A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Construction and Characterization of Novel *Bacillus thuringiensis* *cry1*-type Genes with Improved Insecticidal Activities

나비목 해충에 대한 살충활성이 향상된

신규 ***Bacillus thuringiensis cry1*-type** 유전자의 제작 및 특성 연구

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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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FOR THE DEGREE OF MASTER OF SCIENCE
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ABSTRACT

Bacillus thuringiensis (Bt) is a gram-positive, sporulating bacterium that produces a variety of proteins, in the form of large crystalline (Cry) inclusions, which demonstrate toxicity to a variety of insect and nematode pests. Varieties of Cry proteins have been found around the world, which has been widely used as one of the most successful biological control agent. That is regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects and are used in Organic farming. Thus, *cry* genes encoding these Cry proteins have been widely applied for construction of transgenic crops resistant to pest insects.

In this study, to construct of novel *Bacillus thuringiensis cry1*-type genes for genetically modified crops and to select the most appropriate *cry* gene for transgenic crops with enhanced insecticidal activity as template. For effective expression in a plant, *cry1Ac* gene was modified to plant-preferring codon usage by multi site-directed mutagenesis. Through mutagenesis study for the Mod-Cry1Ac, 7 and 16 amino acid residues from domain I and II, respectively, responsible for its insecticidal activity against larvae of *Plutella xylostella , Spodoptera exigua* and *Ostrinia furnacalis* were identified. For this mutagenesis, 1857 bp of modified *cry1Ac* (Mod-cry1Ac) encoding active domain was used as a template and amplified by polymerase chain reaction with 16 mutagenic primers based on *cry1Ac* sequences. Consequentially, 31 different kinds of mutant cry1Ac genes with improved the insecticidal activity randomly changed 24 amino acid sequences into Cry1Ac were obtained.

For further characterization, these mutant *cry* genes were expressed as a fusion protein with polyhedrin using baculovirus expression system. These mutant *cry* genes encode potent insecticidal proteins in the form of crystalline protoxins of 95 kDa. SDS-PAGE analysis of the recombinant polyhedra revealed that expressed Cry proteins was occluded into polyhedra and activated stably to 65 kDa by trypsin. When the insecticidal activities of these mutant Cry proteins against to larvae of *P. xylostella, S. exigua* and *O. furnacalis* were assayed, they showed higher or similar insecticidal activity compared to those of Cry1Ac and Cry1C. In the bioassay of third-instar larvae of *P. xylostella*, LD_{50} values of these mutant Cry protein was lower about 5-fold than Mod-cry1A, the control Bt protein. In the bioassay of second-instar larvae of *S. exigua*, Mod-Cry1Ac showed no insecticidal activity, on the other hand not as effective as Cry1C but 8 Mut-Cry1Ac showed

insecticidal activity. Also, LD_{50} values of mutant Cry protein through quantitative bioassay was lower about 2-fold than Mod-cry1Ac about *O. furnacalis*. Through quantitative bioassay, several mutant *cry* genes showing higher insecticidal activity, such as Mut-N04, Mut-N06 and Mut-N16 to *P. xylostella* and *O. furnacalis*, might be expected as desirable *cry* genes for the introduction to genetically modified crops.

Key words: *Bacillus thuringiensis*, transgenic plants, *cry* gene, crystal protein, multi site-directed mutagenesis

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INTRODUCTION

Control of insect pests in agriculture and insect vectors of human infectious diseases is mainly accomplished using chemical insecticides. However, nearly all chemical insecticides have the potential to significantly alter ecosystems that are toxic to humans and animals and also concentrated in the food chain. Besides, chemical products provoked outbreak of resistant insect pests. For these reasons, biological insecticides have been recommended for the solution of chemical insecticides problems. The most successful biological insecticide for pests control is the *Bacillus thuringiensis* (Bt)-based insecticides, which presently is about 2% of the total insecticidal market (Raymond et al. 2010).

Bt is a gram-positive, soil-dwelling, spore-forming bacterium showing toxicity against larval stages of several insect orders. The action of Bt relies on insecticidal crystal (Cry) proteins produced during the sporulation phase (Bravo, Gill, and Soberón 2007). Cry proteins were designed to disrupt insect midgut tissues followed by septicemia. Upon ingestion, Cry proteins are solubilized and activated by proteolytic enzymes, which bind to specific receptors on the apical microvillus membrane of the epithelial midgut cell. Subsequent insertion of activated Cry proteins into the membrane leads to ruination of osmotic balance and ultimate lysis of epithelial cells, causing considerable injury to insect midgut and eventually, leading to larval death (de Maagd, Bravo, and Crickmore 2001, Wang et al. 2008, Whalon and Wingerd 2003). Because of these potent insecticidal

activity, Bt products was continuously developed, and widely used for insect pest control in agriculture and also against mosquitoes species.

Although Bt products have beneficial advantage as biological control agents, the conventional use of Bt insecticides have been faced with some limitations such as a narrow spectrum, a short shelf life and development of pest insect resistance. To overcome these problems, many researchers have tried to modify Bt strains and their insecticidal crystal protein-encoding gene (*cry* genes) using genetic manipulation for enhancement of their potency against target insects, faster effects and delay of resistance development (Aronson, Wu, and Zhang 1995, Kalman *et al*. 1995).

Since the first *cry* gene from *B. thuringiensis* was cloned, sequenced, and expressed in *Escherichia coli* in 1981 (Lecadet *et al.*, 1999). This provided the prospects for genetic engineering of *B. thuringiensis* as followed; 1) expression of a different *cry* genes in natural *B. thurigiensis* by transformation or conjugation, 2) expression of *cry* genes in alternative hosts such as *B. subtilis*, *Pseudomonas fluorescens*, plant colonizing bacteria and cyanobacteria, 3) transgenic insecticidal plants transformed with *cry* genes (Murphy and Stevens, 1992; Khasdan *et al.*, 2003; Gao *et al.*, 2004).

Recently, *cry* genes encoding these Cry proteins have been widely applied for construction of transgenic crops, which the aim to manufacture transgenic plant expressing Bt crystal protein is high dose expression of *cry* gene in crops and delay of resistance development of insect pests. Transgenic crops have set a precedent in that the biotech area has grown impressively every single year (James 2012). However, for the conversion of DNA nucleotide sequence to crop-preferring codon usage, Bt *cry* genes

have significant drawbacks; one is AT-rich sequence and the other is the relatively large sizes that they are usually about 1.8 kb in the size of truncated active domain. In this manner, it has been known that the modified Bt genes for transgenic crop limited to representative *cry* genes, such as lepidopteran specific *cry1A* and coleopteran-specific *cry3Aa* (Romeis *et al.*, 2006).

The objective of this study was to construct novel Bt *cry1*-type genes for genetically modified crops and to select the most appropriate *cry* gene for transgenic crops with enhanced insecticidal activity as template. For the variant novel *cry* genes, multi site-directed mutagenesis was performed, manufactured mutant genes were expressed as a fusion protein with polyhedrin using baculovirus expression system. Furthermore, insecticidal activity of Cry proteins was obtained through bioassay measurements against Lepidopteran pests. Based on the result of assay, the most suitable novel *cry* genes are selected for construction of genetically modified plants.

LITERATURE REVIEW

1. General characteristics of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) was first discovered by the Japanese biologist Ishiwata Shigetane in 1901, which was a previously undescribed bacterium as the causative agent of a disease afflicting silkworms. In 1911, the same bacterium was isolated by Ernst Berliner when he isolated a *Bacillus* species from the Mediterranean flour moth, *Ephestia kuehniella*, and named it after the province Thuringia in Germany where the infected moth was found (Roh et al. 2007, Sanahuja et al. 2011). In 1915, the existance of parasporal inclusions in Bt was recorded by Berliner, which was later demonstrated to be proteinaceous in nature and soluble in alkaline solution. The insecticidal properties of the crystal formations were discovered to be soluble in the alkaline digestive fluids of lepidopteran larvae and responsible for toxicity. Spores and crystalline insecticidal proteins produced by Bt have been used to control insect pests since the 1920s and are often applied as liquid sprays (Lemaux 2008). In 1981, strong evidence was reported that correlation existed between loss of crystalline protein production and specific plasmids by González et al (1981). In the same year, the first *cry* gene encording crystal proteins was cloned and expressed by Schnepf and Whiteley(1981). Until the present, 60,000 Bt have been collected by various industries in an effort 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 member of the *Bacillus cereus* group of gram-positive, entomopathology,

endospore-forming that spores of Bt can be isolated from diverse environments such as soil, fresh water, the rhizosphere, the phylloplane, grain dusts and from insects and insectivorous mammals (Raymond et al. 2010).

B. thuringiensis is a sporeforming Gram-positive bacterium capable of producing a number of toxins; insecticidal endotoxins, exotoxins, haemolysins and enterotoxins. Genetically, *B. thuringiensis* is related to *B. cereus* which produces toxins that cause gastroenteritis in humans, but *B. thuringiensis* typically produces one or more proteinaceous inclusions during sporulation as the only notable phenotypic difference (Kolsto *et al.*, 1990). These inclusions can be distinguished as distinctively shaped crystals by phase-contrast microscopy. The inclusions are composed of proteins known as crystal proteins, Cry protein, or δ-endotoxins, which are highly toxic to a wide variety of important agricultural and health-related insect pests. Due to their high specificity and their safety for the environment, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture and forestry and in the home. Insecticidal crystal proteins from *B. thuringiensis* have been used intensively as biopesticides for the several decades. It has been proposed that the rational use of *B. thuringiensis* toxins will provide a variety of alternatives for insect control and for overcoming with the problem of insect resistance to pesticides.

Bt strains are usually classified into serotypes according to their H flagellar antigenic determinants (de Barjac *et al.*, 1981; de Barjac, 1990) To date, up to 69 different serotypes and 13 sub-antigenic groups, giving 82 serovars, have been defined and ranked as subspecies (Lecadet *et al.*, 1999). While serotyping only reflects one characteristic of the species, it is the most common classification method used

throughout the world. However, the flagellar serotyping has limitations, proving unreliable as a predictor of insecticidal activity, even though the technique has greatly aided classification of isolates. For example, Bt subsp. *morrisoni* (H8a8b) includes isolates active against lepidopteran, coleopteran or dipteran insects.

2. Bt crystal proteins

1) Delta-endotoxins

Production of the parasporal body or crystal is the defining feature of Bt. The crystals are proteinaceous in nature and are composed of millions of crystal (Cry) or Cytolytic (Cyt) protein molecules. Production of the crystal(s) is generally concomitant with sporulation. During the process of sporulation, the majority of Bt strains produce crystalline inclusions that contain the insecticidal δ-endotoxins. The proteins comprising these crystals account for 20-30% of the total bacterial protein at sporulation (Boucias and Pendland, 1998). The shape of the crystal is variable depending in the toxins present in crystal and growth conditions, with diverse crystal morphologies sometimes occurring concurrently. Among natural isolates, the most common morphologies are bipyramidal and circular(round) (Martin and Travers 1989, Bernhard et al. 1997). The composite crystalline inclusions are comprised of monomeric protoxins. Degradation of the inclusions by proteolytic enzymes releases the smaller toxic proteins, δ-endotoxins. These vary between strains but in most cases Bt strains produce inclusions that contain a mixture of δ-endotoxins.

The toxins were originally classified into four classes (Höfte and Whiteley 1989), according to their amino acid sequence homology and insecticidal specificities. Type I cry genes encode proteins of 130 kDa, which are usually specific to lepidopteran larvae, type II genes encode for 70 kDa proteins that are specific to lepidopteran and dipteran larvae, and type III genes encode for 70 kDa proteins specific to lepidopteran and dipteran larvae, while type IV genes are specific to the dipteran larvae. The system was further extended to include type V genes that encode for proteins that are effective against lepidopteran and coleopteran larvae(Crickmore et al. 1998, Tailor et al. 1992).

Currently, the toxins are classified on the basis of only amino acid sequence homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers (e.g. Cry25Aa1), depending on its place in a phylogenetic tree. Thus, proteins with less then 45% sequence identity differ in primary rank (Cry1, Cry2, etc.), and 78% and 95% identity constitute the borders for secondary and tertiary rank, respectively. This system replaces the old nomenclature using roman numerals. The similarity in amino acid sequences of all known Cry proteins was taken as the only criterion for distributing them into classes and subclasses. Some classes, such as Cry6 and Cry15, do not show any significant homology with the rest of the Cry proteins.

In addition to Cry toxins, a second type of toxins, Cyt toxins, occurs. These are in the 25-28 kDa mass range and, based on amino acid sequence, are not related to Cry proteins. Two classes (Cyt1 and Cyt2) containing 9 Cyt toxins are known. Interestingly, *cry*-like genes were found recently in the anaerobic bacterium

Clostridium bifermentans. The gene products, Cry16A and Cry17A, showed a remarkable mosquitocidal activity, and this is also the first reported case of a secreted or excreted mosquitocidal toxin derived from an anaerobic bacterium (Barloy *et al.*, 1996).

2) Exoenzymes and exotoxins

2-1) β-exotoxin

The β-exotoxin (thuringiensin, thermostable toxin, fly toxin) is thermostable (70℃, 15 min) low molecular weight toxin and has a broad spectrum of activity, killing various lepidopterans, dipterans, hymenopterans, hemipterans, isopterans, orthopterans, nematodes and mites. β-exotoxin is a specific inhibitor of DNA-dependent RNA polymerases. At high dosages, it may affect the biosynthesis of proteins and DNA. (Sebesta and Horska, 1970; Beebee and Bond, 1973).

2-2) Haemolysins

Haemolysins, which lyse vertebrate erythrocytes, are important virulence factors in several vertebrate bacterial pathogens and are generally thought to be important factors for the establishment of systemic diseases in humans (Honda *et al.*, 1991; Matsuyama *et al.*, 1995).

2-3) Enterotoxins

Bt isolates have been found to produce *B. cereus*-diarrhoeal-type enterotoxins (Carlson *et al.*, 1994). In *B. cereus*, extracellular enterotoxin, 45 kDa protein in typical enterotoxin characteristic, causes diarrhea in higher animals (Asano *et al.*, 1997).

2-4) Exoenzymes

Exoenzymes play a role in its pathogenicity to insects, for example, the release of chitinase and protease by Bt has been proposed to disrupt the peritrophic membrane, providing access to the gut epithelium (Kumar and Venkateswerlu, 1998; Sampson and Gooday, 1998).

2-5) Vegetative insecticidal proteins

A new class of insecticidal toxin, vegetative insecticidal proteins (VIPs) has been isolated from Bt since vip3A was first reported by Estruch in 1996. Approximately 15% of Bt strains has Vip3 homologs, which has showed a wide spectrum of activities against lepidopteran insects, such as black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*) (Estruch *et al.*, 1996).

3. Structure of crystal protein

The tertiary structure of three crystal proteins, Cry3A, Cry1Aa and Cyt2A, has been solved by X-ray crystallography (Li *et al.*, 1991; Grochulski *et al.*, 1995; Li *et*

al., 1996). An analysis in the accompanying review demonstrates that Cry3A and Cry1Aa show about 36% amino acid sequence identity (Crickmore *et al.*, 1998). This similarity is reflected in their three-dimensional structures. However, The structures of three Cyt proteins have been solved, Cyt1Aa, Cyt2Aa and Cyt2Ba showing similar topology displaying a single α-β domain composed of two outer layers of α-helix hairpins wrapped around a β-sheet (Cohen et al. 2008, Cohen et al. 2011, Li, Koni, and Ellar 1996, Soberón, López-Díaz, and Bravo 2013). The α-helices have an amphiphilic character, with the hydrophobic residues packed against the β-sheet. Up to now, three families of Cyt proteins, Cyt1 and Cyt2, and Cyt3, have been identified (Crickmore et al. 2013). Cyt1 and Cyt2 are produced in vivo as protoxins and undergo activation by the removal of small portions of their N-termini and C-termini (Li, Koni, and Ellar 1996).

Cry3A and Cry1Aa possess three domains. Domain I consists of a bundle of seven antiparallel α-helices in which helix 5 is encircled by the remaining helices. Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology, arranged in a so-called β-prism fold. Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich with a "jelly roll" topology. Domain II, especially the highly variable loops in its apex, is involved in specific receptor binding as shown by mutagenesis studies (Schnepf *et al.*, 1998). More recently, domain III was involved in the recognition of the role in receptor binding and thus in insect specificity (Aronson *et al.*, 1995; Lee *et al.*, 1995). Following binding, at least part of domain I inserts into the membrane in an oligomer to form an aqueous pore

with other toxin molecules. Domain I resembles other pore-forming or membrane-translocation domains of bacterial toxins and membrane entry might start by insertion of a hydrophobic two-helix hairpin. In an umbrella-like model, base on mutational and biophysical studies, α-helices four and five of several toxin molecules in an oligomer make up the pore, with the rest of the protein spreading over the membrane surface (Gazit *et al.*, 1998).

4. Crystal proteins intoxication process

Two models have been reported to describe the crystal proteins intoxication process: pore-forming model and signaling pathway model.

1) Pore-forming model

The mode of action of Cry proteins (δ-endotoxins) has been mostly characterized using lepidopteran larvae as model, and the major role of Cry proteins is to disrupt the midgut epithelium barrier to facilitate bacterial invasion of the hemocole. Upon ingestion of the crystal protein by a susceptible insect, the alkaline pH and reducing conditions found in the midgut of lepidoperan larvae allow for solubilization of the protoxins. Solubilization of the protoxin molecules in the crystal renders them available to proteolysis (activation) to yield an active toxin core that is mostly resistant to further proteolysis (Bietlot et al. 1989). Activated toxins traverse the peritrophic matrix before reaching the midgut epithelium. While the size of the matrix pores would allow passage of Cry proteins (Adang and Spence 1983), the

chitinous matrix contains glycoproteins (Adang and Spence 1982) that may bind to Cry proteinsand reduce their passage. After traversing the peritrophic matrix, the Cry proteins bind to the brush border membrane of the midgut cells (Bravo et al. 1992). Reversible protein binding has been proposed to concentrate Cry toxins on the brush border membrane to facilitate irreversible binding (Pacheco et al. 2009). The irreversible binding component is considered synonymous in this region of the toxin insertion on the membrane and is directly correlated to toxicity (Liang, Patel, and Dean 1995). Irreversible binding is mostly dependent on domain I, as mutation in the region of the toxin result in reduced irreversible binding and toxicity (Hussain, Aronson, and Dean 1996). Toxin binding and concentration of aminopeptidase N (APN) and alkaline phosphatase (ALP) on specific membrane regions of a toxin pore that leads to cell death by osmotic shock.

Cyt toxins are generated as protoxins in the form of crystalline inclusions. Unlike lepidopteran-specific crystal proteins, this protein was soluble in alkaline conditions, and then proteolysis is processed by proteases in the mid-gut tracts of susceptible larvae. The conventional model for Cyt proteins suggests that the monomer undergoes conformational changes such that, upon membrane contact, the two outer α-helical layers swing away from the β-sheet that is inserted into the membrane. Oligomerization of Cyt monomers on the cell membrane forms β-barrel pores (Li et al. 2001, Parker and Feil 2005) that induce colloid-osmotic lysis, followed by a net influx of water, cell swelling, and eventual lysis (Bravo, Gill, and Soberón 2007). Cyt toxins induced rapid rounding up and swelling followed by membrane blebbing and cell lysis in mammalian and inset cell cultures, was hemolytic to erythrocytes

(Thammachat et al. 2008). For instance, the toxicity of Cyt1Aa is obtained upon alkaline solubilization and proteolysis from both the N- and the C-termini, which converts the protoxin into its active form (23-24 kDa). This active toxin is carried out by the insect gut proteases. Cyt1Aa's highly hydrophobic nature enable its interaction with unsaturated membrane phospholipids such as phosphatidylchloline, sphingomyeln and cholesterol (Cahan, Friman, and Nitzan 2008). Cyt1Aa forms pores 1-2 nm in diameter in the cell membrane, leading to cell lysis (Knowles et al. 1989).

2) Signaling pathway model

An alternative model proposed that binding provokes cell death in insect cells by activating a previously undescribed signaling pathway involving stimulation of the stimulatory guanine nucleotide-binding protein (G protein) and adenylyl cyclase (AC), increased cyclic adenosine monophosphate (García-Robles et al.) levels, and activation of protein kinase A (PKA). The toxicity of Cry proteins is due to the activation of a Mg^{+2}-dependent signal cascade pathway, PKA is the most key component among others in this pathway. Activated PKA alters downstream effectors that, in turn, actually dismantle the cell by destabilizing both the cytoskeleton and ion channels in the cell membrane. Such impairment of the structural and functional integrity of the cell leads to cell death as manifested by membrane blebbing and cellular swelling (Zhang et al. 2006). That Cry toxin of Bt exacerbate critical

intracellular signaling pathways through cadherin recetor coupled interactions has implications in pest insecticide development.

5. Genetically modified crops expressing Bt crystal protein

Genetically modified crops (GM crops) are plant used in agriculture, the DNA of which has been modified using genetic manipulation for improvement of chemical treatments resistance, enhancement of insecticidal activity, and delay of resistance development. In addition, transgenic plants include non-food crops such as biofuel, pharmaceutical agents, and other industrial products.

The first transgenic crop was constructed using an antibiotic-resistant tobacco in 1982 (Fraley et al. 1983). Moreover, genetically engineered plants with insect tolerance by expressing genes encoding for crystal proteins from Bt was produced in 1987 (Vaeck et al. 1987) and now including many major crop species, such as cotton, maize, potato, tobacco, rice, broccoli, lettuce, walnut, apple, alfalfa, and soybean. These plants are generically referred to as Bt Crops (i.e., Bt corn, Bt cotton, etc.).

An advantage of insect-resistant transgenic crops has brought great economic benefits to growers and significantly reduced the use of chemical insecticides, which are safe to natural environment and human health. GM crops are spreading more rapidly than any other agricultural technology in history, suggesting that farmers perceive important advantages in growing them. GM crops have been deployed for cultivation primarily in the USA, Brazil, Argentina, Canada, India, and China. During the period of 1996 to 2012, transgenic crops have been grown in accumulated hectarage of 3.53 billion hectares (8.72 billion acres). Most of the genetically

modified plants were herbicide-tolerant crops, and biotech crops with Bt genes has covered about 15% of the total transgenic acreage in the world. (James 2012).

However, the wide adoption of Bt crops also places a large selection pressure on the target insect populations and could result in them rapidly evolving a resistance to Bt toxin (Tabashnik et al. 2003, Yang et al. 2011). This is one of the most serious challenges faced y the use of Bt-transgenic crops in the field. In a number of instances, this insect resistance has been attributed to a reduction in the affinity of the proteolytically activated Cry protein to bind to the recepter of insect epithelial midgut cells (Ferré et al. 1991).

Environment Protection Agency (Gleave et al.) has established and generalized guide-line for the use of insecticidal Bt transgenic crops. On the other hand, various studies have been conducted for delay of resistance development such as imposition of high-performance gene by gene fusion technique. Moreover, researches are proceeding for discover renal function and high-efficiency of insecticidal *cry* gene.

MATERIALS AND METHODS

1. Construction of mutant *cry* genes

1) Cloning of pIM-*Mod-cry1Ac* vector

Modified *cry1Ac* gene was provided from Dr. Beom-Seok Park (National Institute of Agricultural Biotechnology, Korea). Modified *cry1Ac* gene was digested with *Eco*RV and *Bam*HI, and introduced to pIM vector digested with same restriction endonucleases, to generate pIM-*Mod-cry1Ac* (Fig. 1).

2) Construction of primers

For the mutation of modified *cry1Ac*, the alignment of amino acid sequence of the modified Cry1Ac was performed using MegAlign (DNASTAR Inc., USA). Sequence of primers was decided according to the codon usage in pooled sequences of Chinese cabbage genes (Table 1). All of the primers used for simultaneous mutagenesis were annealed to the same strand of the template plasmid, and the desired point mutation or degenerate codon was close to the middle of the primer with ~10–15 bases of template-complementary. Primers were designed between 25 and 45 bases in length a melting temperature (Tm) ≥ 75℃, which was calculated with QuikChange® Tm calculator, available online at http://www.stratagene.com. Primers for simultaneous mutagenesis should be added to the mutagenesis reaction in approximately equimolar amounts.

