African Journal of Biotechnology Vol. 11(86), pp. 15236-15240, 25 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3806
ISSN 1684-5315 ©2012 Academic Journals

Full Length Research Paper

Cry3A δ-endotoxin gene mutagenized for enhanced toxicity to spruce bark beetle in a receptor binding loop

Josef VLASÁK[1]*, Jindřich BŘÍZA[1], Daniela PAVINGEROVÁ[1], Roman MODLINGER[2], Miloš KNÍŽEK[2] and Jana MALÁ[2]

[1]Institute of Plant Molecular Biology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic.
[2]The Forestry and Game Management Research Institute, Jíloviště-Strnady 136, 156 04 Praha 5 – Zbraslav, Czech Republic.

Accepted 5 July, 2012

Bacillus thuringiensis Cry3A **gene was redesigned for high expression in Norwegian spruce and the sequence was slightly modified to allow for simple N- and C- terminal deletions and domain II loop 1 exchange for synthetic oligos. Modified Cry3A toxins from 13 variants of the synthetic gene were expressed in** ***Escherichia coli*** **BL21 and their toxicity on spruce bark beetle larvae was tested using spruce bark sandwiches. Mutant toxins with N-terminal deletion and loop 1 duplication showed increased toxicity.**

Key words: *Bacillus thuringiensis*, *Ips typographus*, *Picea abies*, resistance.

INTRODUCTION

Crystal proteins of *Bacillus thuringiensis,* known also as δ-endotoxins or Cry toxins, are long established as insecticidal agents safe for users and environment, which are widely used in agriculture throughout the world. The natural δ-endotoxins comprise a group of nearly 600 related proteins that are effective against many kinds of insects and also nematodes, mites, protozoans, etc. (Crickmore et al., 2011). Individual toxins, however, have rather narrow spectrum of toxicity with a given δ-endotoxin being active against only a few related species. Unfortunately, several important pests seem to be resistant to all known Cry toxins. One of them is spruce bark beetle, (*Ips typographus* L., *Scolytidae*) that attacks massively Norway spruce (*Picea abies* L.) woods in Europe, causing damage of sometimes disastrous dimensions. No specific δ-endotoxin was yet found and experimental attempts to control the pest by application of *Coleoptera*-specific Cry3A based products only proved low efficacy of commercially produced agents (Novotný and Turcani, 2000).

Mechanisms of δ-endotoxin toxicity are intensively studied with the hope that knowledge of biochemical and structural principles will help us to redesign δ-endotoxins to alter their activity and host range (Wu et al., 2000). To be insecticidal, most known δ-endotoxins must first be ingested by the insect, solubilized in the insect gut and proteolytically activated to form an active toxin (Schnepf et al., 1998). The proteases in the insect gut can play a role in toxicity and specificity by determining if and where the δ-endotoxin is processed. Unfortunately, the characteristics of bark beetle gut proteases are unknown. Results in other beetles show, however that extensive N-terminus deletions, up to amino acid 159 in beetle-specific Cry3A protein, retain the insecticidal activity (Carroll et al., 1989) or are perhaps prerequisite for toxicity. For example, Chen and Stacy (2006) inserted cleavage sites of gut protease from resistant insect *Diabrotica* sp. between amino acids 107 and 111 and achieved enhanced toxicity of modified Cry3A protein. Cleavage from the C-terminus of Cry3A is usually supposed to be deleterious for insecticidal activity but Chen and Stacy (2006) achieved enhanced toxicity also by

*Corresponding author. E-mail: vlasak@umbr.cas.cz. Tel: +420 387 775 514. Fax: +420 385 310 356.

