또한, 이 연구는 향상된 살충활성 및 기주범위를 가지는 mutant *Cry* 유전자의 제작에 있어 유용한 정보를 제공할 것이다.

검색어: *Bacillus thuringiensis*, mutant *Cry1Ac* 유전자, multi site-directed mutagenesis, 형질전환 작물, baculovirus expression system

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