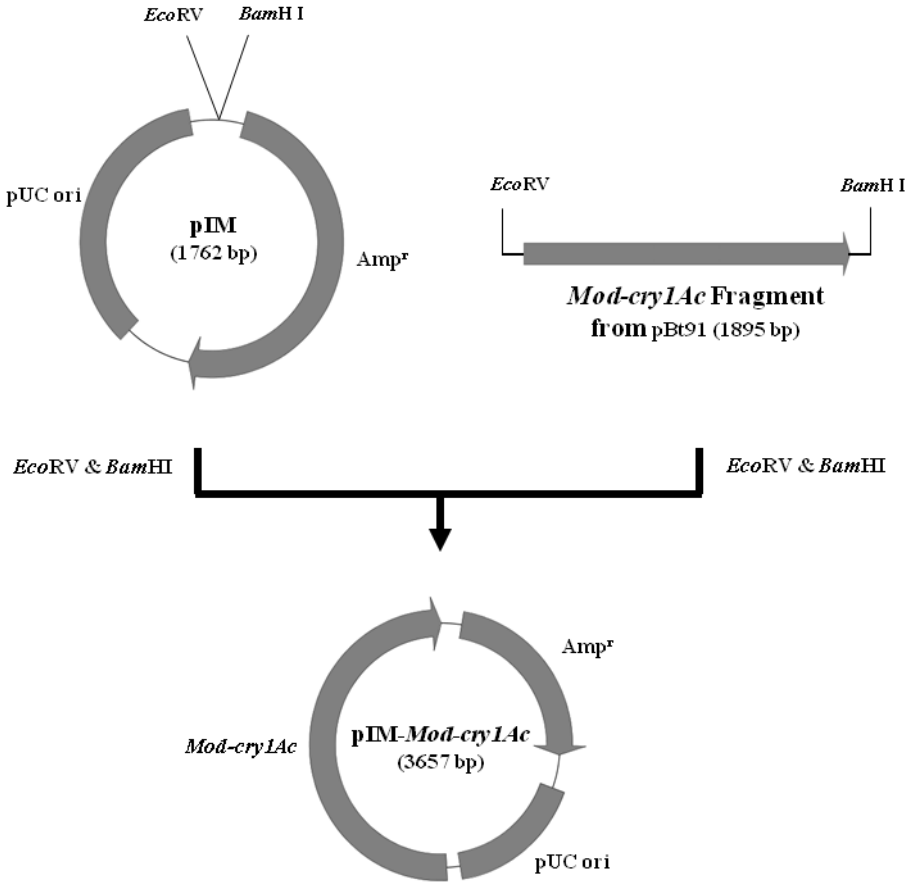


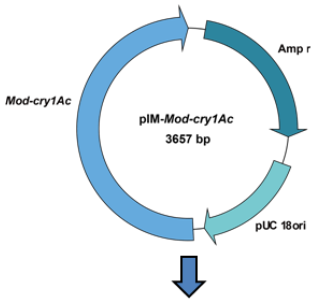
Fig. 1. **Construction map of vector, pIM-*Mod-cry1Ac*.** Modified *cry1Ac* gene was inserted into pIM to obtain the pIM-*Mod-cry1Ac* vector.

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

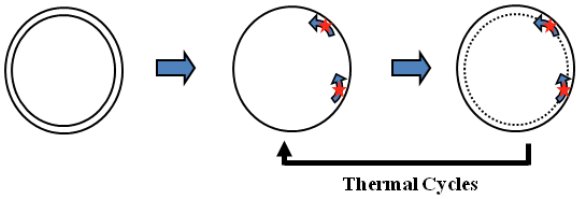
Amino acid	Codon	Preference, %	Amino acid	Codon	Preference, %
ARG (**R**)	CGA	9.4	ASP (**D**)	GAC	41.1
	CGC	13.7		GAU	58.9
	CGG	6.0	ILE (**I**)	AUA	18.5
	CGU	20.5		AUC	45.7
	AGA	25.6		AUU	35.8
	AGG	24.8	SER (**S**)	UCA	19.4
PRO (**P**)	CCA	32.5		UCC	14.7
	CCC	13.3		UCG	11.6
	CCG	19.2		UCU	19.4
	CCU	34.9		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

3) Mutating *mod-cry1Ac* gene through multi site-directed mutagenesis

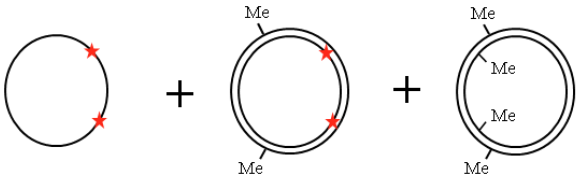
Mod-cry1Ac gene was mutated using the QuickChange® multi site-directed mutagenesis method (Stratagene, USA) (Fig. 2). Template DNA was purified (Qiagen, Germany) and replicated using enzyme blend including QuickChange® DNA polymerase (Stratagene, USA) as following cycling parameters; step 1, 95℃, 1 min; step 2, 95℃, 1 min; step 3, 55℃, 1 min; step 4, 65℃, 17 min (every successive cycle repeats steps 2 to 4, 34 times); step 5, 4℃, unlimited. All DNA amplifications were performed with the DNA Thermal Cycler (BIO-RAD, USA). The disintegration of methylated or hemimethylated template DNA were performed with *Dpn*I (New England Biolab, USA). Mutated single stranded-DNA was transformed into XL-10 Gold Ultracompetent cells (Stratagene, USA). Each mutant was verified by DNA sequencing using specific primers, Mod-cry1Ac-F (5'- ACCGACTACG CTGTTCG) and Mod-cry1Ac-R (5'- AATGTTGTTGCCAGAGC).



Step 1: Mutant Strand Synthesis (Thermal Cycling)



Step 2: *Dpn* I Digestion of methylated and hemimethylated Template DNA



Step 3: Transformation mutated ssDNA into XL-10-Gold Ultracompetent Cells

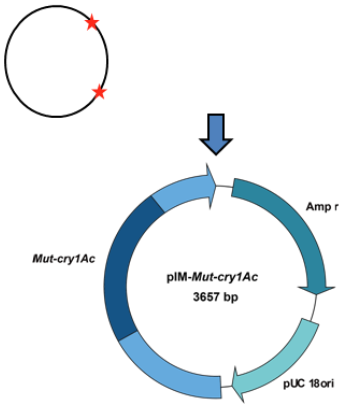


Fig. 2. Schematic diagram for multi site-directed mutagenesis.

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

1) 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, Korea) at 27℃. All insect cell lines were cultured at 27℃ and sub-cultured for every 4-5 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 bApGOZA (Je *et al.*, 2001) was used as a parental virus for construction of recombinant baculoviruses and a wild-type AcMNPV was used as a control virus.

2) Construction of baculovirus transfer vector

For the construction of baculovirus transfer vector expressing *mod-cry1Ac* gene, *mod-cry1Ac* gene was amplified from pIM-*Mod-cry1Ac* vector using specific primers, Mod1Ac-ATG-F containing *Xho*I-F at 5'-end (5'- AAACTCGAGATGGACAACAA CCCAA AC) and Mod1Ac-TAA-R containing *Eco*RI site at 5'-end (5'- TTTGAATT CTTAAAGATTGTACTCAGCCTC). The PCR-amplified *mod-cry1Ac* gene fragment was digested with *Xho*I and *Eco*RI and inserted into pOBI vector digested with same restriction endonucleases to obtain the pOB-*Mod-cry1Ac* (Fig. 3).

In order to construct baculovirus transfer vector, pOB-*Mut-cry1Ac* expressing mutant *cry* genes, mutant *cry1Ac* genes produced through mutagenesis were digested with *Xba*I and *Bgl*II for 821 bp of mutant region cassette fragment, and each cassette

fragment was introduced into pOB-*Mod-cry1Ac* digested with same restriction endonucleases (Fig. 4). Each mutant gene sequence in the constructed pOB-*Mut-cry1Ac* was verified by DNA sequencing using specific primers; Mut-seq-F (5'-ACCGACTACGCTGTTCG) and Mut-seq-R (5'- GGTCAC AGAGGCGTATC).

3) Expression of recombinant protein

The cell culture dish (60-mm diameter) seeded with 5×10^5 Sf9 cells were incubated at 27℃ for 1 h to let the cells attach to the footwall of dish. 500 ng of bApGOZA DNA, 2 ug of transfer vector DNA and 100 ul of the serum-free TC-100 medium were mixed in a polystyrene tube. Twenty ul of CellfectinII ™ (Invitrogen Co., USA) and 100 μl of the incomplete TC-100 medium were mixed in other polystyrene tube. The two solutions were gently mixed and the mixture was incubated at room temperature for 45 min. The attached cells were washed with 3 ml of the incomplete TC-100 medium, and refreshed with 2 ml of the same medium. The CellfectinII-DNA complexes were added dropwise to the media covering the cells while the dish was gently swirled. After incubation at 27℃ for 5 h, 3 ml of the TC-100 medium containing 30 ul antibiotics and 10% FBS were added to each dish and incubation at 27℃ was continued. At 5 days post infection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,500 rpm for 15 min.

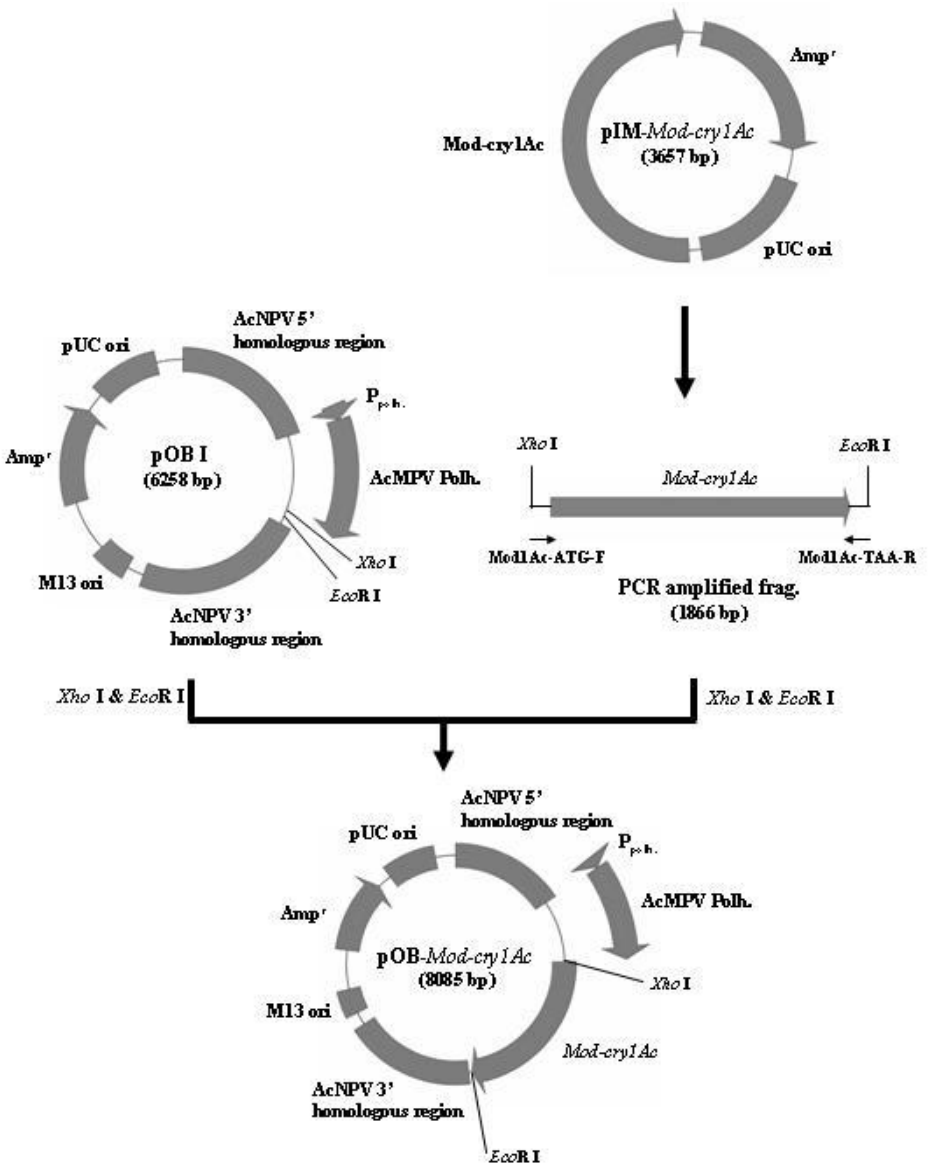


Fig. 3. **Construction map of transfer vector, pOB-*Mod-cry1Ac*, expressing modified *cry1Ac* with polyhedrin.** The PCR-amplified active fragment (about 1.8 kb) from the pIM-*Mod-cry1Ac* gene was cloned into the pOBI vector to give the plasmid pOB-*Mod-cry1Ac*. Solid arrows indicate primer position used in PCR amplification.

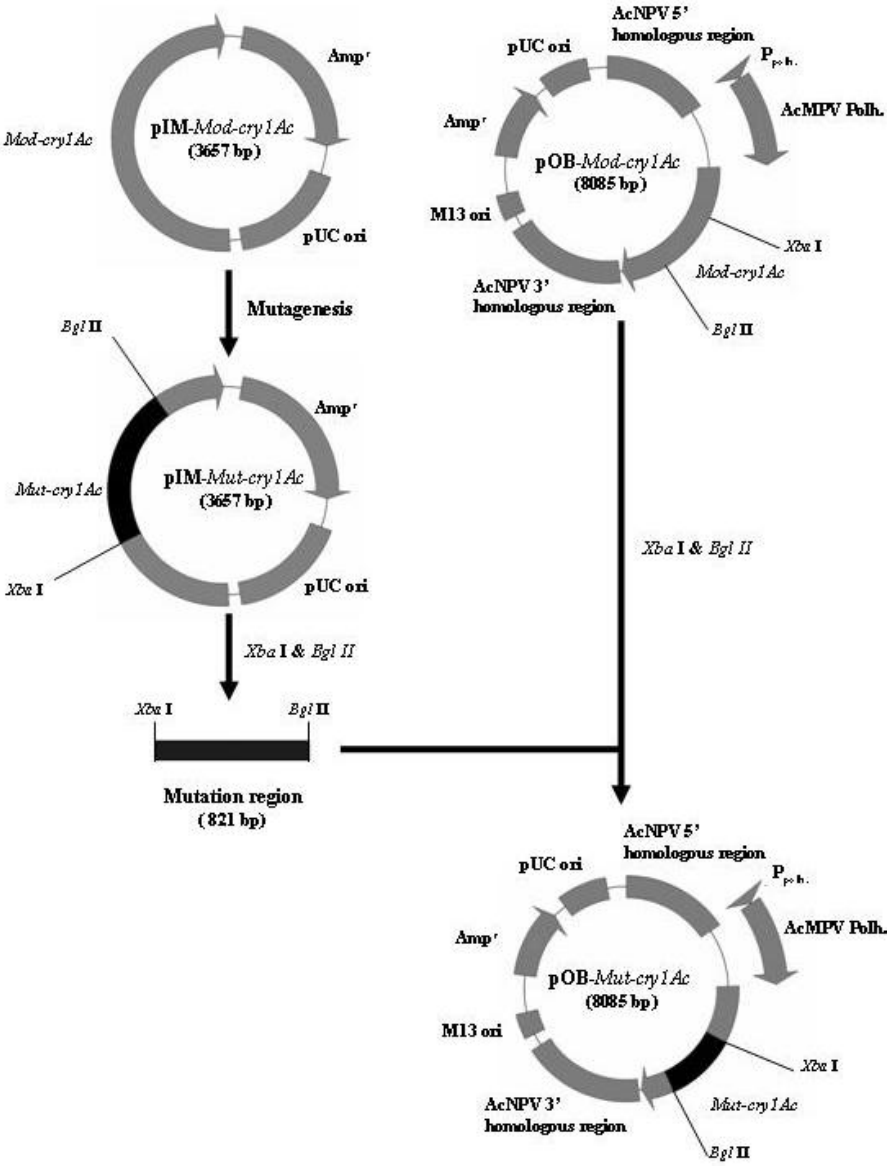


Fig. 4. **Construction map of transfer vector, pOB-*Mut-cry1Ac*, expressing mutant *cry1Ac* with polyhedrin.** The mutant cassette fragments (821 bp) digested with restriction endonucleases from the pIM-*Mut-cry1Ac* gene were inserted into the pOB-*Mod-cry1Ac* to obtain the transfer vector pOB-*Mut-cry1Ac.*

4) Production and purification of polyhedra

Wild-type or recombinant AcMNPV infected SF9 cells were harvested by centrifugation at 1000×g for 5 min. The resulting pellet was resuspended in 0.5% SDS (10 ml of 0.5% SDS per 2×10^8 cells) and centrifuged at 5000×g for 5 min and resuspended the pellet on the same volume of 0.5 M NaCl. The PIBs were pelleted by centrifugation at 5000×g for 5 min. and resuspended in a small volume of 1 M NaCl and 0.01% Triton X-100. To calculate the number of PIBs, PIB stocks were diluted and directly counted using hemocymeter.

5) SDS-PAGE

To analyze the fusion protein expression, Sf9 cells infected with the recombinant baculovirus were washed with Alkaline lysis buffer (0.1 M Na_2CO_3, 0.01 M EDTA, 0.17 M NaCl, pH 10.5) and were subjected to SDS-PAGE. For the analysis of active mutant Cry proteins, purified polyhedra were solubilized with alkaline lysis buffer, and then treated with trypsin at 37℃ for 2 h ~ overnight. Finally, samples treated with trypsin were mixed with an equal volume of 2×SDS-PAGE sample buffer (Sigma, USA), boiling at 100℃ for 10 min, each sample was loaded onto a 12% polyacrylamide separating gel with a 5% stacking gel, as described by Laemmli (Laemmli, 1970), and stained with Coomassie brilliant blue.

For the quantitative analysis of activated Cry proteins in recombinant polyhedra, the activated proteins subjected to 12% SDS-PAGE were determined by 1D-gel analysis system (Kodak Co., USA).

3. Determining toxicity of mutant Cry proteins

1) Screening Bioassay

The insecticidal activity of Mod-Cry1Ac and 8 mutants proteins was determined against to larvae of diamondback moth (*Plutella xylostella*), beet armyworm (*Spodoptera exigua*) and Asian corn borer (*Ostrinia furnacalis*). The recombinant polyhedra were treated on a disc of Chinese cabbage leaf (1.5×1.5 cm^2). For the three times, Ten 3rd-instar larvae of *P. xylostella* and ten 2nd-instar larvae of *S. exigua* were introduced to each leaf surface. To determine the activity against *O. furnacalis*, the recombinant polyhedra were treated on a small slice of artificial diet (2.0×2.0 cm^2) and thirty neonates were laid on each artificial diet. In *P. xylostella*, toxins were treated with 5 ng/larva and their mortality was scored 3 days after inoculation. In this case of *S. exigua*, toxins were treated with 300 ng/larva and their mortality was scored 5 days after inoculation. And about *O. furnacalis*, toxins were treated with 50 ng/larva and their mortality was scored 6 days after inoculation.

2) Quantitative bioassay

The quantitative bioassay of insecticidal activities was determined against to diamondback moth (*P. xylostella*), beet armyworm (*S. exigua*) and Asian corn borer (*O. furnacalis*). 5 different protein concentrations were provided. To determined the median lethal dose (LD_{50}) against *P. xylostella*, and *S. exigua*, , serial dilutions of recombinant polyhedra were treated on a disc of Chinese cabbage leaf pieces

(1.5 x 1.5 cm^2). For the three times, Ten 3rd-instar larvae of *P. xylostella* and ten 2nd-instar larvae of *S. exigua* were introduced to each leaf surface. 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 artificial diet. The mortality for *P. xylostella* was calculated by counting the dead larvae at 24 h interval for 2 days, for *S. exigua* every 24 h for 5 days and for *O. furnacalis* every 24 h for 6 days. And the LD_{50} was calculated by a Probit method (Russell *et al*., 1977) using SPSS statistics 21 (IBM., USA). All assays were performed at 25℃ in 60 to 70% humidity with a 16 h : 8 h light dark cycle.

RESULTS

1. Construction of mutant *cry* genes through multi site-directed mutagenesis

1) Construction of pIM-Mod-*cry1Ac* vector

For the mutagenesis of modified *cry1Ac* gene (1857 bp), modified *cry1Ac* gene digested with *Eco*RV and *Bam*HI was inserted into the pIM vector, a small size vector including pUC origin and ampicillin resistance gene, to construct the pIM-*Mod-cry1Ac* vector. The internal structure of this vector was verified by restriction endonuclease digestion (Fig. 5).

2) Primers for mutagenesis

To compare amino acid sequence of 8 mutant Cry with modified Cry1Ac, the alignment of amino acid sequences was performed. There were 24 residues located on domain I and domain II different from each other. Among them, Among the total 24 different residues, the 9^{th} and 10^{th}, the 12^{th} and 13^{th} residues were very close to share the same primer, so total ten primers were designed (Table 2) for changing amino acid into Mod-Cry1Ac, according to the codon usage of Chinese cabbage. For acquiring random various mutants, repeated mutagenesis using 3 to 5 primers was performed, and then totally 8 different kinds of mutant *cry1Ac* genes randomly

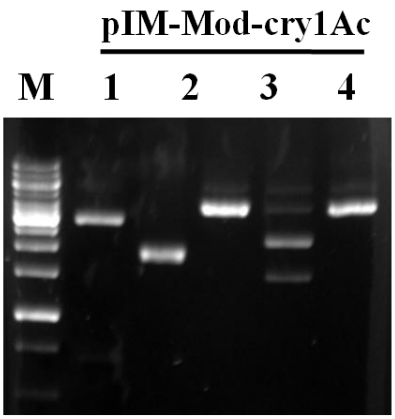


Fig. 5. Construction analysis of the vector, pIM-*Mod-cry1Ac*. The transfer vector pIM-*Mod-cry1Ac* was digested with various restriction endonucleases and electrophoresed on 0.8% agarose gel. Lanes, M, 1 kb DNA Ladder; 1, DraI; 2, EcoRI; 3, EcoRⅤ; 4, ScaI; 5, XbaI.

Table 2. Nucleotide sequences of primers used on *Mod-cry1Ac* for constructing *Mut-cry1Ac* genes.

Domain	Primer	Primer sequence[a] (5′→ 3′)	Converted position of amino acid (Sequence variation)
Domain I	Mod1ac E116A	GCAGAGAGCTTCAGA**GCT**TGGGAAGCCG	116(E→A)
	Mod1ac E128V	CCCAGCTCTCCGC**GTG**GAAA	128(E→V)
	Mod1ac A187T	GGGGATTCGATGCT**ACC**ACCATCAATAGCCG	187(A→T)
	Mod1ac V227I	CTGATTCTAGAGATTGG**ATC**AGATACAACCAGTTCAGG	227(V→I)
	Mod1ac A245S	CAGTTTTGGACATTGTG**TCT**CTCTTCCCGAAC	245(A→S)
	1F	ATTGTGTCTCTCTTC**AGC**AACTATGACTCCAGA	248(P→S)
	Mod1ac R254T	CCCGAACTATGACTCCAGA**ACC**TACCCTATCCGTAC	254(R→T)
Domain I I	S283M	GCTTCCGTGGT**ATG**GCCCAGGGTATCG	283(S→M)
	S283M& G286R	CCGTGGT**ATG**GCCCAG**A**G**G**ATCGAAAGATC	283(S→M) 286(G→R)
	G286R	CGTGGTTCTGCCCAG**A**G**G**ATCGAAAGATCC	286(G→R)
	A309V	GCATAACTATCTACACCGATG**TG**CACAGAGGATACTATTACTGGT	309(A→V)
	M322T& F324S	CTGGACACCAGATCA**CC**GCCT**C**TCCAGTTGG	322(M→T) 324(F→S)
	T334A	CCGGACCTGAGTTT**G**C**T**TTTCCTCTCTATGG	334(T→A)
	I375P	CCCTTCAATATCGGT**CCT**AACAACCAGCAAC	375(I→P)
	E412V	CGTTGATTCCTTGGACG**TG**ATCCCACCACAG	412(E→V)
	Q424A	GTGCCACCCAGG**GCT**GGATTCTCCCAC	424(Q→A)

[a]Underlined sequences indicate mutagenesis site.

changed 24 amino acid sequences into Cry1Ac were obtained. Fig 6 showed which region of amino acid was changed from modified Cry1Ac.

2. Expression of mutant cry genes using baculovirus expression system

bAcGOZA DNA was co-transfected with baculovirus transfer vectors into Sf9 cells (Fig. 7). The supernatant containing budded viruses was collected at 5 days post infection (p.i.), re-infected into a monolayer of Sf9 cells, and infected cells were harvested at 4 days post infection (p.i.). Expressed mutant proteins were named the Mut-N02, Mut-N04, Mut-N05, Mut-N06, Mut-N14, Mut-N16, Mut-N27, and Mut-N44 respectively. 8 mutant genes those domain I and domain II were randomly converted into Mod-cry1Ac. These genes were picked out for expression of mutant Cry proteins using baculovirus expressing system. PCR was performed to confirm the recombinant viruses containing the mutant *cry1Ac* genes and verification of genome structure of the recombinant AcMNPV expressing Mut-cry1Ac by PCR using specific primer sets (Fig. 8). RT-PCR was performed for verification of transcription of polyhedrin-Mut-cry1Ac fusion genes (Fig. 9). 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. 10).