Abbreviations: GMO, Genetically modified organism; **TOXst, TOXX,** synthetic Cry3A genes.

insertion of *Diabrotica* cleavage site between amino acids 536 and 542, allowing deletion of the last 60 amino acids of the Cry3A toxin in the insect gut. The processed toxin binds to specific receptors on the surface of the insects' mid-gut epithelium and subsequently integrates into the lipid bilayer of the brush border membrane. Ion channels then form disruption of the normal function of the midgut and eventually lead to death of the insect (Höfte and Whiteley, 1989). Solving of the three dimensional structure of Cry3A (Li et al., 1991) and similar δ-endotoxins enabled formulation of hypotheses regarding the function of the conserved structures of Cry toxins. Domain I, from residues 1 to 290, consisting of seven alpha helices, is probably responsible for pore formation in the insect gut membrane (Gazit and Shai, 1993), domain II, residues 291 to 500, containing three beta-sheets is responsible for the interaction with the gut receptor (Ge et al., 1991) and the C-terminal domain III, residues 501 to 644, is a beta-sandwich that has stabilizing effect and regulatory impact on ion channel activity (Chen et al., 1993).

Several attempts at engineering the coleopteran-active δ-endotoxins have been reported and most of them involve loop 1 of domain II, responsible for receptor binding. Van Rie et al. (1997) engineered Cry3A by randomly replacing amino acids thought to be important in solvent accessibility with the amino acid alanine. Some of these random replacements reportedly increased western corn rootworm (*Diabrotica virgifera virgifera* LeConte) toxicity. Other replacements in critical loops, however, resulted in disruption of receptor binding or structural instability (Wu and Dean, 1996). Wu et al. (2000) used the site-directed mutagenesis to replace residues R345, Y350 and Y351 in loop 1 and they achieved up to 10 times higher toxicity by deleting critical residues Y350, Y351 or replacing them with hydrophobic phenylalanine. The relative toxicity to different beetle species was also changed. This study objectives were to design and prepare synthetic *Cry3A* gene optimized for the high expression in Norway spruce, allowing simple N- terminal and C-terminal deletions to specified Cry3A regions to test the hypothesis that cleavage of N- and/or C-termini of the protein may enhance toxicity in insect species without specific proteases. At the same time, the sequence was slightly modified to allow simple exchange of critical 16 amino acids in a receptor binding loop 1 with synthetic oligos coding for alternative amino acids. From the original synthetic gene, *TOXX*, 12 modified genes were then prepared, cloned in *Escherichia coli* expression vectors, and modified δ-endotoxins were expressed, purified and tested on bark beetle larvae.

MATERIALS AND METHODS

Construction and synthesis of spruce optimized *Cry3A* genes, *TOXst* and *TOXX*

The sequence of beetle specific *B. thuringiensis* var. *tenebrionis* *Cry3A* gene (Sekar et al., 1987, GenBank J02978) was redesigned with the assistance of commercial firm (GeneArt, Regensburg, Germany) so that all 644 coded amino acids were preserved but the codon usage was adapted to the codon bias of *Picea abies*. In addition, regions of very high (>80%) or very low (<30%) GC content, internal TATA-boxes, ribosomal entry sites and cryptic splice donor/acceptor sites were avoided. Also, unique cloning sites NdeI overlapping Cry3A start codon and SacI behind the stop codon were constructed and the gene was commercially synthesized. Another modified gene, TOXX, starting with Met codon 48 of the original sequence (numbering throughout the paper refers to the original sequence of Sekar et al., 1987) and containing unique restriction sites allowing construction of N-terminal and C- terminal deletions, and also 18 codon deletion of loop 1 in domain II, was constructed in a similar way (Vlasák et al., 2010). The gene contains also the unique NcoI site overlaping both Met48 start codon and KOZAK sequence for eucaryotic translation (ACC*ATG*G), SacI behind the stop codon for cloning into pET *E. coli* expression vectors and flanking XbaI sites for cloning into plant expression vector. In addition, R345 codon was exchanged for A345, which should stabilize the modified protein in *E. coli* without affecting its toxicity (Wu et al., 2000), T41 (second amino acid in the final protein) was exchanged for G41 and S162 for V162 to faciliate N-terminal deletion, Q586 was changed V586 and a sequence coding amino acids Ser, Ala Gly, Ser was added to the very end of the *TOXX* gene to faciliate C-terminal deletion.