In order to examine the expression of fusion protein by recombinant virus in Sf9 cells, the protein synthesis was initially analyzed by SDS-PAGE. Wild-type AcMNPV had an about 30 kDa polyhedrin protein, while recombinant virus mutants showed about 95 kDa fusion protein bands (Fig. 11A). To confirm whether the recombinant polyhedra would be cleaved into the active toxin by proteolytic enzymes and to quantify the amount of toxins, fusion proteins were treated with trypsin, one of the major proteolytic enzymes present in the mid-gut in insects and sequentially subjected to SDS-PAGE. The activated mutant Cry crystal protein bands all were exhibited about 65 kDa (Fig. 11B).

Activated Cry proteins were quantitative measured by using of 1D-gel analysis system (Kodak Co., USA) and used Bovine Serum Albumin (BSA) as the standard. As a result, the amount of activated Cry protein per $1x10^6$ polyhedral inclusion bodies (PIBs) was evaluated (Table 3).

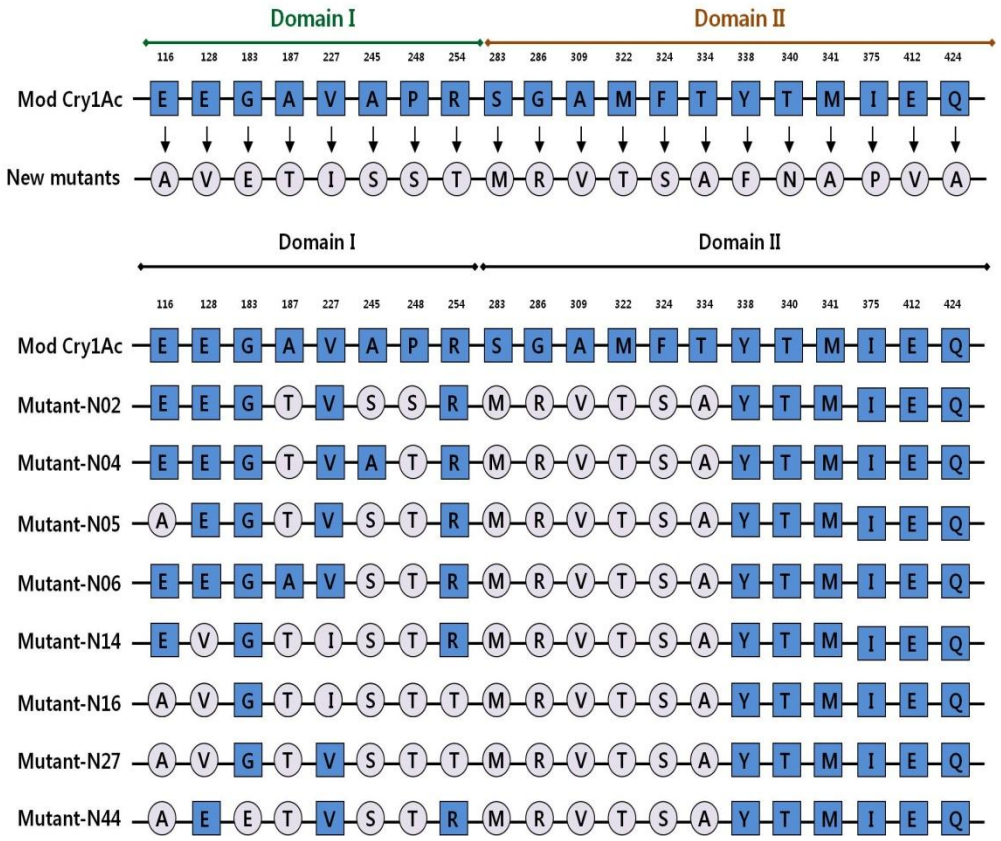


Fig. 6. Mutagenesis for expression of active proteins. The blue indicates the plant codon usage, the white indicates the bacteria codon usage. The rectangle represents the amino acid residues of Cry1Ac. The ellipse represents the amino acid residues of Cry mutant.

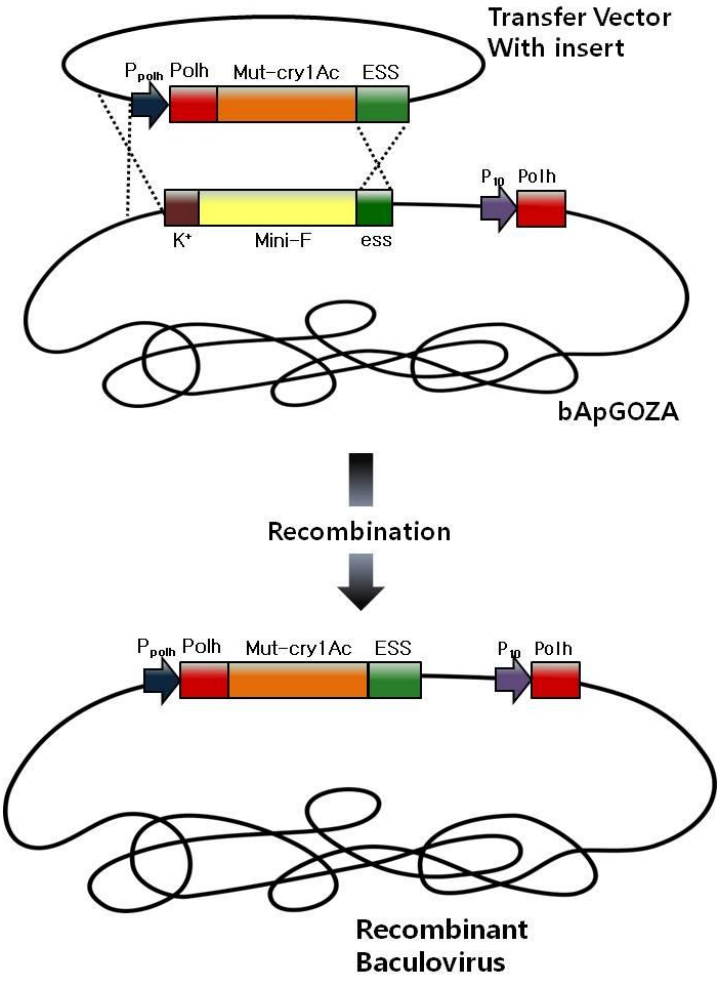


Fig. 7. Schematic diagram of construction of the recombinant baculoviruses expressing novel *Mod-cry1Ac* mutant genes.

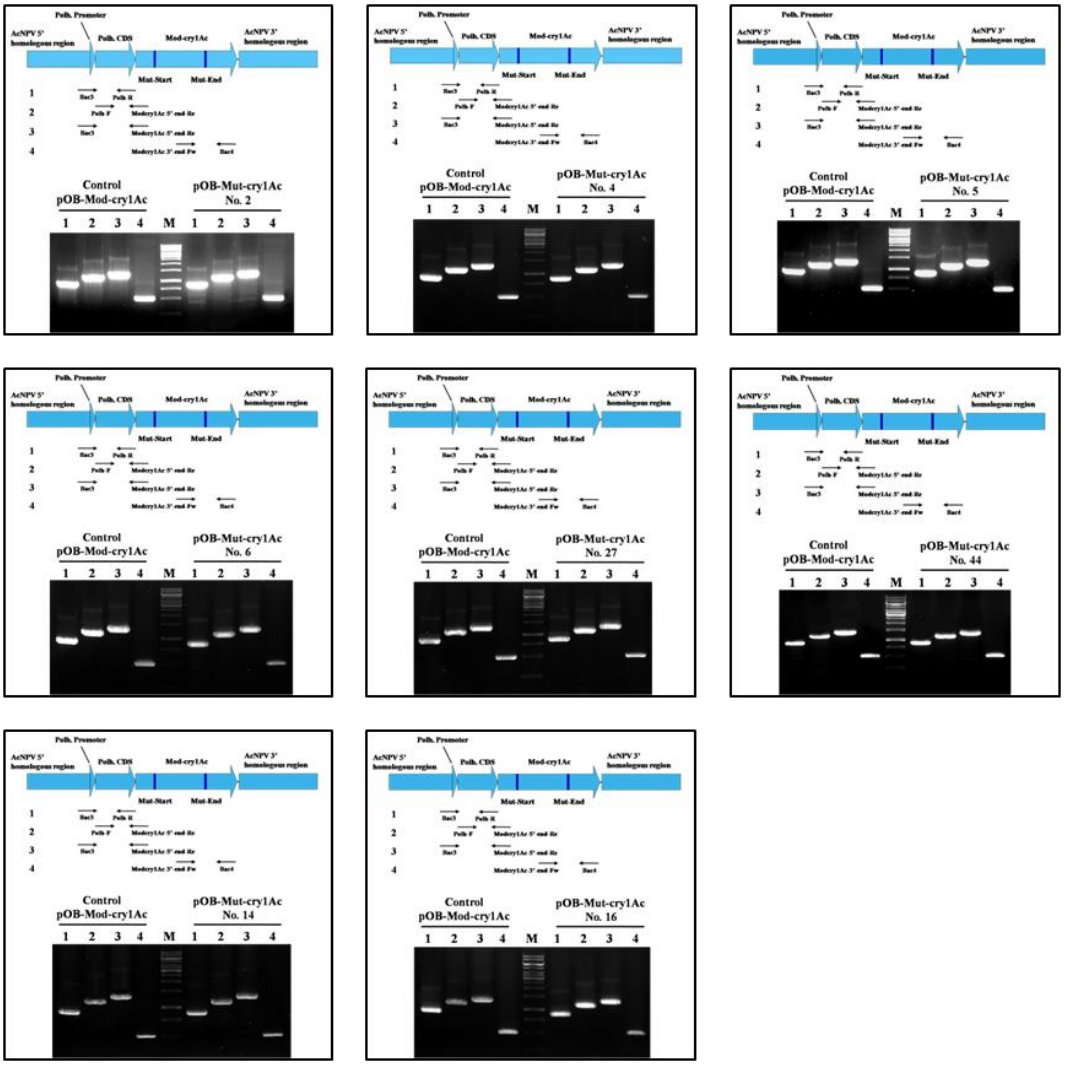


Fig. 8. Verification of genome structure of the recombinant AcMNPV expressing Mut-cry1Ac by PCR using specific primer sets. Solid arrows indicate primer positions used in PCR analysis. Lane: M, 100bp ladder; 1, primers Bac3 and Polh-R; 2, primers Polh-F and Modcry1Ac-5'-end-Re; 3, primers Bac3 and Modcry1Ac-5'-end-Re; 4, primers Modcry1Ac-3'-end-Fw and Bac4.

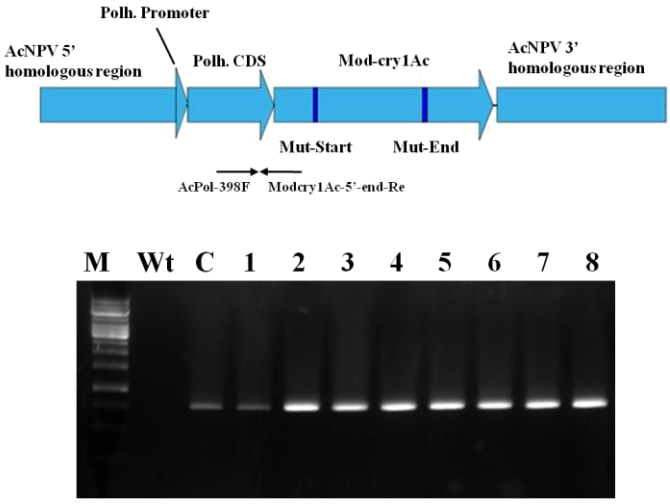


Fig. 9. Verification of transcription of polyhedrin-*Mut-cry1Ac* fusion genes from Sf9 cells infected with recombinant AcMNPVs expressing corresponding *Mut-cry1Ac* gene, respectively. Lane: M, 1kb ladder; Wt, wild-type AcMNPV; C, recombinant AcMNPV expressing *Mod-cry1Ac*; 1, recombinant AcMNPV expressing *Mut-cry1Ac* No.02; 2, recombinant AcMNPV expressing *Mut-cry1Ac* No.04; 3, recombinant AcMNPV expressing *Mut-cry1Ac* No.05; 4, recombinant AcMNPV expressing *Mut-cry1Ac* No.06; 5, recombinant AcMNPV expressing *Mut-cry1Ac* No.14; 6, recombinant AcMNPV expressing *Mut-cry1Ac* No.16; 7, recombinant AcMNPV expressing *Mut-cry1Ac* No.27; 8, recombinant AcMNPVexpressing *Mut-cry1Ac* No. 44.

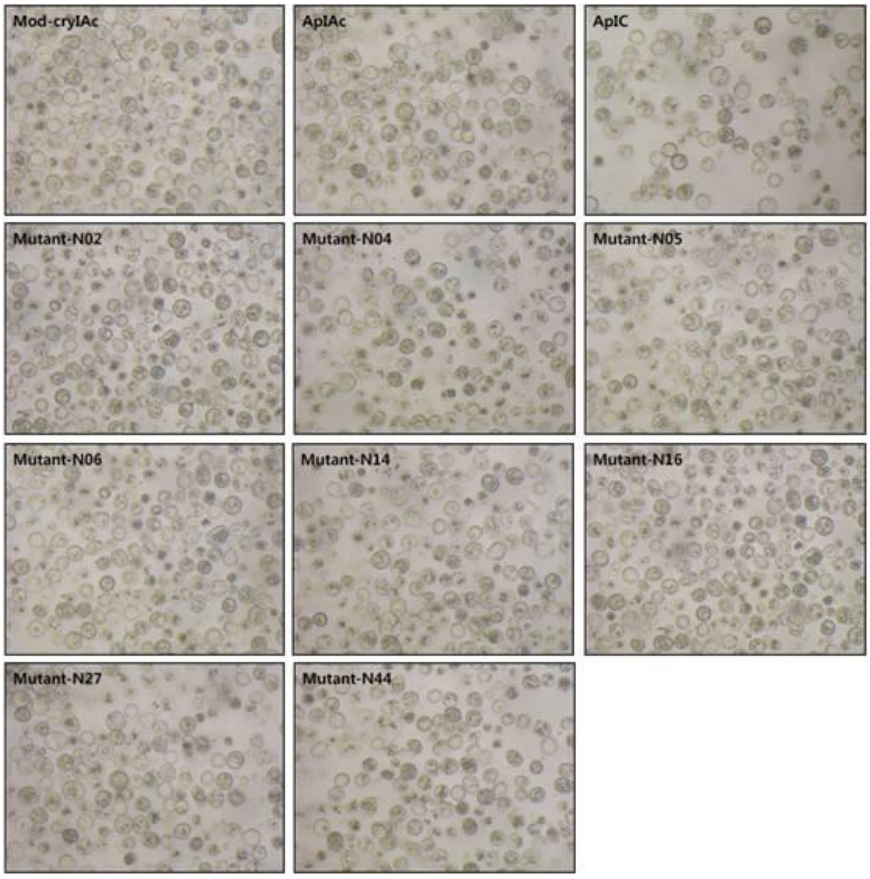


Fig. 10. Phase-contrast microscopy of Sf9 cells, which infected with the recombinant baculoviruses expressing novel *Mod-cry1Ac* mutant genes.

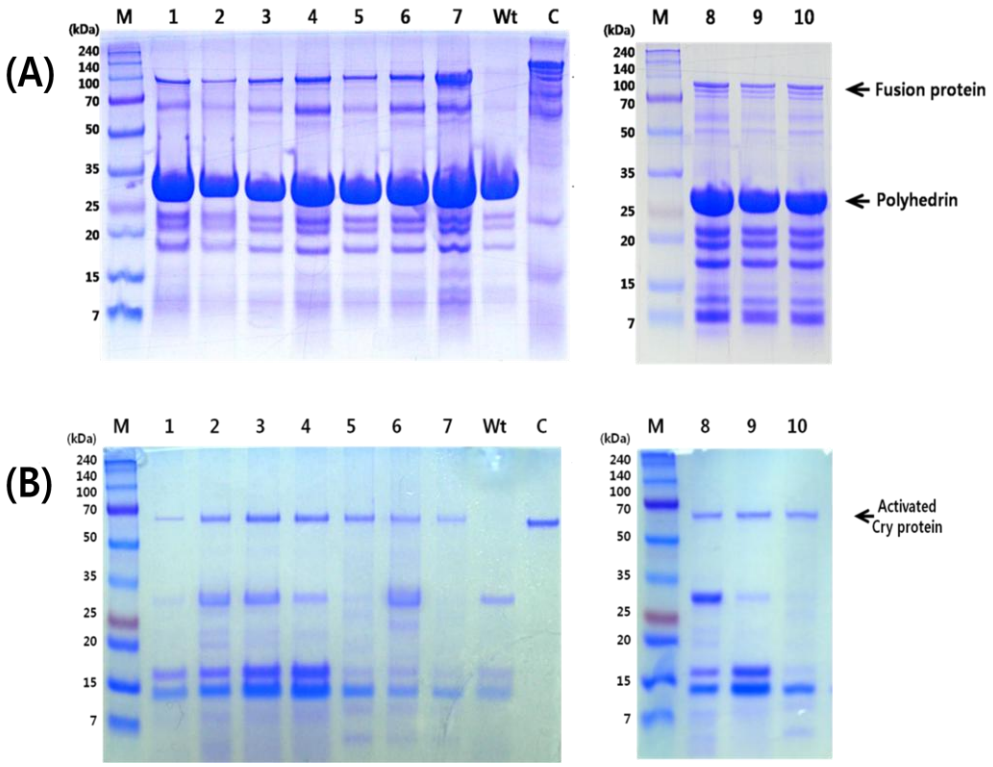


Fig. 11. SDS-PAGE analysis of polyhedra produced by the recombinant baculoviruses which containing novel Mod-cry1Ac mutant proteins in fusion with polyhedrin (A) and activated Cry protein (B) Lanes: M, protein molecular wight marker; 1, ApIAc; 2, ApIc; 3, Mutant N02; 4, Mutant N04; 5, Mutant N05; 6, Mutant N06; 7, Mutant N14; 8, Mutant N16; 9, Mutant N27; 10, Mutant N44; Wt, wild-type AcMNPV; C, Mod-Cry1Ac.

Table 3. Quantification of activated toxins of the novel Mut-Cry1Ac proteins which are occluded in the recombinant polyhedra infusion with polyhedrin

Mutant No.	Bt Toxin (ng/1x10^6 PIBs)	Mutant No.	Bt Toxin (ng/1x10^6 PIBs)
N02	**237.7**	**N14**	**427.4**
N04	**374.1**	**N16**	**550.3**
N05	**296.9**	**N27**	**386.8**
N06	**265.8**	**N44**	**255.9**

3. Expression Insecticidal activity of mutant Bt toxins

1) Bioassay

To evaluate the insecticidal activity of mutant Cry1Ac proteins, bioassays were performed against 3rd instar of *P. xylostella*, 2nd instar of *S. exigua* larvae and neonate of *O. furnacalis*, comparing with modified Cry1Ac and Cry1C as control.

Preliminary bioassay results about Lepidopteran pest (Fig. 12) were showed these mutant Cry proteins have higher or similar insecticidal activity compared to those of Cry1Ac and Cry1C. Based on the results of the experiment results, mutant Cry protein that showed improved pesticidal activity was selected. These mutant Cry proteins were Mut-N04, Mut-N06 and Mut-N16. The LD_{50} values were read representing the lethal dose for 50% larval mortality of *P. xylostella, S. exigua* and *O. furnacalis*. LD_{50} values of these Cry protein through quantitative bioassay was lower about 5-fold than Mod-cry1Ac and about 6-fold than Mut-N16 showing the highest insecticidal activity against *P. xylostella* (Table 4). In *S. exigua* bioassay, Mod-Cry1Ac showed no insecticidal activity, on the other hand not as effective as Cry1C but 8 Mut-Cry1Ac showed insecticidal activity (Table 5). Also, LD_{50} values of mutant Cry protein through quantitative bioassay was lower about 2-fold than Mod-cry1Ac and about 4-fold than Mut-N16 showing the highest insecticidal activity against *O. furnacalis* (Table 6).

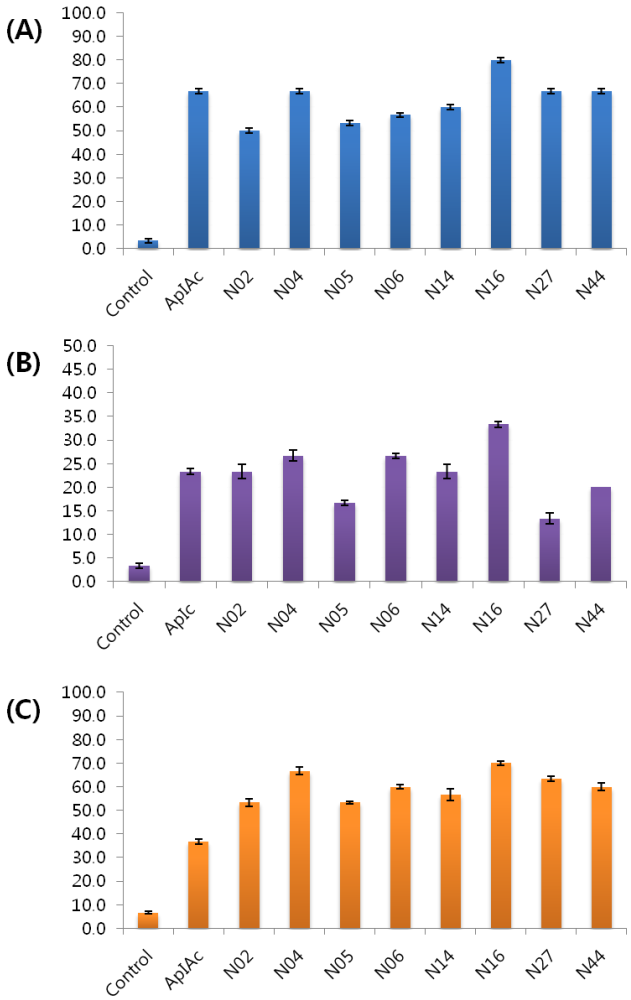


Fig. 12. Insecticidal activity of Mut-Cry1Ac proteins against 3rd instar larvae of *Plutella xylostella* (A), 2nd instar larvae of *Spodoptera exigua* (B), and neonates instar larvae of *Ostrinia furnacalis* (C). In *P. xylostella*, toxins were treated with 5 ng/larva and their mortality was scored 3 days after inoculation. In this case of *S. exigua*, toxins were treated with 500 ng/larva and their mortality was scored 5 days after inoculation. And about *O. furnacalis*, toxins were treated with 50 ng/larva and their mortality was scored 6 days after inoculation.

Table 4. Median lethal dose of mutant Cry proteins against third instar lavae of *P. xylostella*

	LD_{50} (ng/larva)	95% fiducial limits
Ap1Ac	**5.21**	**1.09-12.16**
Ap1C	**>50.00***	**ND**
N04	**0.86**	**0.35-1.81**
N06	**0.93**	**0.33-2.16**
N16	**0.79**	**0.36-1.57**

The mortality was scored 2 days after inoculation.
ND; Not determined0
* The mortality was 30% at 50 ng/larva.
LD_{50} was calculated by IBM SPSS Statistics 21

Table 5. Median lethal dose of mutant Cry proteins against second instar lavae of *S.exigua*

	LD_{50} (ng/larva)	95% fiducial limits
Ap1Ac	**>800.00***	**ND**
Ap1C	**226.88**	**70.64-322.91**
N04	**341.03**	**181.51-168.41**
N06	**321.31**	**182.86-432.41**
N16	**283.70**	**65.47-423.48**

The mortality was scored 5 days after inoculation.
ND; Not determined
* The mortality was 26.7% at 800 ng/larva.
LD_{50} was calculated by IBM SPSS Statistics 21

Table 6. Median lethal dose of mutant Cry proteins against neonates of *0. furnacalis*

	LD_{50} (ng/larva)	95% fiducial limits
Ap1Ac	**67.83**	**50.00-95.58**
Ap1C	**>100.00***	**ND**
N04	**38.77**	**16.65-100.75**
N06	**44.06**	**14.99-197.58**
N16	**15.91**	**5.79-29.30**

The mortality was scored 6 days after inoculation.
ND; Not determined
* The mortality was 28.9% at 100 ng/larva.
LD_{50} was calculated by IBM SPSS Statistics 21

DISCUSSION

The most successful biological insecticide for pest control is the bacterium Bt-based insecticides, which presently is about 2% of the total insecticidal market (Raymond et al. 2010). However, Bt biological insecticides were faced with some limitations such as a narrow spectrum, a short shelf life, and development of pest insect resistance. To overcome these problems, many researchers have tried to modify Bt strains and their insecticidal crystal protein-encoding gene using genetic manipulation for enhancement of their potency against target insects, faster effects, and delay of resistance development. Also, Insect-resistant transgenic plants have an advantage of reduced need for conventional insecticides, providing benefits for human health and the environment. Genetic engineering allows the introduction of several desirable genes in a single event, and can reduce the time to introgress novel genes into elite background (Miller 1997). However, these native *cry* genes were found to be expressed poorly in plants. It was evident that the production of transgenic plants offering effective control over economically significant insect pests would require considerable improvement of *cry* gene expression. The failure of adequate *cry* gene expression in transgenic plants appeared to be due to the *cry* coding sequence, as the genes were transcriptionally fused to highly active promoters and leader sequences which had been used previously to successfully express other prokaryotic genes in plants (Gleave et al. 1998).