Construction of terminal deletions and loop 1 mutants and cloning into pET expression vectors

Unique sites for restriction enzymes creating flush ends were used for terminal deletions in synthetic *TOXX* gene using the techniques of molecular biology performed according to Sambrook et al. (1989): SfoI and PmlI for N-terminal deletion, MscI and AfeI for C-terminal deletion. 54 bp fragment comprising of domain II loop 1 was also deleted or duplicated using unique restriction sites AgeI and BspEI so that original amino acids sequence 346 to 361 FQPGYYGNDSFNYWSG was either deleted or reintegrated as tandem duplication. In some constructs, the fragment was exchanged for synthetic 48 or 54 bp oligos containing the same sequence but with critical codons Y350, Y351 deleted or changed to F350, F351. All constructions were performed in the original vecor pGA4 (GeneArt) and the resulting genes were recloned into *E. coli* expression vector pET-22b (Novagen, Merck) as NcoI-SacI (*TOXX* genes) or NdeI-SacI (TOXst) fragment.

Production of modified δ-endotoxins in *E. coli* BL21

E. coli BL21(DE3) /STRATAGENE #200131/ or BL21-Codon Plus(DE3)-RP /STRATAGENE #230255/ or BL21-CodonPlus (DE3)-RIL /STRATAGENE #230245/ competent cells were transformed with pET plasmids carrying standard and modified synthetic δ endotoxins. 2.5 ml of single colony overnight cultures of transformed clones were used to inoculate 50 ml of pre-warmed Luria Bertani (LB) media and cultivated at 37°C for about 90 min to OD 0.8. 50 µl of isopropyl-β-D-thio-galactoside (IPTG) (200 g/L) were then added (induction), temperature was lowered to 30°C and the culture was cultivated for another 1.5 or 2 h, taking aliquots every 0.5 h. Cells were harvested by centrifugation in Falcon tubes and 25 µl of B-PERII (Pierce) extraction buffer per ml of original culture were added. Cells were resuspended in B-PERII and lysed at room temperature which yielded in positive cultures thick suspension of inclusion bodies that were collected by centrifugation, resuspended in 30% of the original volume of B-PERII with lysozyme 2 mg/ml and incubated on ice for 15 min. Collecting, resuspending and incubation of inclusion bodies with lysozyme was repeated twice, inclusion bodies were washed with 20x diluted

B-PERII and then resuspended in 400 µl of water. Protein content was assayed by Bradford (1976) and all prepared suspensions were adjusted to 2 mg of protein/ml.

Purification of Cry3A crystal protein from the commercial insecticide Novodor FC

100 ml of Novodor FC (Valent Biosciences Corp.) was diluted with water to 1 L and crystals and cells were sedimented at 9000 x*g*, 12 min to remove additives. This washing step was repeated twice. Cry3A crystals in resuspended sediment (1 L) were purified according to Murray and Spencer (1996) using chloroform extraction and Whatman No.1 filtration. The resulting crystal suspension was concentrated by centrifugation in four 13 ml tubes for Beckman 70.1Ti rotor, resuspended in 11 ml water with 7 g of CsCl added and the crystals were purified by isopycnic CsCl centrifugation for 18 h at 60 000 rpm. The crystal zone was collected, dialyzed against water and crystal concentration was adjusted to 2 mg of protein/ml.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting of crude and purified δ-endotoxins

Electrophoresis was performed in OWL 10 x 10 cm elfo unit P8DS according to Laemmli (1970), with 4% PAGE stacking gel and 8% PAGE resolving gel, and proteins were detected by staining with SERVA blue G. For western blotting, proteins separated by SDS-PAGE were electroblotted onto a HYBOND-ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK), incubated with anti-Cry3B antibodies (Biosense Laboratories, Norway) and subsequently, with secondary peroxidase-labeled antibodies (Amersham Biosciences). Blots were stained using the ECL Plus system (Amersham Biosciences).