The objective of this study was to construct novel *Bacillus thuringiensis cry1*-type genes for genetically modified crops and to select the most appropriate *cry* gene for

transgenic crops with enhanced insecticidal activity. For acquiring various random mutants, repeated mutagenesis using three to five primers was performed, and then a total of eight different mutant *cry1Ac* genes randomly changed 24 amino acid sequences to obtain a series of *Mod-cry1Ac* genes. These genes were selected for expression of mutant Cry proteins using baculovirus expressing system. bApGOZA DNA was co-transfected with baculovirus transfer vectors into Sf9 cells, and transfected cells produced polyhedral inclusion bodies made of polyhedron mutant Cry1Ac fusion proteins. To evaluate the insecticidal activity of mutant Cry1Ac proteins, bioassays were performed against lepidopteran pest larvae, comparing with Mod-cry1Ac and Cry1C as control. As a result, constructed mutant Cry proteins have higher or similar insecticidal activity compared to those of Cry1Ac and Cry1C. Therefore, mutant Cry proteins were considered to have the potential for the efficacious biological insecticide. Especially, Mutant-N16 showed the highest insecticidal activity against to lepidopteran pest, which is considered to the appropriate *cry* gene for genetically modified crops.

The mode of action of previously studied Cry proteins (δ -endotoxins) has been mostly characterized using lepidopteran larvae model system, and the major role of Cry proteins is to disrupt the midgut epithelium barrier to facilitate bacterial invasion of the hemocoel. As a result of substitution of amino acid residue, Cry protein structure was influenced, which affected the mode of action of Cry proteins. For examples, site-directed mutagenesis of domain II loop residue has resulted in mutant toxins with increased insecticidal activity, in some cases. Domain II loop mutants with increased insecticidal activity was Cry1Ab toxin where mutations in loop 2

resulted in higher insecticidal activity against Gypsy moth (*Limantria dispar*). More importantly, the increased insecticidal activity correlated with increased binding affinities to brush border membrane vesicle (BBMV) isolated from Gypsy moth (Rajamohan et al. 1996). Similarly, it was shown that mutations of domain II loop regions in the coleopteran specific Cry3Aa resulted in enhanced toxicity to yellow mealworm (*Tenebrio molitor*). A triple domain II loop 1 mutant R345 showed ten-fold higher toxicity to yellow mealworm than Cry3Aa, and two-fold higher toxicity against Colorado potato beetle that correlated with two-fold higher binding affinity to Colorado potato beetle BBMV (Wu et al. 2000). Another example, mutant Cry3Aa toxin displayed decreased toxicity when compared to the wild type toxin, and impaired ability to compete CPB brush border membrane associated cleavage of an a disintegrin and metalloproteinase fluorogenic substrate. Although the proteolytic profile of mutant Cry3Aa toxins generated by brush border membrane associated proteases was similar to that of wild type Cry3Aa toxin, the metalloprotease inhibitor 1,10-phenanthroline was less efficient on the proteolysis of mutants than on that of the wild type toxin (García-Robles et al. 2012). As indicated above, the change of the insecticidal activity by site-directed mutagenesis of the epitope binding regions found in domain I and/or II has a lot of potential for selection of Cry toxins. Cry proteins with improved activity against different insect pests as shown by the different examples of Cry toxins with modifications in these amino acid regions that have resulted in toxins with enhanced insecticidal properties (Bravo et al. 2013).

In conclusion, 8 different kinds of mutant *cry1Ac* genes were constructed, and these gene were introduced into Sf9 cells to express mutant Cry proteins using

baculovirus expressing system. Through quantitative bioassay, several mutant *cry* genes were selected to develope genetically modified crops. To develope high throughput expression system for the expression of mutant *cry* gene with comprehension of the mode of action, this study should be useful means to provide the construction of mutant *cry* gene for transgenic crops.

LITERATURE CITED

Adang, Michael J, and Kemet D Spence. 1982. "Biochemical comparisons of the peritrophic membranes of the lepidopterans *Orgyia pseudotsugata* and *Manduca sexta*." *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* no. 73 (3):645-649.

Adang, MJ, and KD Spence. 1983. "Permeability of the peritrophic membrane of the Douglas fir tussock moth (*Orgyia pseudotsugata*)." *Comparative Biochemistry and Physiology Part A: Physiology* no. 75 (2):233-238.

Aronson, A. 1995. "The protoxin composition of Bacillus thuringiensis insecticidal inclusions affects solubility and toxicity." *Applied and environmental microbiology* no. 61 (11):4057-4060.

Aronson, Arthur I, Dong Wu, and Chunlin Zhang. 1995. "Mutagenesis of specificity and toxicity regions of a Bacillus thuringiensis protoxin gene." *Journal of bacteriology* no. 177 (14):4059-4065.

Ben-Dov, Eitan, Gal Nissan, Nir Pelleg, Robert Manasherob, Sammy Boussiba, and Arieh Zaritsky. 1999. "Refined, Circular Restriction Map of the *Bacillus thuringiensis* subsp. *israelensis* Plasmid Carrying the Mosquito Larvicidal Genes." *Plasmid* no. 42 (3):186-191.

Bernhard, K, P Jarrett, M Meadows, J Butt, DJ Ellis, GM Roberts, S Pauli, P Rodgers, and HD Burges. 1997. "Natural Isolates of *Bacillus thuringiensis*: Worldwide Distribution, Characterization, and Activity against Insect Pests." *Journal of Invertebrate Pathology* no. 70 (1):59-68.

Bietlot, H, PR Carey, C Choma, H Kaplan, T Lessard, and M Pozsgay. 1989. "Facile preparation and characterization of the toxin from Bacillus thuringiensis var. kurstaki." *Biochem. J* no. 260:87-91.

Bourgouin, Catherine, André Klier, and Georges Rapoport. 1986. "Characterization of the genes encoding the haemolytic toxin and the mosquitocidal delta-endotoxin of Bacillus thuringiensis israelensis." *Molecular and General Genetics MGG* no. 205 (3):390-397.

Bravo, Alejandra, Isabel Gómez, Helena Porta, Blanca Ines García-Gómez, Claudia Rodriguez-Almazan, Liliana Pardo, and Mario Soberón. 2013. "Evolution of Bacillus thuringiensis Cry toxins insecticidal activity." *Microbial Biotechnology* no. 6 (1):17-26.

Bravo, Alejandra, Sarjeet S Gill, and Mario Soberón. 2007. "Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control." *Toxicon* no. 49 (4):423-435.

Bravo, Alejandra, Koen Hendrickx, Stefan Jansens, and Marnix Peferoen. 1992. "Immunocytochemical analysis of specific binding of *Bacillus thuringiensis* insecticidal crystal proteins to lepidopteran and coleopteran mudgut membranes." *Journal of Invertebrate Pathology* no. 60 (3):247-253.

Bravo, Alejandra, Supaporn Likitvivatanavong, Sarjeet S Gill, and Mario Soberón. 2011. "*Bacillus thuringiensis*: A story of a successful bioinsecticide." *Insect biochemistry and molecular biology* no. 41 (7):423-431.

Cahan, Rivka, Hen Friman, and Yeshayahu Nitzan. 2008. "Antibacterial activity of Cyt1Aa from Bacillus thuringiensis subsp. israelensis." *Microbiology* no. 154 (11):3529-3536.

Choi, Jae Young, MS Li, HJ Shim, JY Roh, SD Woo, BR Jin, KS Boo, and YH Je. 2007. "Isolation and characterization of strain of Bacillus thuringiensis subsp. kenyae containing two novel cry1-type toxin genes." *Journal of microbiology and biotechnology* no. 17 (9):1498-1503.

Cohen, Shmuel, Shira Albeck, Eitan Ben-Dov, Rivka Cahan, Michael Firer, Arieh Zaritsky, and Orly Dym. 2011. "Cyt1Aa toxin: crystal structure reveals implications for its membrane-perforating function." *Journal of Molecular Biology* no. 413 (4):804-814.

Cohen, Shmuel, Orly Dym, Shira Albeck, Eitan Ben-Dov, Rivka Cahan, Michael Firer, and Arieh Zaritsky. 2008. "High-Resolution Crystal Structure of Activated Cyt2Ba Monomer from *Bacillus thuringiensis* subsp. *israelensis*." *Journal of molecular biology* no. 380 (5):820-827.

Crickmore, N., J. Baum, A. Bravo, D. Lereclus, K. Narva, K. Sampson, E. Schnepf, M. Sun, and D.R. Zeigler. *Bacillus thuringiensis toxin nomenclature* 2013. Available from http://www.btnomenclature.info/

Crickmore, Neil, DR Zeigler, J Feitelson, ESCHERICHIA Schnepf, J Van Rie, D Lereclus, J Baum, and DH Dean. 1998. "Revision of the nomenclature for the Bacillus thuringiensis pesticidal crystal proteins." *Microbiology and Molecular Biology Reviews* no. 62 (3):807-813.

De Maagd, RA, MS Kwa, H Van der Klei, Takashi Yamamoto, Bert Schipper, Just M Vlak, Willem J Stiekema, and Dirk Bosch. 1996. "Domain III substitution in Bacillus thuringiensis delta-endotoxin CryIA (b) results in superior toxicity for Spodoptera exigua and altered membrane protein recognition." *Applied and*

environmental microbiology no. 62 (5):1537-1543.

de Maagd, Ruud A, Alejandra Bravo, and Neil Crickmore. 2001. "How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world." *TRENDS in Genetics* no. 17 (4):193-199.

Donovan, William P, Judith C Donovan, and James T Engleman. 2001. "Gene Knockout Demonstrates That *vip3A* Contributes to the Pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*." *Journal of Invertebrate Pathology* no. 78 (1):45-51.

Ferré, Juan, Maria Dolores Real, Jeroen Van Rie, Stefan Jansens, and Marnix Peferoen. 1991. "Resistance to the Bacillus thuringiensis bioinsecticide in a field population of Plutella xylostella is due to a change in a midgut membrane receptor." *Proceedings of the National Academy of Sciences* no. 88 (12):5119-5123.

Fraley, Robert T, Stephen G Rogers, Robert B Horsch, Patricia R Sanders, Jeffery S Flick, Steven P Adams, Michael L Bittner, Leslie A Brand, Cynthia L Fink, and Joyce S Fry. 1983. "Expression of bacterial genes in plant cells." *Proceedings of the National Academy of Sciences* no. 80 (15):4803-4807.

García-Robles, Inmaculada, Camila Ochoa-Campuzano, Jorge Sánchez, Estefanía Contreras, M Dolores Real, and Carolina Rausell. 2012. "Functional significance of membrane associated proteolysis in the toxicity of *Bacillus thuringiensis* Cry3Aa toxin against Colorado potato beetle." *Toxicon*.

Gleave, Andrew P, Deepali S Mitra, Ngaire P Markwick, Bret AM Morris, and Lesley L Beuning. 1998. "Enhanced expression of the shape Bacillus thuringiensis cry9Aa2 gene in transgenic plants by nucleotide sequence modification confers resistance to potato tuber moth." *Molecular Breeding* no. 4 (5):459-472.

González Jr, JoséM, Howard T Dulmage, and Bruce C Carlton. 1981. "Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*." *Plasmid* no. 5 (3):351-365.

Guerchicoff, Alejandra, Rodolfo A Ugalde, and Clara P Rubinstein. 1997. "Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*." *Applied and environmental microbiology* no. 63 (7):2716-2721.

Höfte, H, and HR Whiteley. 1989. "Insecticidal crystal proteins of Bacillus thuringiensis." *Microbiological reviews* no. 53 (2):242-255.

Hussain, Syed-Rehan A, Arthur I Aronson, and Donald H Dean. 1996. "Substitution of Residues on the Proximal Side of Cry1A *Bacillus thuringiensis* δ-Endotoxins Affects Irreversible Binding to *Manduca sexta* Midgut Membrane." *Biochemical and biophysical research communications* no. 226 (1):8-14.

James, Clive. 2012. Global Status of Commercialized Biotech/GM Crops: 2012, ISAAA Brief No 44 ISAAA: Ithaca, NY, 2012. ISBN 978-1-892456-49-4,[Online], Available: http://www.isaaa.org/resources/publications/briefs/42/executivesummary/default. asp [07.03. 2012].

Kalman, Sue, Kristine L Kiehne, Nicole Cooper, Mitra Shahabi Reynoso, and Takashi Yamamoto. 1995. "Enhanced production of insecticidal proteins in Bacillus thuringiensis strains carrying an additional crystal protein gene in their chromosomes." *Applied and environmental microbiology* no. 61 (8):3063-3068.

Knowles, Barbara H, Michael R Blatt, Mark Tester, Jane M Horsnell, Joe Carroll, Gianfranco Menestrina, and David J Ellar. 1989. "A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers." *FEBS letters* no. 244 (2):259-262.

Lemaux, Peggy G. 2008. "Genetically engineered plants and foods: a scientist's analysis of the issues (Part I)." *Annu. Rev. Plant Biol.* no. 59:771-812.

Li-Ming, Dou, Han Lan-Lan, Zhang Jie, He Kang-lai, Zhao Kui-Jun, Huang Da-Fang, and Song Fu-Ping. 2008. "Cloning, expression and activity of cry1Ia gene from Bacillus thuringiensis isolate." *Chinese Journal of Agricultural Biotechnology* no. 5 (1):49-54.

Li, J, DJ Derbyshire, B Promdonkoy, and DJ Ellar. 2001. "Structural implications for the transformation of the Bacillus thuringiensis delta-endotoxins from water-soluble to membrane-inserted forms." *Biochemical Society Transactions* no. 29 (Pt 4):571-577.

Li, Jade, Pandelakis A Koni, and David J Ellar. 1996. "Structure of the Mosquitocidal δ-Endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and Implications for Membrane Pore Formation." *Journal of molecular biology* no. 257 (1):129-152.

Liang, Yizhi, Smita S Patel, and Donald H Dean. 1995. "Irreversible binding kinetics of Bacillus thuringiensis CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity." *Journal of Biological Chemistry* no. 270 (42):24719-24724.

Manasherob, Robert, Mark Itsko, Nadine Sela-Baranes, Eitan Ben-Dov, Colin Berry, Shmuel Cohen, and Arieh Zaritsky. 2006. "Cyt1Ca from Bacillus thuringiensis subsp. israelensis: production in Escherichia coli and comparison of its biological

activities with those of other Cyt-like proteins." *Microbiology* no. 152 (9):2651-2659.

Martin, Phyllis AW, and Russell S Travers. 1989. "Worldwide abundance and distribution of Bacillus thuringiensis isolates." *Applied and Environmental Microbiology* no. 55 (10):2437-2442.

Miller, Lois K. 1997. *The baculoviruses*: Springer.

Pacheco, Sabino, Isabel Gómez, Ivan Arenas, Gloria Saab-Rincon, Claudia Rodríguez-Almazán, Sarjeet S Gill, Alejandra Bravo, and Mario Soberón. 2009. "Domain II loop 3 of Bacillus thuringiensis Cry1Ab toxin is involved in a "ping pong" binding mechanism with Manduca sexta aminopeptidase-N and cadherin receptors." *Journal of Biological Chemistry* no. 284 (47):32750-32757.

Pardo-Lopez, L, C Munoz-Garay, H Porta, C Rodríguez-Almazán, M Soberón, and A Bravo. 2009. "Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*." *Peptides* no. 30 (3):589-595.

Parker, Michael W, and Susanne C Feil. 2005. "Pore-forming protein toxins: from structure to function." *Progress in biophysics and molecular biology* no. 88 (1):91-142.

Rajamohan, Francis, Oscar Alzate, Jeffrey A Cotrill, April Curtiss, and Donald H Dean. 1996. "Protein engineering of Bacillus thuringiensis δ-endotoxin: mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae." *Proceedings of the National Academy of Sciences* no. 93 (25):14338-14343.

Raymond, Ben, Paul R Johnston, Christina Nielsen-LeRoux, Didier Lereclus, and Neil Crickmore. 2010. " *Bacillus thuringiensis*: an impotent pathogen?" *Trends in microbiology* no. 18 (5):189-194.

Roh, Jong Yul, Jae Young Choi, Ming Shun Li, Byung Rae Jin, and Yeon Ho Je. 2007. "Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control." *Journal of microbiology and biotechnology* no. 17 (4):547-559.

Sanahuja, Georgina, Raviraj Banakar, Richard M Twyman, Teresa Capell, and Paul Christou. 2011. "Bacillus thuringiensis: a century of research, development and commercial applications." *Plant biotechnology journal* no. 9 (3):283-300.

Schnepf, E, N Crickmore, J Van Rie, D Lereclus, J Baum, J Feitelson, DR Zeigler, and DH Dean. 1998. "*Bacillus thuringiensis* and its pesticidal crystal proteins." *Microbiology and molecular biology reviews* no. 62 (3):775-806.

Soberón, Mario, Jazmin A. López-Díaz, and Alejandra Bravo. 2013. "Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms." *Peptides* no. 41 (0):87-93. doi: http://dx.doi.org/10.1016/j.peptides.2012.05.023.

Tabashnik, Bruce E, Yves Carrière, Timothy J Dennehy, Shai Morin, Mark S Sisterson, Richard T Roush, Anthony M Shelton, and Jian-Zhou Zhao. 2003. "Insect resistance to transgenic Bt crops: lessons from the laboratory and field." *Journal of economic entomology* no. 96 (4):1031-1038.

Tailor, Ravi, Jan Tippett, Graham Gibb, Stephen Pells, Linda Jordan, and Susan Ely. 1992. "Identification and characterization of a novel *Bacillus thuringiensis* δ-endotoxin entomocidal to coleopteran and lepidopteran larvae." *Molecular Microbiology* no. 6 (9):1211-1217.

Thammachat, Siriya, Wanwarang Pathaichindachote, Chartchai Krittanai, and Boonhiang Promdonkoy. 2008. "Amino acids at N-and C-termini are required for the efficient production and folding of a cytolytic delta-endotoxin from Bacillus thuringiensis." *BMB. Rep* no. 41:820-825.

Thomas, WENDY E, and DAVID J Ellar. 1983. "*Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*." *Journal of Cell Science* no. 60 (1):181-197.

Vaeck, Mark, Arlette Reynaerts, Herman Höfte, Stefan Jansens, Marc De Beuckeleer, Caroline Dean, Marc Zabeau, Marc Van Montagu, and Jan Leemans. 1987. "Transgenic plants protected from insect attack." *Nature* no. 328:33-37.

Valicente, Fernando H, and André HC Mourão. 2008. "Use of by-products rich in carbon and nitrogen as a nutrient source to produce *Bacillus thuringiensis* (Berliner)-based biopesticide." *Neotropical Entomology* no. 37 (6):702-708.

Wang, G, J Zhang, F Song, A Gu, A Uwais, T Shao, and D Huang. 2008. "Recombinant *Bacillus thuringiensis* strain shows high insecticidal activity against *Plutella xylostella* and Leptinotarsa decemlineata without affecting nontarget species in the field." *Journal of applied microbiology* no. 105 (5):1536-1543.

Whalon, Mark E, and Byron A Wingerd. 2003. "Bt: mode of action and use." *Archives of Insect Biochemistry and Physiology* no. 54 (4):200-211.

Wu, Sheng-Jiun, C Noah Koller, Deborah L Miller, Leah S Bauer, and Donald H Dean. 2000. "Enhanced toxicity of *Bacillus thuringiensis* Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop." *FEBS letters* no. 473

(2):227-232.

Yang, Zhou, Hao Chen, Wei Tang, Hongxia Hua, and Yongjun Lin. 2011. "Development and characterisation of transgenic rice expressing two *Bacillus thuringiensis* genes." *Pest management science* no. 67 (4):414-422.

Zhang, Xuebin, Mehmet Candas, Natalya B Griko, Ronald Taussig, and Lee A Bulla Jr. 2006. "A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of Bacillus thuringiensis." *Science Signaling* no. 103 (26):9897.

Zhong, Chunying, Donghai Peng, Weixing Ye, Lujun Chai, Junliang Qi, Ziniu Yu, Lifang Ruan, and Ming Sun. 2011. "Determination of plasmid copy number reveals the total plasmid DNA amount is greater than the chromosomal DNA amount in *Bacillus thuringiensis* YBT-1520." *PloS one* no. 6 (1):e16025.

나비목 해충에 대한 살충활성이 향상된 신규 *Bacillus thuringiensis cry1*-type 유전자의 제작 및 특성 연구

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초록

Cry 단백질은 그람 양성균이며, 포자를 형성하는 세균 종인 *Bacillus thuringiensis* (Bt)의 살충성 단백질 그룹이다. 전세계에 걸쳐 분리 및 보고 되고 있는 다양한 Cry 단백질들은 오랜 기간 동안 인축과 자연 환경에 가장 안

전한 생물학적 방제 인자들 중 하나로 쓰여지고 있다. 또한, 최근에는 Cry 단백질을 encoding하는 유전자를 이용하여 해충에 저항성이 있는 형질전환 작물을 만들기 위해 *cry* 유전자를 작물에 도입하고 있다.

본 연구에서는 형질전환 작물에 적합하며 보다 향상된 살충성을 갖는 *cry-1* type 유전자를 제작하기 위하여 기존에 이미 식물형질 전환용으로 제작되어 있는 modified *cry1Ac* 유전자를 template으로 하여 multi site-directed mutagenesis를 수행하였다. Active domain을 encoding 하는 1857 bp 의 modified *cry1Ac*를 Mutagenesis하기 위해 식물 선호 codon usage을 바탕으로 제작된 16개의 mutagenic primers를 가지고 PCR을 수행하였다. 그 결과, domain Ⅰ 에서 8개의 residue, domain Ⅱ 에서 6개의 residues에 위치하는 총 14개 아미노산이 여러 조합으로 치환된 모두 8 종류의 다양한 mutant *cry* genes을 확보하였다.

Mutant Cry 단백질의 특성을 알아보기 위하여 8개 mutant *cry* genes을 baculovirus expression system을 이용하여 발현하여 정상적인 크기의 단백질이 생성됨을 확인하였으며, trypsin으로 activation된 mutant 단백질들은 SDS-PAGE 상에서 모두 65 kDa의 안정한 형태를 보여 주었다. 발현된 단백질들은 배추좀나방, 옥수수조명나방 및 파밤나방을 대상으로 하여 생물검정을 수행하였다. 3령 배추좀나방 유충에 대한 생물검정 결과, Mut-N04, Mut-N06 및 Mut-N16의 LD_{50} 값이 대조구 단백질인 Mod-Cry1Ac 보다 5-6배 낮았다. 갓 부

화한 옥수수조명나방에 대한 생물검정에서는 Mut-N04, Mut-N06 및 Mut-N16의 LD_{50} 값이 Mod-Cry1Ac 보다 약 2-4배 낮았다. 한편, 2령 파밤나방을 대상으로 한 생물검정에서 Mod-Cry1Ac은 살충성을 보이지 않았으나, Mut-N04, Mut-N06 및 Mut-N16의 LD_{50} 값은 Cry1C에 비해서는 비슷하거나 낮은 살충성을 보였다.

이상의 결과를 통해 배추좀나방, 파밤나방 및 옥수수조명나방과 같은 나비목 해충에 대해 높은 살충성을 가지는 Mut-N16과 같은 mutant *cry* genes을 선발하였고, 이 후에 선발된 유전자를 형질전환 작물에 도입함으로써 보다 효과적인 살충성 형질전환 작물 개발에 기여할 수 있을 것으로 생각된다.

검색어: *Bacillus thuringiensis*, **형질전환 작물**, *cry* gene, crystal protein, **베큘로바이러스발현계**, multi site-directed mutagenesis

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A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Construction and Characterization of Novel *Bacillus thuringiensis* *cry1*-type Genes with Improved Insecticidal Activities

나비목 해충에 대한 살충활성이 향상된

신규 ***Bacillus thuringiensis cry1*-type** 유전자의 제작 및 특성 연구

By

Song-Eun Kim

Major in Entomology

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Construction and Characterization of Novel *Bacillus thuringiensis* *cry1*-type Genes with Improved Insecticidal Activities

나비목 해충에 대한 살충활성이 향상된

신규 ***Bacillus thuringiensis cry1*-type** 유전자의 제작 및 특성 연구

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

by

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APPROVED AS A QUALIFIED THESIS OF SONG-EUN KIM
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ABSTRACT

Bacillus thuringiensis (Bt) is a gram-positive, sporulating bacterium that produces a variety of proteins, in the form of large crystalline (Cry) inclusions, which demonstrate toxicity to a variety of insect and nematode pests. Varieties of Cry proteins have been found around the world, which has been widely used as one of the most successful biological control agent. That is regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects and are used in Organic farming. Thus, *cry* genes encoding these Cry proteins have been widely applied for construction of transgenic crops resistant to pest insects.

In this study, to construct of novel *Bacillus thuringiensis cry1*-type genes for genetically modified crops and to select the most appropriate *cry* gene for transgenic crops with enhanced insecticidal activity as template. For effective expression in a plant, *cry1Ac* gene was modified to plant-preferring codon usage by multi site-directed mutagenesis. Through mutagenesis study for the Mod-Cry1Ac, 7 and 16 amino acid residues from domain I and II, respectively, responsible for its insecticidal activity against larvae of *Plutella xylostella , Spodoptera exigua* and *Ostrinia furnacalis* were identified. For this mutagenesis, 1857 bp of modified *cry1Ac* (Mod-cry1Ac) encoding active domain was used as a template and amplified by polymerase chain reaction with 16 mutagenic primers based on *cry1Ac* sequences. Consequentially, 31 different kinds of mutant cry1Ac genes with improved the insecticidal activity randomly changed 24 amino acid sequences into Cry1Ac were obtained.

For further characterization, these mutant *cry* genes were expressed as a fusion protein with polyhedrin using baculovirus expression system. These mutant *cry* genes encode potent insecticidal proteins in the form of crystalline protoxins of 95 kDa. SDS-PAGE analysis of the recombinant polyhedra revealed that expressed Cry proteins was occluded into polyhedra and activated stably to 65 kDa by trypsin. When the insecticidal activities of these mutant Cry proteins against to larvae of *P. xylostella, S. exigua* and *O. furnacalis* were assayed, they showed higher or similar insecticidal activity compared to those of Cry1Ac and Cry1C. In the bioassay of third-instar larvae of *P. xylostella*, LD_{50} values of these mutant Cry protein was lower about 5-fold than Mod-cry1A, the control Bt protein. In the bioassay of second-instar larvae of *S. exigua*, Mod-Cry1Ac showed no insecticidal activity, on the other hand not as effective as Cry1C but 8 Mut-Cry1Ac showed

insecticidal activity. Also, LD_{50} values of mutant Cry protein through quantitative bioassay was lower about 2-fold than Mod-cry1Ac about *O. furnacalis*. Through quantitative bioassay, several mutant *cry* genes showing higher insecticidal activity, such as Mut-N04, Mut-N06 and Mut-N16 to *P. xylostella* and *O. furnacalis*, might be expected as desirable *cry* genes for the introduction to genetically modified crops.

Key words: *Bacillus thuringiensis*, transgenic plants, *cry* gene, crystal protein, multi site-directed mutagenesis

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INTRODUCTION

Control of insect pests in agriculture and insect vectors of human infectious diseases is mainly accomplished using chemical insecticides. However, nearly all chemical insecticides have the potential to significantly alter ecosystems that are toxic to humans and animals and also concentrated in the food chain. Besides, chemical products provoked outbreak of resistant insect pests. For these reasons, biological insecticides have been recommended for the solution of chemical insecticides problems. The most successful biological insecticide for pests control is the *Bacillus thuringiensis* (Bt)-based insecticides, which presently is about 2% of the total insecticidal market (Raymond et al. 2010).

Bt is a gram-positive, soil-dwelling, spore-forming bacterium showing toxicity against larval stages of several insect orders. The action of Bt relies on insecticidal crystal (Cry) proteins produced during the sporulation phase (Bravo, Gill, and Soberón 2007). Cry proteins were designed to disrupt insect midgut tissues followed by septicemia. Upon ingestion, Cry proteins are solubilized and activated by proteolytic enzymes, which bind to specific receptors on the apical microvillus membrane of the epithelial midgut cell. Subsequent insertion of activated Cry proteins into the membrane leads to ruination of osmotic balance and ultimate lysis of epithelial cells, causing considerable injury to insect midgut and eventually, leading to larval death (de Maagd, Bravo, and Crickmore 2001, Wang et al. 2008, Whalon and Wingerd 2003). Because of these potent insecticidal

activity, Bt products was continuously developed, and widely used for insect pest control in agriculture and also against mosquitoes species.

Although Bt products have beneficial advantage as biological control agents, the conventional use of Bt insecticides have been faced with some limitations such as a narrow spectrum, a short shelf life and development of pest insect resistance. To overcome these problems, many researchers have tried to modify Bt strains and their insecticidal crystal protein-encoding gene (*cry* genes) using genetic manipulation for enhancement of their potency against target insects, faster effects and delay of resistance development (Aronson, Wu, and Zhang 1995, Kalman *et al*. 1995).

Since the first *cry* gene from *B. thuringiensis* was cloned, sequenced, and expressed in *Escherichia coli* in 1981 (Lecadet *et al.*, 1999). This provided the prospects for genetic engineering of *B. thuringiensis* as followed; 1) expression of a different *cry* genes in natural *B. thurigiensis* by transformation or conjugation, 2) expression of *cry* genes in alternative hosts such as *B. subtilis*, *Pseudomonas fluorescens*, plant colonizing bacteria and cyanobacteria, 3) transgenic insecticidal plants transformed with *cry* genes (Murphy and Stevens, 1992; Khasdan *et al.*, 2003; Gao *et al.*, 2004).

Recently, *cry* genes encoding these Cry proteins have been widely applied for construction of transgenic crops, which the aim to manufacture transgenic plant expressing Bt crystal protein is high dose expression of *cry* gene in crops and delay of resistance development of insect pests. Transgenic crops have set a precedent in that the biotech area has grown impressively every single year (James 2012). However, for the conversion of DNA nucleotide sequence to crop-preferring codon usage, Bt *cry* genes

have significant drawbacks; one is AT-rich sequence and the other is the relatively large sizes that they are usually about 1.8 kb in the size of truncated active domain. In this manner, it has been known that the modified Bt genes for transgenic crop limited to representative *cry* genes, such as lepidopteran specific *cry1A* and coleopteran-specific *cry3Aa* (Romeis *et al.*, 2006).

The objective of this study was to construct novel Bt *cry1*-type genes for genetically modified crops and to select the most appropriate *cry* gene for transgenic crops with enhanced insecticidal activity as template. For the variant novel *cry* genes, multi site-directed mutagenesis was performed, manufactured mutant genes were expressed as a fusion protein with polyhedrin using baculovirus expression system. Furthermore, insecticidal activity of Cry proteins was obtained through bioassay measurements against Lepidopteran pests. Based on the result of assay, the most suitable novel *cry* genes are selected for construction of genetically modified plants.

LITERATURE REVIEW

1. General characteristics of *Bacillus thuringiensis*

Bacillus thuringiensis (Bt) was first discovered by the Japanese biologist Ishiwata Shigetane in 1901, which was a previously undescribed bacterium as the causative agent of a disease afflicting silkworms. In 1911, the same bacterium was isolated by Ernst Berliner when he isolated a *Bacillus* species from the Mediterranean flour moth, *Ephestia kuehniella*, and named it after the province Thuringia in Germany where the infected moth was found (Roh et al. 2007, Sanahuja et al. 2011). In 1915, the existance of parasporal inclusions in Bt was recorded by Berliner, which was later demonstrated to be proteinaceous in nature and soluble in alkaline solution. The insecticidal properties of the crystal formations were discovered to be soluble in the alkaline digestive fluids of lepidopteran larvae and responsible for toxicity. Spores and crystalline insecticidal proteins produced by Bt have been used to control insect pests since the 1920s and are often applied as liquid sprays (Lemaux 2008). In 1981, strong evidence was reported that correlation existed between loss of crystalline protein production and specific plasmids by González et al (1981). In the same year, the first *cry* gene encording crystal proteins was cloned and expressed by Schnepf and Whiteley(1981). Until the present, 60,000 Bt have been collected by various industries in an effort 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 member of the *Bacillus cereus* group of gram-positive, entomopathology,

endospore-forming that spores of Bt can be isolated from diverse environments such as soil, fresh water, the rhizosphere, the phylloplane, grain dusts and from insects and insectivorous mammals (Raymond et al. 2010).

B. thuringiensis is a sporeforming Gram-positive bacterium capable of producing a number of toxins; insecticidal endotoxins, exotoxins, haemolysins and enterotoxins. Genetically, *B. thuringiensis* is related to *B. cereus* which produces toxins that cause gastroenteritis in humans, but *B. thuringiensis* typically produces one or more proteinaceous inclusions during sporulation as the only notable phenotypic difference (Kolsto *et al.*, 1990). These inclusions can be distinguished as distinctively shaped crystals by phase-contrast microscopy. The inclusions are composed of proteins known as crystal proteins, Cry protein, or δ-endotoxins, which are highly toxic to a wide variety of important agricultural and health-related insect pests. Due to their high specificity and their safety for the environment, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture and forestry and in the home. Insecticidal crystal proteins from *B. thuringiensis* have been used intensively as biopesticides for the several decades. It has been proposed that the rational use of *B. thuringiensis* toxins will provide a variety of alternatives for insect control and for overcoming with the problem of insect resistance to pesticides.

Bt strains are usually classified into serotypes according to their H flagellar antigenic determinants (de Barjac *et al.*, 1981; de Barjac, 1990) To date, up to 69 different serotypes and 13 sub-antigenic groups, giving 82 serovars, have been defined and ranked as subspecies (Lecadet *et al.*, 1999). While serotyping only reflects one characteristic of the species, it is the most common classification method used

throughout the world. However, the flagellar serotyping has limitations, proving unreliable as a predictor of insecticidal activity, even though the technique has greatly aided classification of isolates. For example, Bt subsp. *morrisoni* (H8a8b) includes isolates active against lepidopteran, coleopteran or dipteran insects.

2. Bt crystal proteins

1) Delta-endotoxins

Production of the parasporal body or crystal is the defining feature of Bt. The crystals are proteinaceous in nature and are composed of millions of crystal (Cry) or Cytolytic (Cyt) protein molecules. Production of the crystal(s) is generally concomitant with sporulation. During the process of sporulation, the majority of Bt strains produce crystalline inclusions that contain the insecticidal δ-endotoxins. The proteins comprising these crystals account for 20-30% of the total bacterial protein at sporulation (Boucias and Pendland, 1998). The shape of the crystal is variable depending in the toxins present in crystal and growth conditions, with diverse crystal morphologies sometimes occurring concurrently. Among natural isolates, the most common morphologies are bipyramidal and circular(round) (Martin and Travers 1989, Bernhard et al. 1997). The composite crystalline inclusions are comprised of monomeric protoxins. Degradation of the inclusions by proteolytic enzymes releases the smaller toxic proteins, δ-endotoxins. These vary between strains but in most cases Bt strains produce inclusions that contain a mixture of δ-endotoxins.

The toxins were originally classified into four classes (Höfte and Whiteley 1989), according to their amino acid sequence homology and insecticidal specificities. Type I cry genes encode proteins of 130 kDa, which are usually specific to lepidopteran larvae, type II genes encode for 70 kDa proteins that are specific to lepidopteran and dipteran larvae, and type III genes encode for 70 kDa proteins specific to lepidopteran and dipteran larvae, while type IV genes are specific to the dipteran larvae. The system was further extended to include type V genes that encode for proteins that are effective against lepidopteran and coleopteran larvae(Crickmore et al. 1998, Tailor et al. 1992).

Currently, the toxins are classified on the basis of only amino acid sequence homology, where each protoxin acquired a name consisting of the mnemonic Cry (or Cyt) and four hierarchical ranks consisting of numbers (e.g. Cry25Aa1), depending on its place in a phylogenetic tree. Thus, proteins with less then 45% sequence identity differ in primary rank (Cry1, Cry2, etc.), and 78% and 95% identity constitute the borders for secondary and tertiary rank, respectively. This system replaces the old nomenclature using roman numerals. The similarity in amino acid sequences of all known Cry proteins was taken as the only criterion for distributing them into classes and subclasses. Some classes, such as Cry6 and Cry15, do not show any significant homology with the rest of the Cry proteins.

In addition to Cry toxins, a second type of toxins, Cyt toxins, occurs. These are in the 25-28 kDa mass range and, based on amino acid sequence, are not related to Cry proteins. Two classes (Cyt1 and Cyt2) containing 9 Cyt toxins are known. Interestingly, *cry*-like genes were found recently in the anaerobic bacterium

Clostridium bifermentans. The gene products, Cry16A and Cry17A, showed a remarkable mosquitocidal activity, and this is also the first reported case of a secreted or excreted mosquitocidal toxin derived from an anaerobic bacterium (Barloy *et al.*, 1996).

2) Exoenzymes and exotoxins

2-1) β-exotoxin

The β-exotoxin (thuringiensin, thermostable toxin, fly toxin) is thermostable (70℃, 15 min) low molecular weight toxin and has a broad spectrum of activity, killing various lepidopterans, dipterans, hymenopterans, hemipterans, isopterans, orthopterans, nematodes and mites. β-exotoxin is a specific inhibitor of DNA-dependent RNA polymerases. At high dosages, it may affect the biosynthesis of proteins and DNA. (Sebesta and Horska, 1970; Beebee and Bond, 1973).

2-2) Haemolysins

Haemolysins, which lyse vertebrate erythrocytes, are important virulence factors in several vertebrate bacterial pathogens and are generally thought to be important factors for the establishment of systemic diseases in humans (Honda *et al.*, 1991; Matsuyama *et al.*, 1995).

2-3) Enterotoxins

Bt isolates have been found to produce *B. cereus*-diarrhoeal-type enterotoxins (Carlson *et al.*, 1994). In *B. cereus*, extracellular enterotoxin, 45 kDa protein in typical enterotoxin characteristic, causes diarrhea in higher animals (Asano *et al.*, 1997).

2-4) Exoenzymes

Exoenzymes play a role in its pathogenicity to insects, for example, the release of chitinase and protease by Bt has been proposed to disrupt the peritrophic membrane, providing access to the gut epithelium (Kumar and Venkateswerlu, 1998; Sampson and Gooday, 1998).

2-5) Vegetative insecticidal proteins

A new class of insecticidal toxin, vegetative insecticidal proteins (VIPs) has been isolated from Bt since vip3A was first reported by Estruch in 1996. Approximately 15% of Bt strains has Vip3 homologs, which has showed a wide spectrum of activities against lepidopteran insects, such as black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*) (Estruch *et al.*, 1996).

3. Structure of crystal protein

The tertiary structure of three crystal proteins, Cry3A, Cry1Aa and Cyt2A, has been solved by X-ray crystallography (Li *et al.*, 1991; Grochulski *et al.*, 1995; Li *et*

al., 1996). An analysis in the accompanying review demonstrates that Cry3A and Cry1Aa show about 36% amino acid sequence identity (Crickmore *et al.*, 1998). This similarity is reflected in their three-dimensional structures. However, The structures of three Cyt proteins have been solved, Cyt1Aa, Cyt2Aa and Cyt2Ba showing similar topology displaying a single α-β domain composed of two outer layers of α-helix hairpins wrapped around a β-sheet (Cohen et al. 2008, Cohen et al. 2011, Li, Koni, and Ellar 1996, Soberón, López-Díaz, and Bravo 2013). The α-helices have an amphiphilic character, with the hydrophobic residues packed against the β-sheet. Up to now, three families of Cyt proteins, Cyt1 and Cyt2, and Cyt3, have been identified (Crickmore et al. 2013). Cyt1 and Cyt2 are produced in vivo as protoxins and undergo activation by the removal of small portions of their N-termini and C-termini (Li, Koni, and Ellar 1996).

Cry3A and Cry1Aa possess three domains. Domain I consists of a bundle of seven antiparallel α-helices in which helix 5 is encircled by the remaining helices. Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology, arranged in a so-called β-prism fold. Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich with a "jelly roll" topology. Domain II, especially the highly variable loops in its apex, is involved in specific receptor binding as shown by mutagenesis studies (Schnepf *et al.*, 1998). More recently, domain III was involved in the recognition of the role in receptor binding and thus in insect specificity (Aronson *et al.*, 1995; Lee *et al.*, 1995). Following binding, at least part of domain I inserts into the membrane in an oligomer to form an aqueous pore

with other toxin molecules. Domain I resembles other pore-forming or membrane-translocation domains of bacterial toxins and membrane entry might start by insertion of a hydrophobic two-helix hairpin. In an umbrella-like model, base on mutational and biophysical studies, α-helices four and five of several toxin molecules in an oligomer make up the pore, with the rest of the protein spreading over the membrane surface (Gazit *et al.*, 1998).

4. Crystal proteins intoxication process

Two models have been reported to describe the crystal proteins intoxication process: pore-forming model and signaling pathway model.

1) Pore-forming model

The mode of action of Cry proteins (δ-endotoxins) has been mostly characterized using lepidopteran larvae as model, and the major role of Cry proteins is to disrupt the midgut epithelium barrier to facilitate bacterial invasion of the hemocole. Upon ingestion of the crystal protein by a susceptible insect, the alkaline pH and reducing conditions found in the midgut of lepidoperan larvae allow for solubilization of the protoxins. Solubilization of the protoxin molecules in the crystal renders them available to proteolysis (activation) to yield an active toxin core that is mostly resistant to further proteolysis (Bietlot et al. 1989). Activated toxins traverse the peritrophic matrix before reaching the midgut epithelium. While the size of the matrix pores would allow passage of Cry proteins (Adang and Spence 1983), the

chitinous matrix contains glycoproteins (Adang and Spence 1982) that may bind to Cry proteinsand reduce their passage. After traversing the peritrophic matrix, the Cry proteins bind to the brush border membrane of the midgut cells (Bravo et al. 1992). Reversible protein binding has been proposed to concentrate Cry toxins on the brush border membrane to facilitate irreversible binding (Pacheco et al. 2009). The irreversible binding component is considered synonymous in this region of the toxin insertion on the membrane and is directly correlated to toxicity (Liang, Patel, and Dean 1995). Irreversible binding is mostly dependent on domain I, as mutation in the region of the toxin result in reduced irreversible binding and toxicity (Hussain, Aronson, and Dean 1996). Toxin binding and concentration of aminopeptidase N (APN) and alkaline phosphatase (ALP) on specific membrane regions of a toxin pore that leads to cell death by osmotic shock.

Cyt toxins are generated as protoxins in the form of crystalline inclusions. Unlike lepidopteran-specific crystal proteins, this protein was soluble in alkaline conditions, and then proteolysis is processed by proteases in the mid-gut tracts of susceptible larvae. The conventional model for Cyt proteins suggests that the monomer undergoes conformational changes such that, upon membrane contact, the two outer α-helical layers swing away from the β-sheet that is inserted into the membrane. Oligomerization of Cyt monomers on the cell membrane forms β-barrel pores (Li et al. 2001, Parker and Feil 2005) that induce colloid-osmotic lysis, followed by a net influx of water, cell swelling, and eventual lysis (Bravo, Gill, and Soberón 2007). Cyt toxins induced rapid rounding up and swelling followed by membrane blebbing and cell lysis in mammalian and inset cell cultures, was hemolytic to erythrocytes

(Thammachat et al. 2008). For instance, the toxicity of Cyt1Aa is obtained upon alkaline solubilization and proteolysis from both the N- and the C-termini, which converts the protoxin into its active form (23-24 kDa). This active toxin is carried out by the insect gut proteases. Cyt1Aa's highly hydrophobic nature enable its interaction with unsaturated membrane phospholipids such as phosphatidylchloline, sphingomyeln and cholesterol (Cahan, Friman, and Nitzan 2008). Cyt1Aa forms pores 1-2 nm in diameter in the cell membrane, leading to cell lysis (Knowles et al. 1989).

2) Signaling pathway model

An alternative model proposed that binding provokes cell death in insect cells by activating a previously undescribed signaling pathway involving stimulation of the stimulatory guanine nucleotide-binding protein (G protein) and adenylyl cyclase (AC), increased cyclic adenosine monophosphate (García-Robles et al.) levels, and activation of protein kinase A (PKA). The toxicity of Cry proteins is due to the activation of a Mg^{+2}-dependent signal cascade pathway, PKA is the most key component among others in this pathway. Activated PKA alters downstream effectors that, in turn, actually dismantle the cell by destabilizing both the cytoskeleton and ion channels in the cell membrane. Such impairment of the structural and functional integrity of the cell leads to cell death as manifested by membrane blebbing and cellular swelling (Zhang et al. 2006). That Cry toxin of Bt exacerbate critical

intracellular signaling pathways through cadherin recetor coupled interactions has implications in pest insecticide development.

5. Genetically modified crops expressing Bt crystal protein

Genetically modified crops (GM crops) are plant used in agriculture, the DNA of which has been modified using genetic manipulation for improvement of chemical treatments resistance, enhancement of insecticidal activity, and delay of resistance development. In addition, transgenic plants include non-food crops such as biofuel, pharmaceutical agents, and other industrial products.

The first transgenic crop was constructed using an antibiotic-resistant tobacco in 1982 (Fraley et al. 1983). Moreover, genetically engineered plants with insect tolerance by expressing genes encoding for crystal proteins from Bt was produced in 1987 (Vaeck et al. 1987) and now including many major crop species, such as cotton, maize, potato, tobacco, rice, broccoli, lettuce, walnut, apple, alfalfa, and soybean. These plants are generically referred to as Bt Crops (i.e., Bt corn, Bt cotton, etc.).

An advantage of insect-resistant transgenic crops has brought great economic benefits to growers and significantly reduced the use of chemical insecticides, which are safe to natural environment and human health. GM crops are spreading more rapidly than any other agricultural technology in history, suggesting that farmers perceive important advantages in growing them. GM crops have been deployed for cultivation primarily in the USA, Brazil, Argentina, Canada, India, and China. During the period of 1996 to 2012, transgenic crops have been grown in accumulated hectarage of 3.53 billion hectares (8.72 billion acres). Most of the genetically

modified plants were herbicide-tolerant crops, and biotech crops with Bt genes has covered about 15% of the total transgenic acreage in the world. (James 2012).

However, the wide adoption of Bt crops also places a large selection pressure on the target insect populations and could result in them rapidly evolving a resistance to Bt toxin (Tabashnik et al. 2003, Yang et al. 2011). This is one of the most serious challenges faced y the use of Bt-transgenic crops in the field. In a number of instances, this insect resistance has been attributed to a reduction in the affinity of the proteolytically activated Cry protein to bind to the recepter of insect epithelial midgut cells (Ferré et al. 1991).

Environment Protection Agency (Gleave et al.) has established and generalized guide-line for the use of insecticidal Bt transgenic crops. On the other hand, various studies have been conducted for delay of resistance development such as imposition of high-performance gene by gene fusion technique. Moreover, researches are proceeding for discover renal function and high-efficiency of insecticidal *cry* gene.

MATERIALS AND METHODS

1. Construction of mutant *cry* genes

1) Cloning of pIM-*Mod-cry1Ac* vector

Modified *cry1Ac* gene was provided from Dr. Beom-Seok Park (National Institute of Agricultural Biotechnology, Korea). Modified *cry1Ac* gene was digested with *Eco*RV and *Bam*HI, and introduced to pIM vector digested with same restriction endonucleases, to generate pIM-*Mod-cry1Ac* (Fig. 1).

2) Construction of primers

For the mutation of modified *cry1Ac*, the alignment of amino acid sequence of the modified Cry1Ac was performed using MegAlign (DNASTAR Inc., USA). Sequence of primers was decided according to the codon usage in pooled sequences of Chinese cabbage genes (Table 1). All of the primers used for simultaneous mutagenesis were annealed to the same strand of the template plasmid, and the desired point mutation or degenerate codon was close to the middle of the primer with ~10–15 bases of template-complementary. Primers were designed between 25 and 45 bases in length a melting temperature (Tm) ≥ 75℃, which was calculated with QuikChange® Tm calculator, available online at http://www.stratagene.com. Primers for simultaneous mutagenesis should be added to the mutagenesis reaction in approximately equimolar amounts.