Spruce bark beetle toxicity bioassays

Spruce bark sandwiches were prepared according to Wermelinger and Seifert (1998) and Modlinger and Knizek (2010) that consisted of a piece of spruce bark 6 x 6 cm with eight holes 8 mm in diameter, 3 mm deep. 1 ml of TOX protein water suspension with 20 µg of protein was equally applied on the phloem surface and allowed to dry; water was used as a control. 2nd instar larvae of spruce bark beetle were then individually deposited in the phloem holes with the help of wet brush and covered with a glass plane, sandwiched with the bark using spring clamps. 10 larvae on one sandwich were used in each assay. Larvae mortality was checked on 1st, 4th and finally on 18th day; dead larvae from the 1st and 4th day and those showing no feeding were not included in the results. Feeding of larvae was minotored according to fresh excrements production. 14 TOX proteins were tested in two experiments, with five replicas each.

RESULTS

Expression of optimized *Cry3A* genes and their terminally deleted and loop 1 mutated variants in *E. coli* BL21

Norway spruce codon-optimized *Cry3A* gene (Sekar et al., 1987), *TOXst* was synthesized and integrated into *E. coli* BL21 expression vector pET-22b. Similar synthetic *TOXX* gene with slightly modified sequence (GenBank JN989558) allowing simple construction of terminal deletions and/or domain II loop 1 deletions and mutations was also integrated in pET-22b. *TOXst* gene was used as standard in all experiments but also TOXX can be considered a complete *Cry* gene though it starts with Met48 of the natural Cry3A, because Adang et al. (1993) and others had shown that the first 47 amino acids of Cry3A have no effect on Cry3A toxicity and Met 48 is probably used as an alternative start codon. Cloning experiments with TOXX yielded another 12 mutated *Cry3A* genes (Table 1).

The production of synthetic δ-endotoxins in *E. coli* BL21 (DE3) was rather low, but as shown already by Kumar et al. (2005), it was much improved when using the “Codon Plus” *E. coli* strains containing additional *tRNA* genes for efficient translation of rare *E. coli* codons AGA, AGG, CCC (RP cells) and still more with RIL cells containing additional tRNAs for AGA, AGG, ATA and CTA codons. These codons are preferred in plant systems and were abundantly present in optimized genes and their variants. Using Codon Plus RIL cells resulted in significant protein expression half an hour after induction which achieved its maximum between one and half and two hours when the reaction was stopped. All modified δ-endotoxines were isolated in the form of inclusion bodies that could be readily purified. Higher expression was repeatedly obtained for toxins with deleted N- terminal part (Figure 1). Isolated proteins were quantified using western blot with Cry3B antibodies using commercial Cry3B protein as standard, because Cry3A antibodies for blotting purposes were not available. 10 ng of both Cry3A and Cry3B proteins were detectable after blotting the PAGE gels (Figure 2), and 0.5 mg to 2 mg of modified δ-endotoxins could be prepared from 50 ml of *E. coli* culture.

Toxicity assays of modified Cry3A proteins on spruce bark beetle larvae

Modified Cry3 protein TOXX was less toxic than standard TOXst protein expressed in *E. coli* or purified Cry3A crystals produced by *B. thuringiensis* var. tenebrionis. However, the N-terminal deletion of 112 amino acids increased the toxicity of TOXX to the level of TOXst or higher in some cases (Table 1). Deletion of critical residues Y350, Y351 in loop 1 that was suggested by Wu et al. (2000) as a way of increasing Cry3A toxicity to *Tenebrio molitor* showed some additional increase of toxicity that was though comparable with the unexpected toxic effect of the whole loop 1 deletion. Exchange of Y350, Y351 for more hydrophobic F350, F351 that was most efficient in *T. molitor* experiments (Wu et al., 2000) was moderately effective against *Ips typographus*. On the other hand, heterodimers of loop 1FF and loop 1YY (standard) showed surprisingly high toxicity, especially in N-terminal deleted TOX protein (Table 1). Due to a rather

Table 1. Synthetic Cry3A (TOXX) terminal deletions and loop 1 mutants and toxicity assays against *I. typographus* larvae.

Mutant	Construction	Expression in *E. coli*	Dead[e] (%)	Relative toxicity[f]
TOXst	Standard Cry3A	++	55±9	1
Cry3A crystals, purified	Standard Cry3A	-	59±24	1.07
TOXX	Modified Cry3A	++	25±16	0.45
NdTOXX	Δ1(A50-H161)[a]	+++	55±13	1
TOXXCd	Δ2(Y587-N644)[b]	+++	25±16	0.45
TOXX-Δloop 1	Δ3(F346-G361)	++	50±10	0.91
NdTOXX-Δloop 1	Δ1+Δ3	+++	63±21	1.14
TOXX-loop 1ΔYY	Δ4(Y350-Y351)[c]	++	54±9	0.98
NdTOXX-loop 1ΔYY	Δ1+Δ4	+++	67±9	1.21
TOXX-loop 1FF	Y350-F, Y351-F[d]	++	45±24	0.82
NdTOXX-loop 1FF	Δ1, Y350-F, Y351-F	+++	51±18	0.93
TOXX-(loop 1)2	Loop 1 homodimer	++	28±15	0.51
NdTOXX-(loop 1)2	Δ1, loop 1 homodimer	++++	49±15	0.89
TOXX-(loop 1FF-loop 1)	Loop 1 heterodimer	++	43±17	0.78
NdTOXX-(loop 1FF-loop 1)	Δ1, loop 1 heterodimer	+++	80±13	1.45

[a]N-terminal deletion from amino acid 3 to 114 of the TOXX protein (A50-H161 in the original sequence of Sekar et al., 1987); [b]C-terminal deletion from amino acid 540 (Y587 Sekar et al., 1987); [c]deletion of Y350, Y351 in loop 1; [d]both Y350 and Y351 in loop 1 exchanged for F; [e]% of feeding larvae found dead after 18 days ± standard deviation; [f]Control sample with applied water showed dead (%) 13 ± 14 (relative toxicity 0.23).

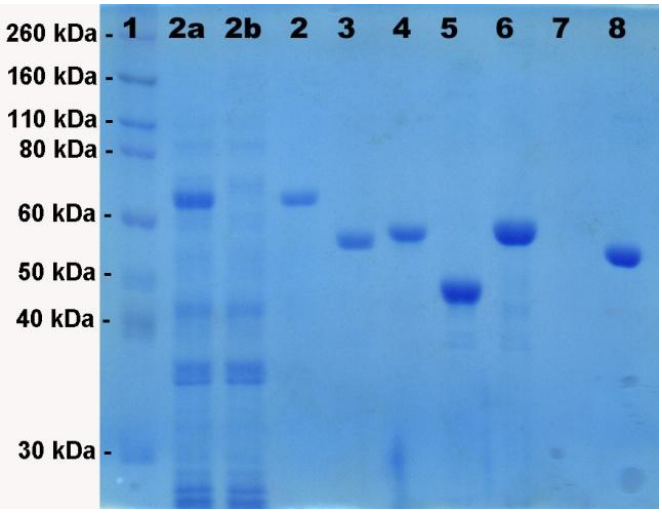

Figure 1. Production of TOXX and its mutants in *E. coli.* SDS-PAGE gel of selected samples is shown with 20 µg of protein loaded in lanes 2 to 8. Lane 1, protein standards Novex (Invitrogen**)**; lanes 2a, 2b, 2, TOXX: induced *E. coli* extract, uninduced *E. coli* extract and purified inclusion bodies from induced *E. coli* extract; lanes 3, 4, 5, 6, 7, purified inclusion bodies from induced NdTOXX, TOXXCd, NdTOXX loop 1, NdTOXX loop 1 dimer and pET-22b; 8, BSA 2 µg.