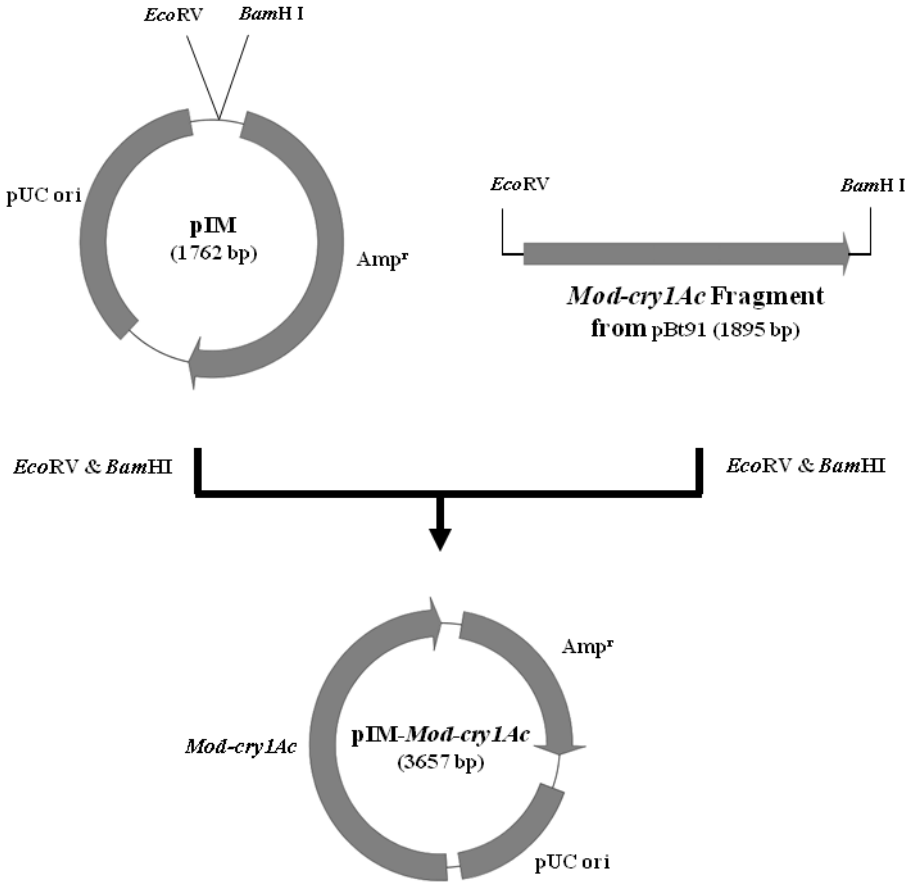


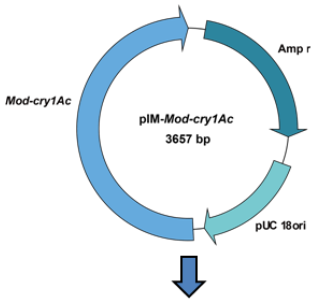
Fig. 1. **Construction map of vector, pIM-*Mod-cry1Ac*.** Modified *cry1Ac* gene was inserted into pIM to obtain the pIM-*Mod-cry1Ac* vector.

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

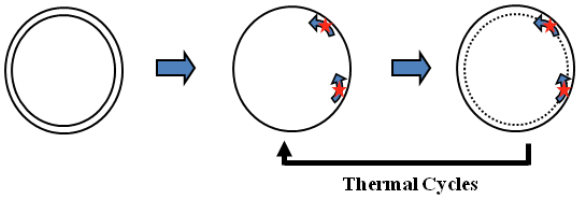
Amino acid	Codon	Preference, %	Amino acid	Codon	Preference, %
ARG (**R**)	CGA	9.4	ASP (**D**)	GAC	41.1
	CGC	13.7		GAU	58.9
	CGG	6.0	ILE (**I**)	AUA	18.5
	CGU	20.5		AUC	45.7
	AGA	25.6		AUU	35.8
	AGG	24.8	SER (**S**)	UCA	19.4
PRO (**P**)	CCA	32.5		UCC	14.7
	CCC	13.3		UCG	11.6
	CCG	19.2		UCU	19.4
	CCU	34.9		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

3) Mutating *mod-cry1Ac* gene through multi site-directed mutagenesis

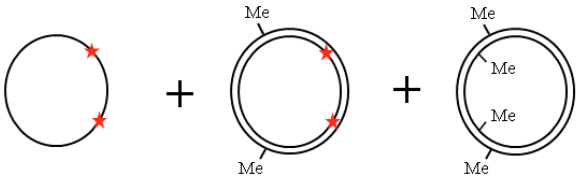
Mod-cry1Ac gene was mutated using the QuickChange® multi site-directed mutagenesis method (Stratagene, USA) (Fig. 2). Template DNA was purified (Qiagen, Germany) and replicated using enzyme blend including QuickChange® DNA polymerase (Stratagene, USA) as following cycling parameters; step 1, 95℃, 1 min; step 2, 95℃, 1 min; step 3, 55℃, 1 min; step 4, 65℃, 17 min (every successive cycle repeats steps 2 to 4, 34 times); step 5, 4℃, unlimited. All DNA amplifications were performed with the DNA Thermal Cycler (BIO-RAD, USA). The disintegration of methylated or hemimethylated template DNA were performed with *Dpn*I (New England Biolab, USA). Mutated single stranded-DNA was transformed into XL-10 Gold Ultracompetent cells (Stratagene, USA). Each mutant was verified by DNA sequencing using specific primers, Mod-cry1Ac-F (5'- ACCGACTACG CTGTTCG) and Mod-cry1Ac-R (5'- AATGTTGTTGCCAGAGC).



Step 1: Mutant Strand Synthesis (Thermal Cycling)



Step 2: *Dpn* I Digestion of methylated and hemimethylated Template DNA



Step 3: Transformation mutated ssDNA into XL-10-Gold Ultracompetent Cells

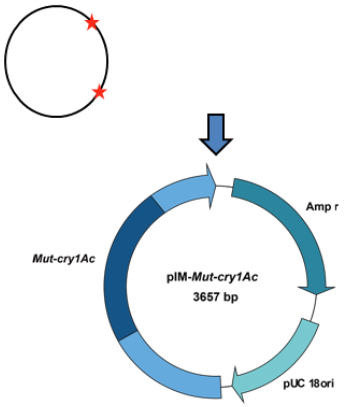


Fig. 2. Schematic diagram for multi site-directed mutagenesis.

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

1) 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, Korea) at 27℃. All insect cell lines were cultured at 27℃ and sub-cultured for every 4-5 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 bApGOZA (Je *et al.*, 2001) was used as a parental virus for construction of recombinant baculoviruses and a wild-type AcMNPV was used as a control virus.

2) Construction of baculovirus transfer vector

For the construction of baculovirus transfer vector expressing *mod-cry1Ac* gene, *mod-cry1Ac* gene was amplified from pIM-*Mod-cry1Ac* vector using specific primers, Mod1Ac-ATG-F containing *Xho*I-F at 5'-end (5'- AAACTCGAGATGGACAACAA CCCAA AC) and Mod1Ac-TAA-R containing *Eco*RI site at 5'-end (5'- TTTGAATT CTTAAAGATTGTACTCAGCCTC). The PCR-amplified *mod-cry1Ac* gene fragment was digested with *Xho*I and *Eco*RI and inserted into pOBI vector digested with same restriction endonucleases to obtain the pOB-*Mod-cry1Ac* (Fig. 3).

In order to construct baculovirus transfer vector, pOB-*Mut-cry1Ac* expressing mutant *cry* genes, mutant *cry1Ac* genes produced through mutagenesis were digested with *Xba*I and *Bgl*II for 821 bp of mutant region cassette fragment, and each cassette

fragment was introduced into pOB-*Mod-cry1Ac* digested with same restriction endonucleases (Fig. 4). Each mutant gene sequence in the constructed pOB-*Mut-cry1Ac* was verified by DNA sequencing using specific primers; Mut-seq-F (5'-ACCGACTACGCTGTTCG) and Mut-seq-R (5'- GGTCAC AGAGGCGTATC).

3) Expression of recombinant protein

The cell culture dish (60-mm diameter) seeded with 5×10^5 Sf9 cells were incubated at 27℃ for 1 h to let the cells attach to the footwall of dish. 500 ng of bApGOZA DNA, 2 ug of transfer vector DNA and 100 ul of the serum-free TC-100 medium were mixed in a polystyrene tube. Twenty ul of CellfectinII ™ (Invitrogen Co., USA) and 100 μl of the incomplete TC-100 medium were mixed in other polystyrene tube. The two solutions were gently mixed and the mixture was incubated at room temperature for 45 min. The attached cells were washed with 3 ml of the incomplete TC-100 medium, and refreshed with 2 ml of the same medium. The CellfectinII-DNA complexes were added dropwise to the media covering the cells while the dish was gently swirled. After incubation at 27℃ for 5 h, 3 ml of the TC-100 medium containing 30 ul antibiotics and 10% FBS were added to each dish and incubation at 27℃ was continued. At 5 days post infection (p.i.), the supernatant was harvested, clarified by centrifugation at 2,500 rpm for 15 min.

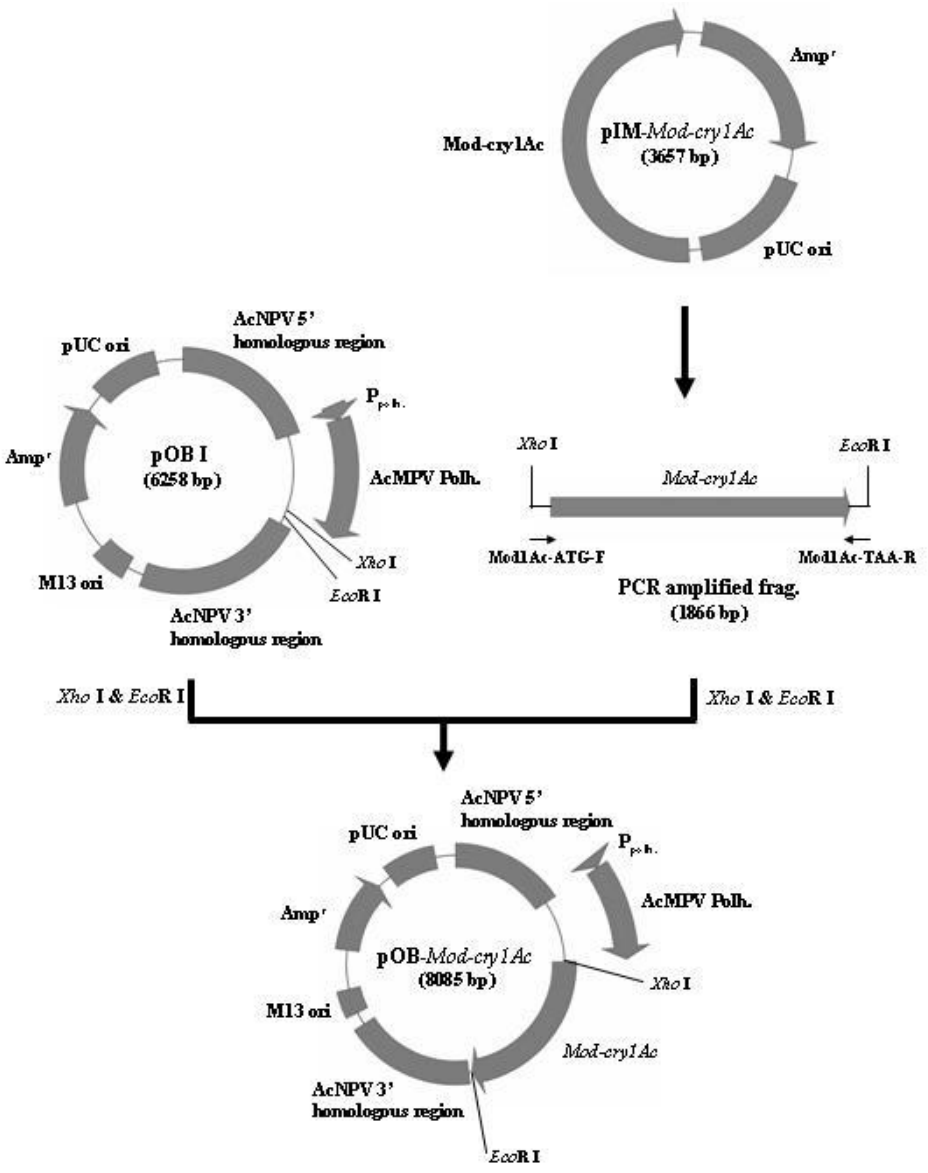


Fig. 3. **Construction map of transfer vector, pOB-*Mod-cry1Ac*, expressing modified *cry1Ac* with polyhedrin.** The PCR-amplified active fragment (about 1.8 kb) from the pIM-*Mod-cry1Ac* gene was cloned into the pOBI vector to give the plasmid pOB-*Mod-cry1Ac*. Solid arrows indicate primer position used in PCR amplification.

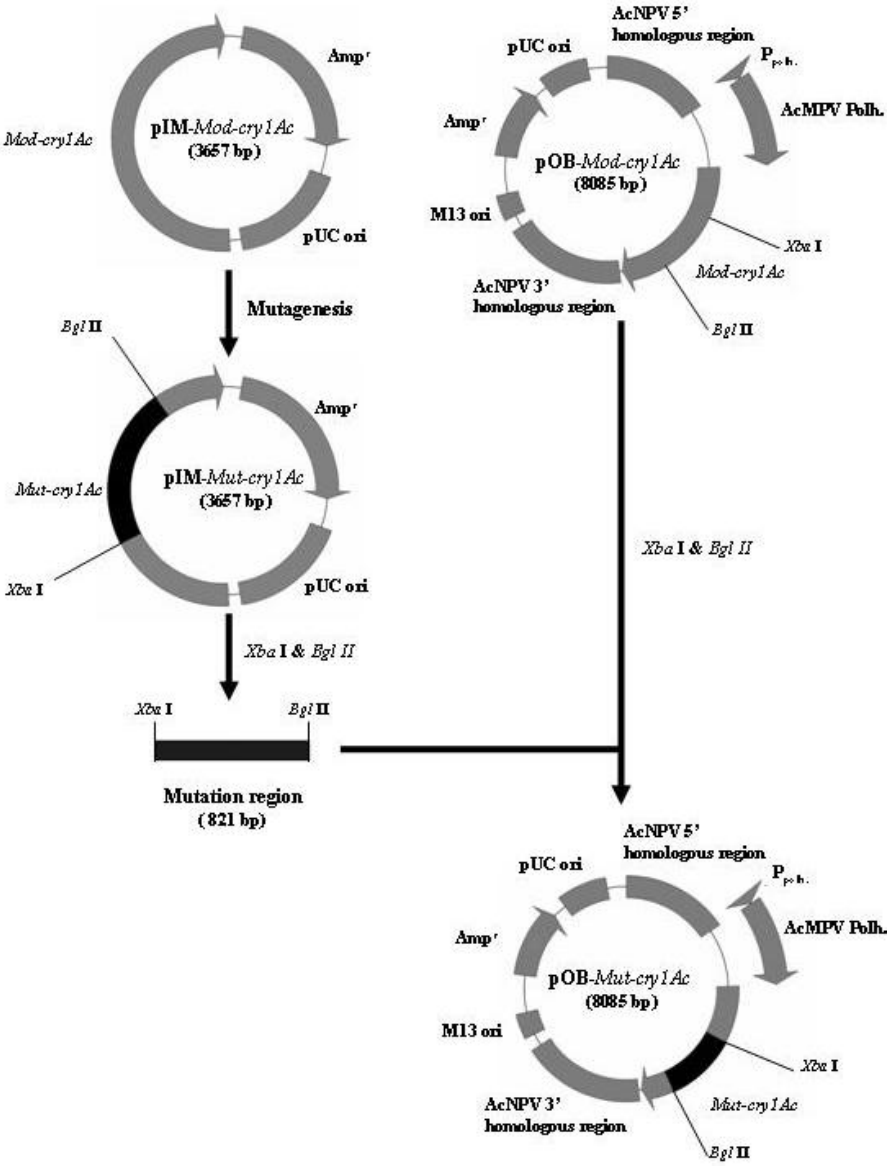


Fig. 4. **Construction map of transfer vector, pOB-*Mut-cry1Ac*, expressing mutant *cry1Ac* with polyhedrin.** The mutant cassette fragments (821 bp) digested with restriction endonucleases from the pIM-*Mut-cry1Ac* gene were inserted into the pOB-*Mod-cry1Ac* to obtain the transfer vector pOB-*Mut-cry1Ac.*

4) Production and purification of polyhedra

Wild-type or recombinant AcMNPV infected SF9 cells were harvested by centrifugation at 1000×g for 5 min. The resulting pellet was resuspended in 0.5% SDS (10 ml of 0.5% SDS per 2×10^8 cells) and centrifuged at 5000×g for 5 min and resuspended the pellet on the same volume of 0.5 M NaCl. The PIBs were pelleted by centrifugation at 5000×g for 5 min. and resuspended in a small volume of 1 M NaCl and 0.01% Triton X-100. To calculate the number of PIBs, PIB stocks were diluted and directly counted using hemocymeter.

5) SDS-PAGE

To analyze the fusion protein expression, Sf9 cells infected with the recombinant baculovirus were washed with Alkaline lysis buffer (0.1 M Na_2CO_3, 0.01 M EDTA, 0.17 M NaCl, pH 10.5) and were subjected to SDS-PAGE. For the analysis of active mutant Cry proteins, purified polyhedra were solubilized with alkaline lysis buffer, and then treated with trypsin at 37℃ for 2 h ~ overnight. Finally, samples treated with trypsin were mixed with an equal volume of 2×SDS-PAGE sample buffer (Sigma, USA), boiling at 100℃ for 10 min, each sample was loaded onto a 12% polyacrylamide separating gel with a 5% stacking gel, as described by Laemmli (Laemmli, 1970), and stained with Coomassie brilliant blue.

For the quantitative analysis of activated Cry proteins in recombinant polyhedra, the activated proteins subjected to 12% SDS-PAGE were determined by 1D-gel analysis system (Kodak Co., USA).

3. Determining toxicity of mutant Cry proteins

1) Screening Bioassay

The insecticidal activity of Mod-Cry1Ac and 8 mutants proteins was determined against to larvae of diamondback moth (*Plutella xylostella*), beet armyworm (*Spodoptera exigua*) and Asian corn borer (*Ostrinia furnacalis*). The recombinant polyhedra were treated on a disc of Chinese cabbage leaf (1.5×1.5 cm^2). For the three times, Ten 3rd-instar larvae of *P. xylostella* and ten 2nd-instar larvae of *S. exigua* were introduced to each leaf surface. To determine the activity against *O. furnacalis*, the recombinant polyhedra were treated on a small slice of artificial diet (2.0×2.0 cm^2) and thirty neonates were laid on each artificial diet. In *P. xylostella*, toxins were treated with 5 ng/larva and their mortality was scored 3 days after inoculation. In this case of *S. exigua*, toxins were treated with 300 ng/larva and their mortality was scored 5 days after inoculation. And about *O. furnacalis*, toxins were treated with 50 ng/larva and their mortality was scored 6 days after inoculation.

2) Quantitative bioassay

The quantitative bioassay of insecticidal activities was determined against to diamondback moth (*P. xylostella*), beet armyworm (*S. exigua*) and Asian corn borer (*O. furnacalis*). 5 different protein concentrations were provided. To determined the median lethal dose (LD_{50}) against *P. xylostella*, and *S. exigua*, , serial dilutions of recombinant polyhedra were treated on a disc of Chinese cabbage leaf pieces

(1.5 x 1.5 cm^2). For the three times, Ten 3rd-instar larvae of *P. xylostella* and ten 2nd-instar larvae of *S. exigua* were introduced to each leaf surface. 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 artificial diet. The mortality for *P. xylostella* was calculated by counting the dead larvae at 24 h interval for 2 days, for *S. exigua* every 24 h for 5 days and for *O. furnacalis* every 24 h for 6 days. And the LD_{50} was calculated by a Probit method (Russell *et al*., 1977) using SPSS statistics 21 (IBM., USA). All assays were performed at 25℃ in 60 to 70% humidity with a 16 h : 8 h light dark cycle.

RESULTS

1. Construction of mutant *cry* genes through multi site-directed mutagenesis

1) Construction of pIM-Mod-*cry1Ac* vector

For the mutagenesis of modified *cry1Ac* gene (1857 bp), modified *cry1Ac* gene digested with *Eco*RV and *Bam*HI was inserted into the pIM vector, a small size vector including pUC origin and ampicillin resistance gene, to construct the pIM-*Mod-cry1Ac* vector. The internal structure of this vector was verified by restriction endonuclease digestion (Fig. 5).

2) Primers for mutagenesis

To compare amino acid sequence of 8 mutant Cry with modified Cry1Ac, the alignment of amino acid sequences was performed. There were 24 residues located on domain I and domain II different from each other. Among them, Among the total 24 different residues, the 9^{th} and 10^{th}, the 12^{th} and 13^{th} residues were very close to share the same primer, so total ten primers were designed (Table 2) for changing amino acid into Mod-Cry1Ac, according to the codon usage of Chinese cabbage. For acquiring random various mutants, repeated mutagenesis using 3 to 5 primers was performed, and then totally 8 different kinds of mutant *cry1Ac* genes randomly

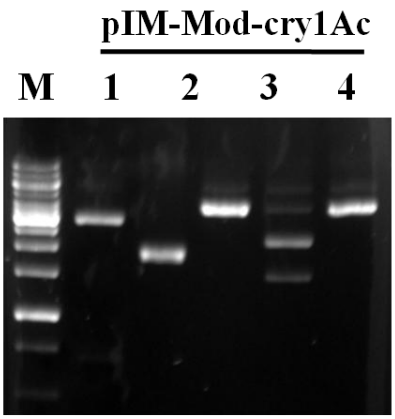


Fig. 5. Construction analysis of the vector, pIM-*Mod-cry1Ac*. The transfer vector pIM-*Mod-cry1Ac* was digested with various restriction endonucleases and electrophoresed on 0.8% agarose gel. Lanes, M, 1 kb DNA Ladder; 1, DraI; 2, EcoRI; 3, EcoRⅤ; 4, ScaI; 5, XbaI.

Table 2. Nucleotide sequences of primers used on *Mod-cry1Ac* for constructing *Mut-cry1Ac* genes.

Domain	Primer	Primer sequence[a] (5′→ 3′)	Converted position of amino acid (Sequence variation)
Domain I	Mod1ac E116A	GCAGAGAGCTTCAGA**GCT**TGGGAAGCCG	116(E→A)
	Mod1ac E128V	CCCAGCTCTCCGC**GTG**GAAA	128(E→V)
	Mod1ac A187T	GGGGATTCGATGCT**ACC**ACCATCAATAGCCG	187(A→T)
	Mod1ac V227I	CTGATTCTAGAGATTGG**ATC**AGATACAACCAGTTCAGG	227(V→I)
	Mod1ac A245S	CAGTTTTGGACATTGTG**TCT**CTCTTCCCGAAC	245(A→S)
	1F	ATTGTGTCTCTCTTC**AGC**AACTATGACTCCAGA	248(P→S)
	Mod1ac R254T	CCCGAACTATGACTCCAGA**ACC**TACCCTATCCGTAC	254(R→T)
Domain I I	S283M	GCTTCCGTGGT**ATG**GCCCAGGGTATCG	283(S→M)
	S283M& G286R	CCGTGGT**ATG**GCCCAG**A**G**G**ATCGAAAGATC	283(S→M) 286(G→R)
	G286R	CGTGGTTCTGCCCAG**A**G**G**ATCGAAAGATCC	286(G→R)
	A309V	GCATAACTATCTACACCGATG**TG**CACAGAGGATACTATTACTGGT	309(A→V)
	M322T& F324S	CTGGACACCAGATCA**CC**GCCT**C**TCCAGTTGG	322(M→T) 324(F→S)
	T334A	CCGGACCTGAGTTT**G**C**T**TTTCCTCTCTATGG	334(T→A)
	I375P	CCCTTCAATATCGGT**CCT**AACAACCAGCAAC	375(I→P)
	E412V	CGTTGATTCCTTGGACG**TG**ATCCCACCACAG	412(E→V)
	Q424A	GTGCCACCCAGG**GCT**GGATTCTCCCAC	424(Q→A)

[a]Underlined sequences indicate mutagenesis site.

changed 24 amino acid sequences into Cry1Ac were obtained. Fig 6 showed which region of amino acid was changed from modified Cry1Ac.

2. Expression of mutant cry genes using baculovirus expression system

bAcGOZA DNA was co-transfected with baculovirus transfer vectors into Sf9 cells (Fig. 7). The supernatant containing budded viruses was collected at 5 days post infection (p.i.), re-infected into a monolayer of Sf9 cells, and infected cells were harvested at 4 days post infection (p.i.). Expressed mutant proteins were named the Mut-N02, Mut-N04, Mut-N05, Mut-N06, Mut-N14, Mut-N16, Mut-N27, and Mut-N44 respectively. 8 mutant genes those domain I and domain II were randomly converted into Mod-cry1Ac. These genes were picked out for expression of mutant Cry proteins using baculovirus expressing system. PCR was performed to confirm the recombinant viruses containing the mutant *cry1Ac* genes and verification of genome structure of the recombinant AcMNPV expressing Mut-cry1Ac by PCR using specific primer sets (Fig. 8). RT-PCR was performed for verification of transcription of polyhedrin-Mut-cry1Ac fusion genes (Fig. 9). 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. 10).