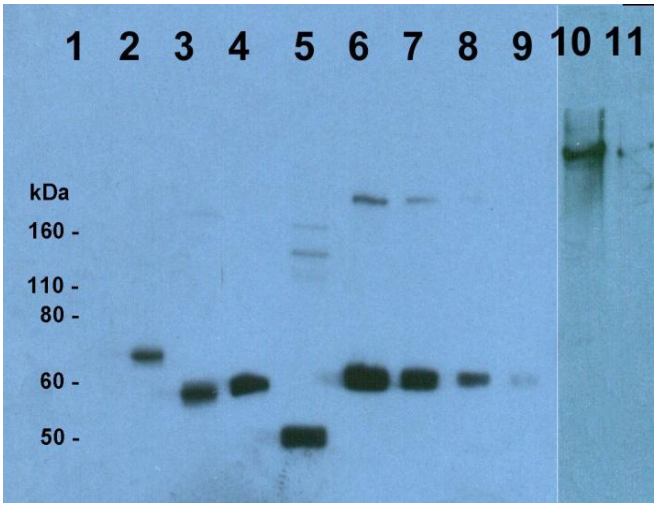

Figure 2. Western blot of TOXX and its mutants produced in *E. coli.* Lanes 1, 2, 3, 4, 5, 6, as in Figure 1; lane 7, NdTOXX loop 1 dimer 40 ng; lane 8, 20 ng; lane 9, 10 ng; lane 10, Cry3B-30 ng; lane 11, Cry3B-10 ng.

high mortality of control larvae and resulting higher error rate, only this last modified TOX protein could be shown as significantly more toxic than standard isolated TOXst or commercial Cry3A using the Dunnett's Upper One-Sided Multiple-Comparison Test (Dunnet, 1955).

DISCUSSION

Constructed phloem sandwiches have been suggested as optimal tools for regulated rearing of bark beetle larvae in relatively natural coditions (Taylor et al., 1992; Wermelinger and Seifert, 1998). They can be used also for determining how the particular species larvae respond to various toxic substances. Due to possible instability of toxic proteins and relatively long duration of the egg

stage, our sandwiches were loaded with the already hatched larvae instead of more naturally colonized by beetles. The chancy procedure of larvae application led to rather high death rate of control larvae that caused equalization of toxicity results, because even if nearly all larvae were dead after 18 days with some of the most toxic proteins, the caculated death rate showed only six times higher mortality than controls. Due to the uneven and difficult to control distribution of toxic substance in phloem, only one concentration of toxic proteins of about 2 µg per cm^3 of phloem was used in all experiments which prevented us from discriminating between individual toxic proteins with higher accuracy. Nevertheless, five times repeated experiments revealed clearly some general rules regarding modified TOX proteins toxicity to spruce bark beetle larvae: original Cry3A as well as modified TOXX protein were surprisingly efficient to spruce bark beetle causing rather high mortality, taking into account that only about one microgramm of protein was fed by individual larvae (calculated from the amount of fed material and toxin concentration in phloem). The deletion of critical residues Y350, Y351 or their exchange for more hydrophobic F350, F351 that was shown to cause highest relative toxicity in *T. molitor* (Wu et al., 2000) had smaller effect in spruce bark beetle, but the combination of these modifications with N- terminal Cry3A deletion resulted in more toxic proteins. In addition and surprisingly, combined with N-terminal deletion, both loop 1 deletion and duplication in the modified TOXX protein resulted in significantly increased toxicity.

The most efficient *TOXX* protein genes were integrated into *Agrobacterium* plant vector and spruce embryonic cultures were transformed. Transformed plants were regenerated and they are presently tranferred from serile cultures to soil.

ACKNOWLEDGEMENT

This work was supported by Ministry of Agriculture of the Czech Republic (Project No. QH71290) and the Czech Science Foundation of the Czech Republic Grant P502/11/1471).

REFERENCES

Adang MJ, Brody MS, Cardineau G, Eagan N, Roush RT, Shewmaker CK, Jones A, Oakes JV, McBride KE (1993). The reconstruction and expression of a Bacillus thuringiensis cryIIIA gene in protoplasts and potato plants. Plant Mol. Biol. 21 (6): 1131–1145.

Bradford MM (1976). A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Carroll J, Li J, Ellar DJ (1989). Proteolytic processing of a coleopteran-specific δ-endotoxin produced by Bacillus thuringiensis var. tenebrionis. Biochem. J. 261: 99–105.

Chen E, Stacy C (2006). Modified Cry3A toxins and nucleic acid sequences coding therefor. US Patent 7,030,295.

Chen XJ, Lee MK, Dean DH (1993). Site-directed mutations in a highly conserved region of *Bacillus thuringiensis* d-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. Proc. Natl. Acad. Sci. USA. 90: 9041–9045.

Crickmore N, Zeigler DR, Schnepf E, Van Rie J, Lereclus D, Baum J, Bravo A, Dean DH (2011). "*Bacillus thuringiensis* toxin nomenclature". http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore /Bt/.

Dunnett CW (1955). A multiple-comparison procedure for comparing several treatments with a controll. J. Am. Stat. Assoc. 50 (272): 1096–1121.

Gazit E, Shai Y (1993). Structural and functional characterization of the α5 segment of *Bacillus thuringiensis* δ-endotoxin. Biochemistry 32: 3429–3436.

Ge AZ, Rivers D, Milne R, Dean DH (1991). Functional domains of *Bacillus thuringiensis* insecticidal crystal proteins: refinement of the *Heliothis virescens* and *Trichoplusia ni* specificity domains on CrylAc. J. Biol. Chem. 266: 17954–17958.

Höfte H, Whiteley HR (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242–255.

Kumar S, Birah A, Chaudhary B, Burma PK, Gupta GP, Pental D (2005). Plant codon optimized cry genes of Bacillus thuringiensis can be expressed as soluble proteins in Escherichia coli BL21 Codon Plus strain as NusA-Cry protein fusions. J. Inverteb. Pathol. 88: 83–86.

Laemmli UK (1970). Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature 227: 680-685.

Modlinger R, Knížek M (2010). Spruce bark beetle *Ips typographus* (L.) breeding methods for substances feeding toxicity assays. Rep. Forestry Res. 55(4): 331–336.

Murray ED, Spencer EY (1966). A simplified purification technique for parasporal inclusions from certain varieties of Bacillus thuringiensis. J. Invertebr. Pathol. 8: 418–420.

Novotný J, Turčáni M (2000). The results of experimental application of the insecticide based on Bacillus thuringiensis var. tenebrionis against spruce bark beetle Ips typogragphus L. (in Czech). Lesnícky časopis 46: 303–310.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, Cold-Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA. Vol. 1, 2, 3.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler, DR, Dean DH (1998). Microbiol. Mol. Biol. Rev. 62, 775–806.

Sekar V, Thompson DV, Maroney MJ, Bookland RG, Adang MJ (1987). Molecular cloning and characterization of the insecticidal crystal protein gene of Bacillus thuringiensis var. tenebrionis. Proc. Natl. Acad. Sci. USA. 84 (20): 7036–7040.

Taylor AD, Hayes JL, Roton L, Moser JC (1992). A Phloem Sandwich Allowing Attack and Colonization by Bark Beetles (Coleoptera: Scolytidae) and Associates. J. Entomol. Sci. 27(4): 311–316.

Van Rie J, Jansens S, Peferoen M (1997). Diabrotica toxins. US Patent 5,659,123.

Vlasák J, Pavingerová D, Bříza J, Sehnal F, Malá J (2010). Modified insecticidal Cry3A protein against spruce bark beetle and red flour beetle and its production. Czech Republic Patent application PV 2010-912.

Wermelinger B, Seifert M (1998). Analysis of the temperature dependent development of the spruce bark beetle Ips typographus (L.)(Col., Scolytidae). J. Appl. Entomol. 122: 185–191.

Wu SJ, Dean DH (1996). Functional Significance of Loops in The Receptor Binding Domain of *Bacillus thuringiensis* CrylIIA d-Endotoxin. Mol. Biol. 255: 628–640.

Wu SJ, Koller CN, Miller DL, Bauer LS, Dean DH (2000). Enhanced toxicity of Bacillus thuringiensis Cry3A N-endotoxin in coleopterans by mutagenesis in a receptor binding loop FEBS Letters 473: 227–232.