In order to examine the expression of fusion protein by recombinant virus in Sf9 cells, the protein synthesis was initially analyzed by SDS-PAGE. Wild-type AcMNPV had an about 30 kDa polyhedrin protein, while recombinant virus mutants showed about 95 kDa fusion protein bands (Fig. 11A). To confirm whether the recombinant polyhedra would be cleaved into the active toxin by proteolytic enzymes and to quantify the amount of toxins, fusion proteins were treated with trypsin, one of the major proteolytic enzymes present in the mid-gut in insects and sequentially subjected to SDS-PAGE. The activated mutant Cry crystal protein bands all were exhibited about 65 kDa (Fig. 11B).

Activated Cry proteins were quantitative measured by using of 1D-gel analysis system (Kodak Co., USA) and used Bovine Serum Albumin (BSA) as the standard. As a result, the amount of activated Cry protein per $1x10^6$ polyhedral inclusion bodies (PIBs) was evaluated (Table 3).

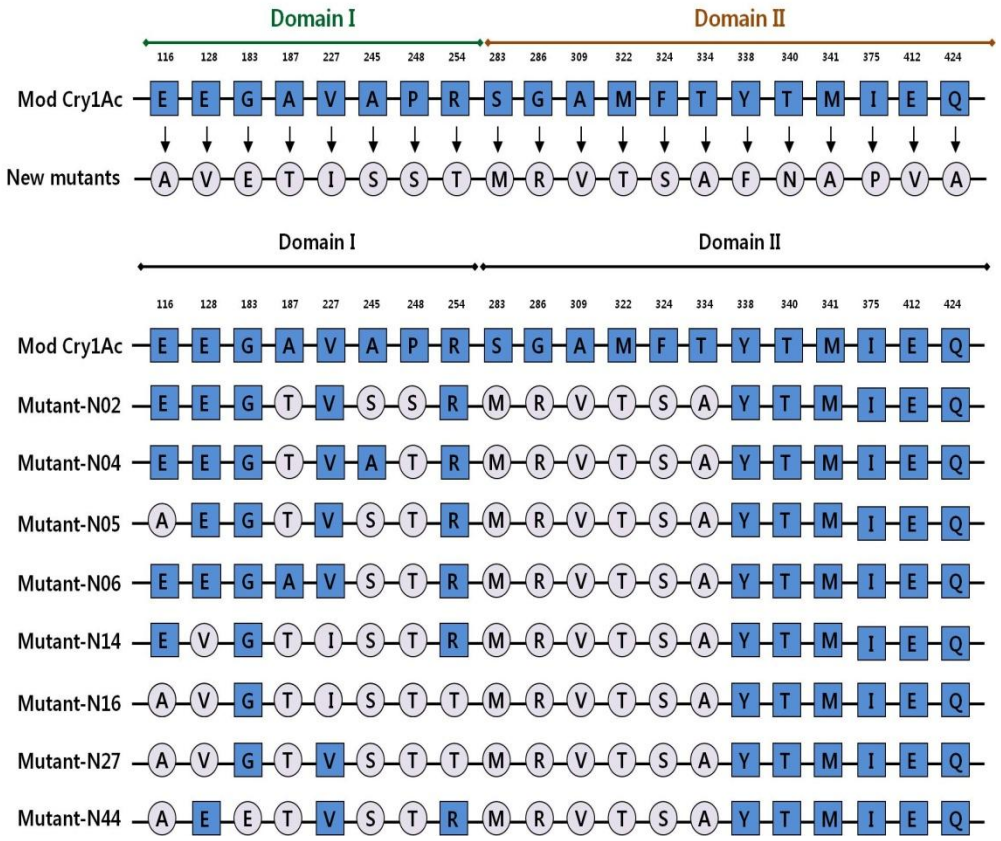


Fig. 6. Mutagenesis for expression of active proteins. The blue indicates the plant codon usage, the white indicates the bacteria codon usage. The rectangle represents the amino acid residues of Cry1Ac. The ellipse represents the amino acid residues of Cry mutant.

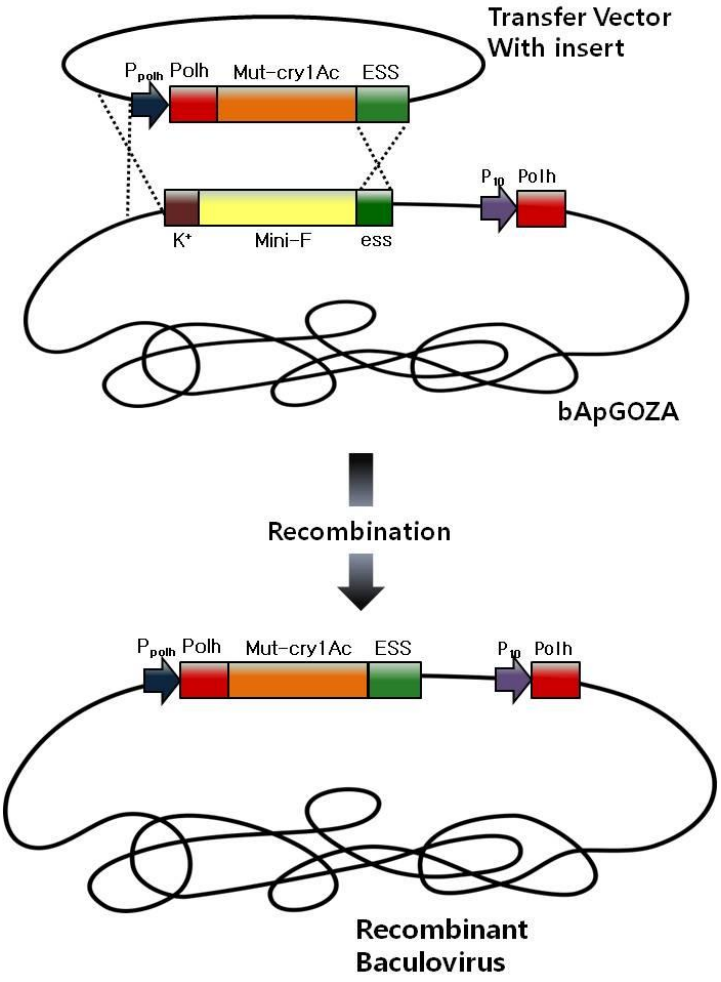


Fig. 7. Schematic diagram of construction of the recombinant baculoviruses expressing novel *Mod-cry1Ac* mutant genes.

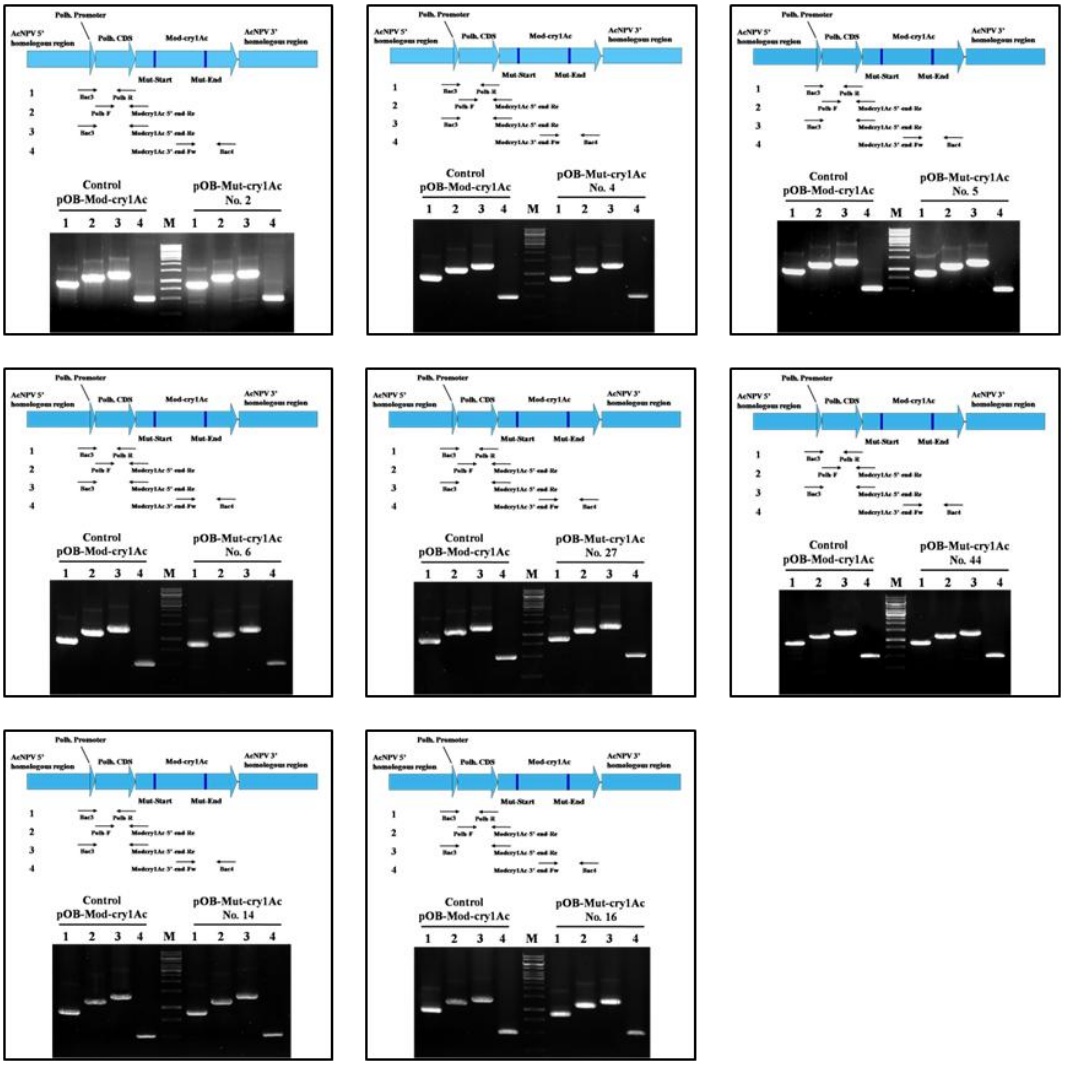


Fig. 8. Verification of genome structure of the recombinant AcMNPV expressing Mut-cry1Ac by PCR using specific primer sets. Solid arrows indicate primer positions used in PCR analysis. Lane: M, 100bp ladder; 1, primers Bac3 and Polh-R; 2, primers Polh-F and Modcry1Ac-5'-end-Re; 3, primers Bac3 and Modcry1Ac-5'-end-Re; 4, primers Modcry1Ac-3'-end-Fw and Bac4.

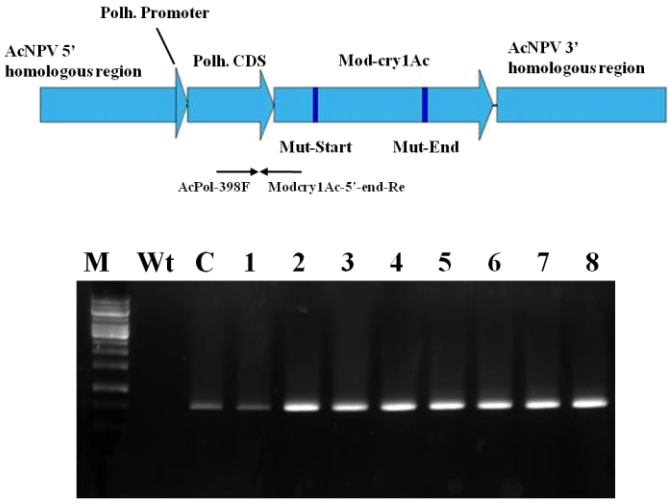


Fig. 9. Verification of transcription of polyhedrin-*Mut-cry1Ac* fusion genes from Sf9 cells infected with recombinant AcMNPVs expressing corresponding *Mut-cry1Ac* gene, respectively. Lane: M, 1kb ladder; Wt, wild-type AcMNPV; C, recombinant AcMNPV expressing *Mod-cry1Ac*; 1, recombinant AcMNPV expressing *Mut-cry1Ac* No.02; 2, recombinant AcMNPV expressing *Mut-cry1Ac* No.04; 3, recombinant AcMNPV expressing *Mut-cry1Ac* No.05; 4, recombinant AcMNPV expressing *Mut-cry1Ac* No.06; 5, recombinant AcMNPV expressing *Mut-cry1Ac* No.14; 6, recombinant AcMNPV expressing *Mut-cry1Ac* No.16; 7, recombinant AcMNPV expressing *Mut-cry1Ac* No.27; 8, recombinant AcMNPVexpressing *Mut-cry1Ac* No. 44.

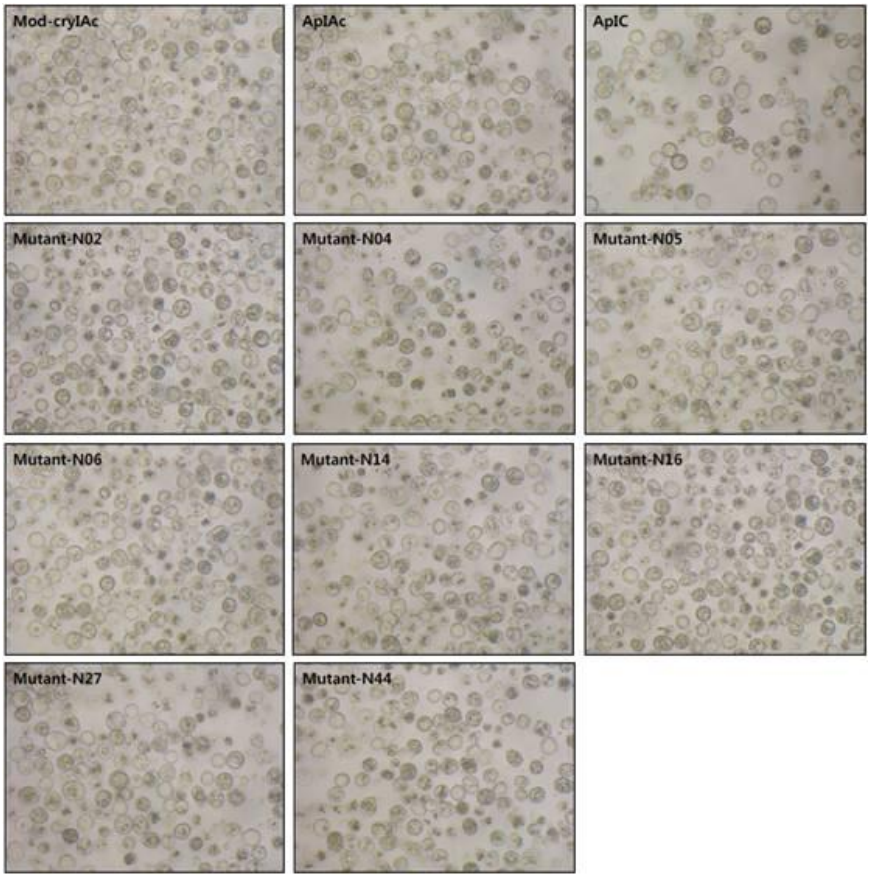


Fig. 10. Phase-contrast microscopy of Sf9 cells, which infected with the recombinant baculoviruses expressing novel *Mod-cry1Ac* mutant genes.

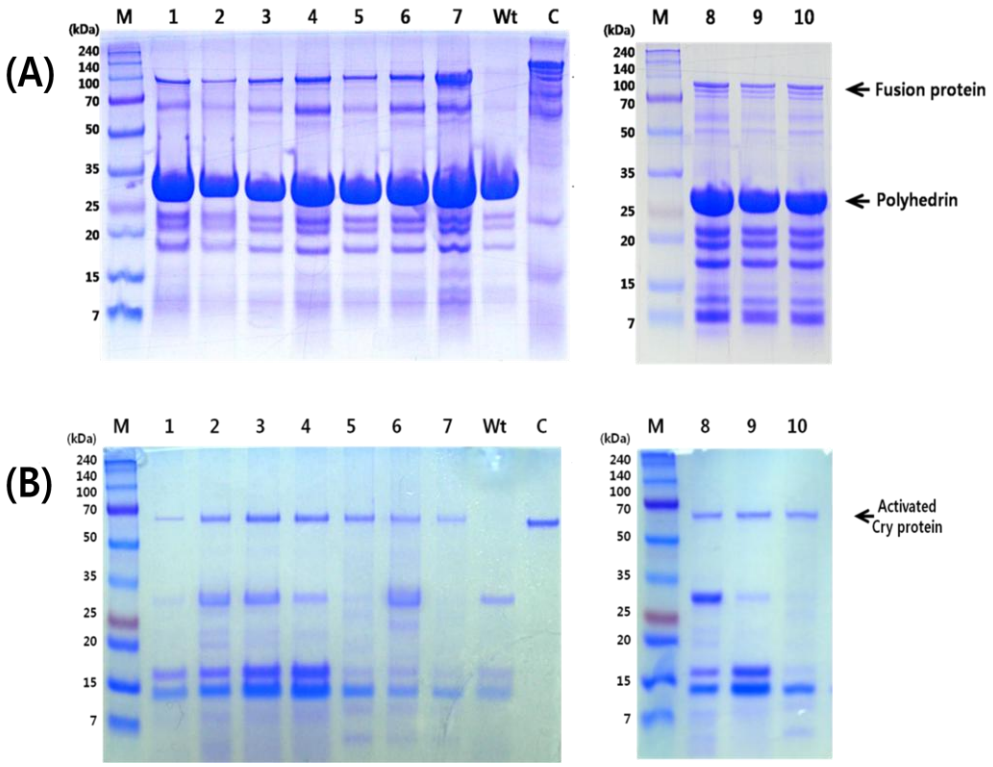


Fig. 11. SDS-PAGE analysis of polyhedra produced by the recombinant baculoviruses which containing novel Mod-cry1Ac mutant proteins in fusion with polyhedrin (A) and activated Cry protein (B) Lanes: M, protein molecular wight marker; 1, ApIAc; 2, ApIc; 3, Mutant N02; 4, Mutant N04; 5, Mutant N05; 6, Mutant N06; 7, Mutant N14; 8, Mutant N16; 9, Mutant N27; 10, Mutant N44; Wt, wild-type AcMNPV; C, Mod-Cry1Ac.

Table 3. Quantification of activated toxins of the novel Mut-Cry1Ac proteins which are occluded in the recombinant polyhedra infusion with polyhedrin

Mutant No.	Bt Toxin (ng/1x10^6 PIBs)	Mutant No.	Bt Toxin (ng/1x10^6 PIBs)
N02	**237.7**	**N14**	**427.4**
N04	**374.1**	**N16**	**550.3**
N05	**296.9**	**N27**	**386.8**
N06	**265.8**	**N44**	**255.9**

3. Expression Insecticidal activity of mutant Bt toxins

1) Bioassay

To evaluate the insecticidal activity of mutant Cry1Ac proteins, bioassays were performed against 3rd instar of *P. xylostella*, 2nd instar of *S. exigua* larvae and neonate of *O. furnacalis*, comparing with modified Cry1Ac and Cry1C as control.

Preliminary bioassay results about Lepidopteran pest (Fig. 12) were showed these mutant Cry proteins have higher or similar insecticidal activity compared to those of Cry1Ac and Cry1C. Based on the results of the experiment results, mutant Cry protein that showed improved pesticidal activity was selected. These mutant Cry proteins were Mut-N04, Mut-N06 and Mut-N16. The LD_{50} values were read representing the lethal dose for 50% larval mortality of *P. xylostella, S. exigua* and *O. furnacalis*. LD_{50} values of these Cry protein through quantitative bioassay was lower about 5-fold than Mod-cry1Ac and about 6-fold than Mut-N16 showing the highest insecticidal activity against *P. xylostella* (Table 4). In *S. exigua* bioassay, Mod-Cry1Ac showed no insecticidal activity, on the other hand not as effective as Cry1C but 8 Mut-Cry1Ac showed insecticidal activity (Table 5). Also, LD_{50} values of mutant Cry protein through quantitative bioassay was lower about 2-fold than Mod-cry1Ac and about 4-fold than Mut-N16 showing the highest insecticidal activity against *O. furnacalis* (Table 6).

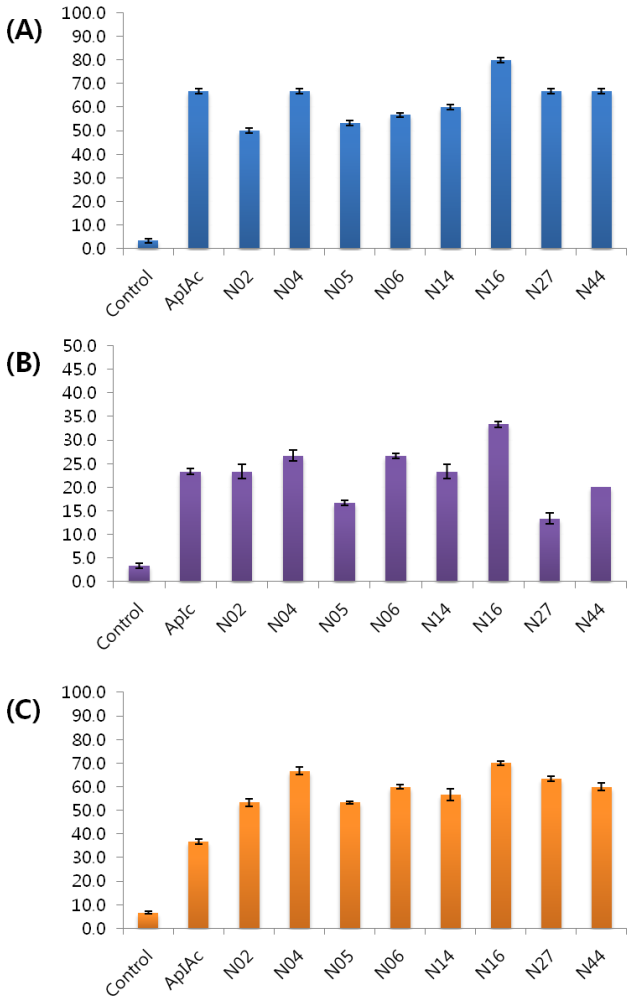


Fig. 12. Insecticidal activity of Mut-Cry1Ac proteins against 3rd instar larvae of *Plutella xylostella* (A), 2nd instar larvae of *Spodoptera exigua* (B), and neonates instar larvae of *Ostrinia furnacalis* (C). In *P. xylostella*, toxins were treated with 5 ng/larva and their mortality was scored 3 days after inoculation. In this case of *S. exigua*, toxins were treated with 500 ng/larva and their mortality was scored 5 days after inoculation. And about *O. furnacalis*, toxins were treated with 50 ng/larva and their mortality was scored 6 days after inoculation.

Table 4. Median lethal dose of mutant Cry proteins against third instar lavae of *P. xylostella*

	LD_{50} (ng/larva)	95% fiducial limits
Ap1Ac	**5.21**	**1.09-12.16**
Ap1C	**>50.00***	**ND**
N04	**0.86**	**0.35-1.81**
N06	**0.93**	**0.33-2.16**
N16	**0.79**	**0.36-1.57**

The mortality was scored 2 days after inoculation.
ND; Not determined0
* The mortality was 30% at 50 ng/larva.
LD_{50} was calculated by IBM SPSS Statistics 21

Table 5. Median lethal dose of mutant Cry proteins against second instar lavae of *S.exigua*

	LD_{50} (ng/larva)	95% fiducial limits
Ap1Ac	**>800.00***	**ND**
Ap1C	**226.88**	**70.64-322.91**
N04	**341.03**	**181.51-168.41**
N06	**321.31**	**182.86-432.41**
N16	**283.70**	**65.47-423.48**

The mortality was scored 5 days after inoculation.
ND; Not determined
* The mortality was 26.7% at 800 ng/larva.
LD_{50} was calculated by IBM SPSS Statistics 21

Table 6. Median lethal dose of mutant Cry proteins against neonates of *0. furnacalis*

	LD_{50} (ng/larva)	95% fiducial limits
Ap1Ac	**67.83**	**50.00-95.58**
Ap1C	**>100.00***	**ND**
N04	**38.77**	**16.65-100.75**
N06	**44.06**	**14.99-197.58**
N16	**15.91**	**5.79-29.30**

The mortality was scored 6 days after inoculation.
ND; Not determined
* The mortality was 28.9% at 100 ng/larva.
LD_{50} was calculated by IBM SPSS Statistics 21

DISCUSSION

The most successful biological insecticide for pest control is the bacterium Bt-based insecticides, which presently is about 2% of the total insecticidal market (Raymond et al. 2010). However, Bt biological insecticides were faced with some limitations such as a narrow spectrum, a short shelf life, and development of pest insect resistance. To overcome these problems, many researchers have tried to modify Bt strains and their insecticidal crystal protein-encoding gene using genetic manipulation for enhancement of their potency against target insects, faster effects, and delay of resistance development. Also, Insect-resistant transgenic plants have an advantage of reduced need for conventional insecticides, providing benefits for human health and the environment. Genetic engineering allows the introduction of several desirable genes in a single event, and can reduce the time to introgress novel genes into elite background (Miller 1997). However, these native *cry* genes were found to be expressed poorly in plants. It was evident that the production of transgenic plants offering effective control over economically significant insect pests would require considerable improvement of *cry* gene expression. The failure of adequate *cry* gene expression in transgenic plants appeared to be due to the *cry* coding sequence, as the genes were transcriptionally fused to highly active promoters and leader sequences which had been used previously to successfully express other prokaryotic genes in plants (Gleave et al. 1998).

The objective of this study was to construct novel *Bacillus thuringiensis cry1*-type genes for genetically modified crops and to select the most appropriate *cry* gene for

transgenic crops with enhanced insecticidal activity. For acquiring various random mutants, repeated mutagenesis using three to five primers was performed, and then a total of eight different mutant *cry1Ac* genes randomly changed 24 amino acid sequences to obtain a series of *Mod-cry1Ac* genes. These genes were selected for expression of mutant Cry proteins using baculovirus expressing system. bApGOZA DNA was co-transfected with baculovirus transfer vectors into Sf9 cells, and transfected cells produced polyhedral inclusion bodies made of polyhedron mutant Cry1Ac fusion proteins. To evaluate the insecticidal activity of mutant Cry1Ac proteins, bioassays were performed against lepidopteran pest larvae, comparing with Mod-cry1Ac and Cry1C as control. As a result, constructed mutant Cry proteins have higher or similar insecticidal activity compared to those of Cry1Ac and Cry1C. Therefore, mutant Cry proteins were considered to have the potential for the efficacious biological insecticide. Especially, Mutant-N16 showed the highest insecticidal activity against to lepidopteran pest, which is considered to the appropriate *cry* gene for genetically modified crops.

The mode of action of previously studied Cry proteins (δ -endotoxins) has been mostly characterized using lepidopteran larvae model system, and the major role of Cry proteins is to disrupt the midgut epithelium barrier to facilitate bacterial invasion of the hemocoel. As a result of substitution of amino acid residue, Cry protein structure was influenced, which affected the mode of action of Cry proteins. For examples, site-directed mutagenesis of domain II loop residue has resulted in mutant toxins with increased insecticidal activity, in some cases. Domain II loop mutants with increased insecticidal activity was Cry1Ab toxin where mutations in loop 2

resulted in higher insecticidal activity against Gypsy moth (*Limantria dispar*). More importantly, the increased insecticidal activity correlated with increased binding affinities to brush border membrane vesicle (BBMV) isolated from Gypsy moth (Rajamohan et al. 1996). Similarly, it was shown that mutations of domain II loop regions in the coleopteran specific Cry3Aa resulted in enhanced toxicity to yellow mealworm (*Tenebrio molitor*). A triple domain II loop 1 mutant R345 showed ten-fold higher toxicity to yellow mealworm than Cry3Aa, and two-fold higher toxicity against Colorado potato beetle that correlated with two-fold higher binding affinity to Colorado potato beetle BBMV (Wu et al. 2000). Another example, mutant Cry3Aa toxin displayed decreased toxicity when compared to the wild type toxin, and impaired ability to compete CPB brush border membrane associated cleavage of an a disintegrin and metalloproteinase fluorogenic substrate. Although the proteolytic profile of mutant Cry3Aa toxins generated by brush border membrane associated proteases was similar to that of wild type Cry3Aa toxin, the metalloprotease inhibitor 1,10-phenanthroline was less efficient on the proteolysis of mutants than on that of the wild type toxin (García-Robles et al. 2012). As indicated above, the change of the insecticidal activity by site-directed mutagenesis of the epitope binding regions found in domain I and/or II has a lot of potential for selection of Cry toxins. Cry proteins with improved activity against different insect pests as shown by the different examples of Cry toxins with modifications in these amino acid regions that have resulted in toxins with enhanced insecticidal properties (Bravo et al. 2013).

In conclusion, 8 different kinds of mutant *cry1Ac* genes were constructed, and these gene were introduced into Sf9 cells to express mutant Cry proteins using

baculovirus expressing system. Through quantitative bioassay, several mutant *cry* genes were selected to develope genetically modified crops. To develope high throughput expression system for the expression of mutant *cry* gene with comprehension of the mode of action, this study should be useful means to provide the construction of mutant *cry* gene for transgenic crops.

LITERATURE CITED

Adang, Michael J, and Kemet D Spence. 1982. "Biochemical comparisons of the peritrophic membranes of the lepidopterans *Orgyia pseudotsugata* and *Manduca sexta*." *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* no. 73 (3):645-649.

Adang, MJ, and KD Spence. 1983. "Permeability of the peritrophic membrane of the Douglas fir tussock moth (*Orgyia pseudotsugata*)." *Comparative Biochemistry and Physiology Part A: Physiology* no. 75 (2):233-238.

Aronson, A. 1995. "The protoxin composition of Bacillus thuringiensis insecticidal inclusions affects solubility and toxicity." *Applied and environmental microbiology* no. 61 (11):4057-4060.

Aronson, Arthur I, Dong Wu, and Chunlin Zhang. 1995. "Mutagenesis of specificity and toxicity regions of a Bacillus thuringiensis protoxin gene." *Journal of bacteriology* no. 177 (14):4059-4065.

Ben-Dov, Eitan, Gal Nissan, Nir Pelleg, Robert Manasherob, Sammy Boussiba, and Arieh Zaritsky. 1999. "Refined, Circular Restriction Map of the *Bacillus thuringiensis* subsp. *israelensis* Plasmid Carrying the Mosquito Larvicidal Genes." *Plasmid* no. 42 (3):186-191.

Bernhard, K, P Jarrett, M Meadows, J Butt, DJ Ellis, GM Roberts, S Pauli, P Rodgers, and HD Burges. 1997. "Natural Isolates of *Bacillus thuringiensis*: Worldwide Distribution, Characterization, and Activity against Insect Pests." *Journal of Invertebrate Pathology* no. 70 (1):59-68.

Bietlot, H, PR Carey, C Choma, H Kaplan, T Lessard, and M Pozsgay. 1989. "Facile preparation and characterization of the toxin from Bacillus thuringiensis var. kurstaki." *Biochem. J* no. 260:87-91.

Bourgouin, Catherine, André Klier, and Georges Rapoport. 1986. "Characterization of the genes encoding the haemolytic toxin and the mosquitocidal delta-endotoxin of Bacillus thuringiensis israelensis." *Molecular and General Genetics MGG* no. 205 (3):390-397.

Bravo, Alejandra, Isabel Gómez, Helena Porta, Blanca Ines García-Gómez, Claudia Rodriguez-Almazan, Liliana Pardo, and Mario Soberón. 2013. "Evolution of Bacillus thuringiensis Cry toxins insecticidal activity." *Microbial Biotechnology* no. 6 (1):17-26.

Bravo, Alejandra, Sarjeet S Gill, and Mario Soberón. 2007. "Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control." *Toxicon* no. 49 (4):423-435.

Bravo, Alejandra, Koen Hendrickx, Stefan Jansens, and Marnix Peferoen. 1992. "Immunocytochemical analysis of specific binding of *Bacillus thuringiensis* insecticidal crystal proteins to lepidopteran and coleopteran mudgut membranes." *Journal of Invertebrate Pathology* no. 60 (3):247-253.

Bravo, Alejandra, Supaporn Likitvivatanavong, Sarjeet S Gill, and Mario Soberón. 2011. "*Bacillus thuringiensis*: A story of a successful bioinsecticide." *Insect biochemistry and molecular biology* no. 41 (7):423-431.

Cahan, Rivka, Hen Friman, and Yeshayahu Nitzan. 2008. "Antibacterial activity of Cyt1Aa from Bacillus thuringiensis subsp. israelensis." *Microbiology* no. 154 (11):3529-3536.

Choi, Jae Young, MS Li, HJ Shim, JY Roh, SD Woo, BR Jin, KS Boo, and YH Je. 2007. "Isolation and characterization of strain of Bacillus thuringiensis subsp. kenyae containing two novel cry1-type toxin genes." *Journal of microbiology and biotechnology* no. 17 (9):1498-1503.

Cohen, Shmuel, Shira Albeck, Eitan Ben-Dov, Rivka Cahan, Michael Firer, Arieh Zaritsky, and Orly Dym. 2011. "Cyt1Aa toxin: crystal structure reveals implications for its membrane-perforating function." *Journal of Molecular Biology* no. 413 (4):804-814.

Cohen, Shmuel, Orly Dym, Shira Albeck, Eitan Ben-Dov, Rivka Cahan, Michael Firer, and Arieh Zaritsky. 2008. "High-Resolution Crystal Structure of Activated Cyt2Ba Monomer from *Bacillus thuringiensis* subsp. *israelensis*." *Journal of molecular biology* no. 380 (5):820-827.

Crickmore, N., J. Baum, A. Bravo, D. Lereclus, K. Narva, K. Sampson, E. Schnepf, M. Sun, and D.R. Zeigler. *Bacillus thuringiensis toxin nomenclature* 2013. Available from http://www.btnomenclature.info/

Crickmore, Neil, DR Zeigler, J Feitelson, ESCHERICHIA Schnepf, J Van Rie, D Lereclus, J Baum, and DH Dean. 1998. "Revision of the nomenclature for the Bacillus thuringiensis pesticidal crystal proteins." *Microbiology and Molecular Biology Reviews* no. 62 (3):807-813.

De Maagd, RA, MS Kwa, H Van der Klei, Takashi Yamamoto, Bert Schipper, Just M Vlak, Willem J Stiekema, and Dirk Bosch. 1996. "Domain III substitution in Bacillus thuringiensis delta-endotoxin CryIA (b) results in superior toxicity for Spodoptera exigua and altered membrane protein recognition." *Applied and*

environmental microbiology no. 62 (5):1537-1543.

de Maagd, Ruud A, Alejandra Bravo, and Neil Crickmore. 2001. "How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world." *TRENDS in Genetics* no. 17 (4):193-199.

Donovan, William P, Judith C Donovan, and James T Engleman. 2001. "Gene Knockout Demonstrates That *vip3A* Contributes to the Pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*." *Journal of Invertebrate Pathology* no. 78 (1):45-51.

Ferré, Juan, Maria Dolores Real, Jeroen Van Rie, Stefan Jansens, and Marnix Peferoen. 1991. "Resistance to the Bacillus thuringiensis bioinsecticide in a field population of Plutella xylostella is due to a change in a midgut membrane receptor." *Proceedings of the National Academy of Sciences* no. 88 (12):5119-5123.

Fraley, Robert T, Stephen G Rogers, Robert B Horsch, Patricia R Sanders, Jeffery S Flick, Steven P Adams, Michael L Bittner, Leslie A Brand, Cynthia L Fink, and Joyce S Fry. 1983. "Expression of bacterial genes in plant cells." *Proceedings of the National Academy of Sciences* no. 80 (15):4803-4807.

García-Robles, Inmaculada, Camila Ochoa-Campuzano, Jorge Sánchez, Estefanía Contreras, M Dolores Real, and Carolina Rausell. 2012. "Functional significance of membrane associated proteolysis in the toxicity of *Bacillus thuringiensis* Cry3Aa toxin against Colorado potato beetle." *Toxicon*.

Gleave, Andrew P, Deepali S Mitra, Ngaire P Markwick, Bret AM Morris, and Lesley L Beuning. 1998. "Enhanced expression of the shape Bacillus thuringiensis cry9Aa2 gene in transgenic plants by nucleotide sequence modification confers resistance to potato tuber moth." *Molecular Breeding* no. 4 (5):459-472.

González Jr, JoséM, Howard T Dulmage, and Bruce C Carlton. 1981. "Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*." *Plasmid* no. 5 (3):351-365.

Guerchicoff, Alejandra, Rodolfo A Ugalde, and Clara P Rubinstein. 1997. "Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*." *Applied and environmental microbiology* no. 63 (7):2716-2721.

Höfte, H, and HR Whiteley. 1989. "Insecticidal crystal proteins of Bacillus thuringiensis." *Microbiological reviews* no. 53 (2):242-255.

Hussain, Syed-Rehan A, Arthur I Aronson, and Donald H Dean. 1996. "Substitution of Residues on the Proximal Side of Cry1A *Bacillus thuringiensis* δ-Endotoxins Affects Irreversible Binding to *Manduca sexta* Midgut Membrane." *Biochemical and biophysical research communications* no. 226 (1):8-14.

James, Clive. 2012. Global Status of Commercialized Biotech/GM Crops: 2012, ISAAA Brief No 44 ISAAA: Ithaca, NY, 2012. ISBN 978-1-892456-49-4,[Online], Available: http://www.isaaa.org/resources/publications/briefs/42/executivesummary/default. asp [07.03. 2012].

Kalman, Sue, Kristine L Kiehne, Nicole Cooper, Mitra Shahabi Reynoso, and Takashi Yamamoto. 1995. "Enhanced production of insecticidal proteins in Bacillus thuringiensis strains carrying an additional crystal protein gene in their chromosomes." *Applied and environmental microbiology* no. 61 (8):3063-3068.

Knowles, Barbara H, Michael R Blatt, Mark Tester, Jane M Horsnell, Joe Carroll, Gianfranco Menestrina, and David J Ellar. 1989. "A cytolytic δ-endotoxin from *Bacillus thuringiensis* var. *israelensis* forms cation-selective channels in planar lipid bilayers." *FEBS letters* no. 244 (2):259-262.

Lemaux, Peggy G. 2008. "Genetically engineered plants and foods: a scientist's analysis of the issues (Part I)." *Annu. Rev. Plant Biol.* no. 59:771-812.

Li-Ming, Dou, Han Lan-Lan, Zhang Jie, He Kang-lai, Zhao Kui-Jun, Huang Da-Fang, and Song Fu-Ping. 2008. "Cloning, expression and activity of cry1Ia gene from Bacillus thuringiensis isolate." *Chinese Journal of Agricultural Biotechnology* no. 5 (1):49-54.

Li, J, DJ Derbyshire, B Promdonkoy, and DJ Ellar. 2001. "Structural implications for the transformation of the Bacillus thuringiensis delta-endotoxins from water-soluble to membrane-inserted forms." *Biochemical Society Transactions* no. 29 (Pt 4):571-577.

Li, Jade, Pandelakis A Koni, and David J Ellar. 1996. "Structure of the Mosquitocidal δ-Endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and Implications for Membrane Pore Formation." *Journal of molecular biology* no. 257 (1):129-152.

Liang, Yizhi, Smita S Patel, and Donald H Dean. 1995. "Irreversible binding kinetics of Bacillus thuringiensis CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity." *Journal of Biological Chemistry* no. 270 (42):24719-24724.

Manasherob, Robert, Mark Itsko, Nadine Sela-Baranes, Eitan Ben-Dov, Colin Berry, Shmuel Cohen, and Arieh Zaritsky. 2006. "Cyt1Ca from Bacillus thuringiensis subsp. israelensis: production in Escherichia coli and comparison of its biological

activities with those of other Cyt-like proteins." *Microbiology* no. 152 (9):2651-2659.

Martin, Phyllis AW, and Russell S Travers. 1989. "Worldwide abundance and distribution of Bacillus thuringiensis isolates." *Applied and Environmental Microbiology* no. 55 (10):2437-2442.

Miller, Lois K. 1997. *The baculoviruses*: Springer.

Pacheco, Sabino, Isabel Gómez, Ivan Arenas, Gloria Saab-Rincon, Claudia Rodríguez-Almazán, Sarjeet S Gill, Alejandra Bravo, and Mario Soberón. 2009. "Domain II loop 3 of Bacillus thuringiensis Cry1Ab toxin is involved in a "ping pong" binding mechanism with Manduca sexta aminopeptidase-N and cadherin receptors." *Journal of Biological Chemistry* no. 284 (47):32750-32757.

Pardo-Lopez, L, C Munoz-Garay, H Porta, C Rodríguez-Almazán, M Soberón, and A Bravo. 2009. "Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*." *Peptides* no. 30 (3):589-595.

Parker, Michael W, and Susanne C Feil. 2005. "Pore-forming protein toxins: from structure to function." *Progress in biophysics and molecular biology* no. 88 (1):91-142.

Rajamohan, Francis, Oscar Alzate, Jeffrey A Cotrill, April Curtiss, and Donald H Dean. 1996. "Protein engineering of Bacillus thuringiensis δ-endotoxin: mutations at domain II of CryIAb enhance receptor affinity and toxicity toward gypsy moth larvae." *Proceedings of the National Academy of Sciences* no. 93 (25):14338-14343.

Raymond, Ben, Paul R Johnston, Christina Nielsen-LeRoux, Didier Lereclus, and Neil Crickmore. 2010. " *Bacillus thuringiensis*: an impotent pathogen?" *Trends in microbiology* no. 18 (5):189-194.

Roh, Jong Yul, Jae Young Choi, Ming Shun Li, Byung Rae Jin, and Yeon Ho Je. 2007. "Bacillus thuringiensis as a specific, safe, and effective tool for insect pest control." *Journal of microbiology and biotechnology* no. 17 (4):547-559.

Sanahuja, Georgina, Raviraj Banakar, Richard M Twyman, Teresa Capell, and Paul Christou. 2011. "Bacillus thuringiensis: a century of research, development and commercial applications." *Plant biotechnology journal* no. 9 (3):283-300.

Schnepf, E, N Crickmore, J Van Rie, D Lereclus, J Baum, J Feitelson, DR Zeigler, and DH Dean. 1998. "*Bacillus thuringiensis* and its pesticidal crystal proteins." *Microbiology and molecular biology reviews* no. 62 (3):775-806.

Soberón, Mario, Jazmin A. López-Díaz, and Alejandra Bravo. 2013. "Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms." *Peptides* no. 41 (0):87-93. doi: http://dx.doi.org/10.1016/j.peptides.2012.05.023.

Tabashnik, Bruce E, Yves Carrière, Timothy J Dennehy, Shai Morin, Mark S Sisterson, Richard T Roush, Anthony M Shelton, and Jian-Zhou Zhao. 2003. "Insect resistance to transgenic Bt crops: lessons from the laboratory and field." *Journal of economic entomology* no. 96 (4):1031-1038.

Tailor, Ravi, Jan Tippett, Graham Gibb, Stephen Pells, Linda Jordan, and Susan Ely. 1992. "Identification and characterization of a novel *Bacillus thuringiensis* δ-endotoxin entomocidal to coleopteran and lepidopteran larvae." *Molecular Microbiology* no. 6 (9):1211-1217.

Thammachat, Siriya, Wanwarang Pathaichindachote, Chartchai Krittanai, and Boonhiang Promdonkoy. 2008. "Amino acids at N-and C-termini are required for the efficient production and folding of a cytolytic delta-endotoxin from Bacillus thuringiensis." *BMB. Rep* no. 41:820-825.

Thomas, WENDY E, and DAVID J Ellar. 1983. "*Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*." *Journal of Cell Science* no. 60 (1):181-197.

Vaeck, Mark, Arlette Reynaerts, Herman Höfte, Stefan Jansens, Marc De Beuckeleer, Caroline Dean, Marc Zabeau, Marc Van Montagu, and Jan Leemans. 1987. "Transgenic plants protected from insect attack." *Nature* no. 328:33-37.

Valicente, Fernando H, and André HC Mourão. 2008. "Use of by-products rich in carbon and nitrogen as a nutrient source to produce *Bacillus thuringiensis* (Berliner)-based biopesticide." *Neotropical Entomology* no. 37 (6):702-708.

Wang, G, J Zhang, F Song, A Gu, A Uwais, T Shao, and D Huang. 2008. "Recombinant *Bacillus thuringiensis* strain shows high insecticidal activity against *Plutella xylostella* and Leptinotarsa decemlineata without affecting nontarget species in the field." *Journal of applied microbiology* no. 105 (5):1536-1543.

Whalon, Mark E, and Byron A Wingerd. 2003. "Bt: mode of action and use." *Archives of Insect Biochemistry and Physiology* no. 54 (4):200-211.

Wu, Sheng-Jiun, C Noah Koller, Deborah L Miller, Leah S Bauer, and Donald H Dean. 2000. "Enhanced toxicity of *Bacillus thuringiensis* Cry3A δ-endotoxin in coleopterans by mutagenesis in a receptor binding loop." *FEBS letters* no. 473

(2):227-232.

Yang, Zhou, Hao Chen, Wei Tang, Hongxia Hua, and Yongjun Lin. 2011. "Development and characterisation of transgenic rice expressing two *Bacillus thuringiensis* genes." *Pest management science* no. 67 (4):414-422.

Zhang, Xuebin, Mehmet Candas, Natalya B Griko, Ronald Taussig, and Lee A Bulla Jr. 2006. "A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of Bacillus thuringiensis." *Science Signaling* no. 103 (26):9897.

Zhong, Chunying, Donghai Peng, Weixing Ye, Lujun Chai, Junliang Qi, Ziniu Yu, Lifang Ruan, and Ming Sun. 2011. "Determination of plasmid copy number reveals the total plasmid DNA amount is greater than the chromosomal DNA amount in *Bacillus thuringiensis* YBT-1520." *PloS one* no. 6 (1):e16025.

나비목 해충에 대한 살충활성이 향상된 신규 *Bacillus thuringiensis cry1*-type 유전자의 제작 및 특성 연구

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초록

Cry 단백질은 그람 양성균이며, 포자를 형성하는 세균 종인 *Bacillus thuringiensis* (Bt)의 살충성 단백질 그룹이다. 전세계에 걸쳐 분리 및 보고 되고 있는 다양한 Cry 단백질들은 오랜 기간 동안 인축과 자연 환경에 가장 안

전한 생물학적 방제 인자들 중 하나로 쓰여지고 있다. 또한, 최근에는 Cry 단백질을 encoding하는 유전자를 이용하여 해충에 저항성이 있는 형질전환 작물을 만들기 위해 *cry* 유전자를 작물에 도입하고 있다.

본 연구에서는 형질전환 작물에 적합하며 보다 향상된 살충성을 갖는 *cry-1* type 유전자를 제작하기 위하여 기존에 이미 식물형질 전환용으로 제작되어 있는 modified *cry1Ac* 유전자를 template으로 하여 multi site-directed mutagenesis를 수행하였다. Active domain을 encoding 하는 1857 bp 의 modified *cry1Ac*를 Mutagenesis하기 위해 식물 선호 codon usage을 바탕으로 제작된 16개의 mutagenic primers를 가지고 PCR을 수행하였다. 그 결과, domain Ⅰ 에서 8개의 residue, domain Ⅱ 에서 6개의 residues에 위치하는 총 14개 아미노산이 여러 조합으로 치환된 모두 8 종류의 다양한 mutant *cry* genes을 확보하였다.

Mutant Cry 단백질의 특성을 알아보기 위하여 8개 mutant *cry* genes을 baculovirus expression system을 이용하여 발현하여 정상적인 크기의 단백질이 생성됨을 확인하였으며, trypsin으로 activation된 mutant 단백질들은 SDS-PAGE 상에서 모두 65 kDa의 안정한 형태를 보여 주었다. 발현된 단백질들은 배추좀나방, 옥수수조명나방 및 파밤나방을 대상으로 하여 생물검정을 수행하였다. 3령 배추좀나방 유충에 대한 생물검정 결과, Mut-N04, Mut-N06 및 Mut-N16의 LD_{50} 값이 대조구 단백질인 Mod-Cry1Ac 보다 5-6배 낮았다. 갓 부

화한 옥수수조명나방에 대한 생물검정에서는 Mut-N04, Mut-N06 및 Mut-N16의 LD_{50} 값이 Mod-Cry1Ac 보다 약 2-4배 낮았다. 한편, 2령 파밤나방을 대상으로 한 생물검정에서 Mod-Cry1Ac은 살충성을 보이지 않았으나, Mut-N04, Mut-N06 및 Mut-N16의 LD_{50} 값은 Cry1C에 비해서는 비슷하거나 낮은 살충성을 보였다.

이상의 결과를 통해 배추좀나방, 파밤나방 및 옥수수조명나방과 같은 나비목 해충에 대해 높은 살충성을 가지는 Mut-N16과 같은 mutant *cry* genes을 선발하였고, 이 후에 선발된 유전자를 형질전환 작물에 도입함으로써 보다 효과적인 살충성 형질전환 작물 개발에 기여할 수 있을 것으로 생각된다.

검색어: *Bacillus thuringiensis*, **형질전환 작물**, *cry* gene, crystal protein, **베큘로바이러스발현계**, multi site-directed mutagenesis

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