

A Novel *cry2Ab* Gene from the Indigenous Isolate *Bacillus thuringiensis* subsp. *kurstaki*

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A novel *cry2Ab* gene was cloned and sequenced from the indigenous isolate of *Bacillus thuringiensis* subsp. *kurstaki*. This gene was designated as *cry2Ab25* and its sequence revealed an open reading frame of 1,902 bp encoding a 633 aa protein with calculated molecular mass of 70 kDa and pI value of 8.98. The amino acid sequence of the Cry2Ab25 protein was compared with previously known Cry2Ab toxins, and the phylogenetic relationships among them were determined. The deduced amino acid sequence of the Cry2Ab25 protein showed 99% homology to the known Cry2Ab proteins, except for Cry2Ab10 and Cry2Ab12 with 97% homology, and a variation in one amino acid residue in comparison with all known Cry2Ab proteins. The *cry2Ab25* gene was expressed in *Escherichia coli* BL21(DE3) cells. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that the Cry2Ab25 protein is about 70 kDa. The toxin expressed in BL21(DE3) exhibited high toxicity against *Malacosoma neustria* and *Rhagoletis cerasi* with 73% and 75% mortality after 5 days of treatment, respectively.

Keywords: *cry2Ab*, cloning, virulence, *Malacosoma neustria*, *Rhagoletis cerasi*

Bacillus thuringiensis (*Bt*), is a ubiquitous Gram-positive, spore-forming bacterium that forms parasporal inclusion bodies called insecticidal crystal protein (ICPs) or delta-endotoxin during the stationary phase of its growth cycle [31, 35]. These crystal proteins are toxic to susceptible larvae of Lepidopteran, Coleopteran, Dipteran, Hymenopteran, Homopteran, Mallophaga, and Orthopteran species of insects (as well as some invertebrates upon ingestion). However, they are benign to the environment [23, 35, 42, 44]. Delta-endotoxins, encoded by *cry* genes, have been

classified as Cry proteins, depending on their host specificity and their amino acid sequences [35]. Up to now, more than 300 *cry* genes have been reported and classified into *cry1–cry68* according to the degree of amino acid sequence homology and their host range (a full list is at http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

The *cry2A*-type gene encodes approximately 60–70 kDa proteins, which form cuboidal crystals and exhibit a wide spectrum of toxicity to Lepidopteran and Dipteran pests [33, 35, 45, 47]. Up to now, nine *cry2A* genes, namely, *cry2Aa* [14, 45], *cry2Ab* [13, 45], *cry2Ac* [33, 46], *cry2Ad* [33], *cry2Ae* (unpublished), *cry2Af* [5], *cry2Ag* [48], *cry2Ah* (unpublished), and *cry2Ai* (unpublished), have been characterized (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Among these, 27 types of *cry2Ab* genes have been identified (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

B. thuringiensis crystalline inclusions range in molecular mass from 27 to 140 kDa [28]. These endotoxins are actually protoxins activated by proteolytic cleavage in the insect midgut after ingestion. The activated toxins (55–70 kDa) destroy midgut epithelial cells, killing sensitive insects within a day or two of ingestion [8, 15].

B. thuringiensis is widely used as a bioinsecticide for the control of many agricultural pests and vectors of human diseases, and constitutes the basis of over 90% of commercially available biopesticides [11]. This is typically owing to its ability to produce δ -endotoxins (Cry proteins) [31, 35]. *Bt* microbial products have been, and continue to be, the preferred insect control choice for organic growers. In addition to the use of *Bt* isolates and their toxins in microbial control of insect pests, *cry* protein-encoding genes were an obvious choice for plant expression as a means to protect crops against insect pests [7]. In 1981, the first *cry* gene was cloned and expressed in *Escherichia coli* [36], followed a few years later by the production of the first genetically modified *Bt*-protected tomato, tobacco,

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and cotton plants [7, 16, 29, 43]. Today, genetically modified plants (GMP) based on *B. thuringiensis* toxin genes represent about 19% of the total transgenic acreage in the world [30].

Investigations for new *cry* genes are an on-going effort worldwide, and it is always desirable to find new *cry* genes because of insect resistance against Cry proteins. In the present study, we have cloned and sequenced a novel *cry2Ab* gene and studied its expression in *E. coli* BL21(DE3) cells. Cry2Ab25 protein showed significant insecticidal activity against *Malacosoma neustria* and *Rhagoletis cerasi*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The MnD strain of *B. thuringiensis* subsp. *kurstaki* was isolated from *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae) at the Microbiology Laboratory, Department of Biology at Karadeniz Technical University, Trabzon, Turkey. The strain was previously identified based on biochemical and molecular characteristics and H-serotyping [20]. *E. coli* strain DH5- α was used for the cloning of the *cry2Ab25* gene, and *E. coli* strain BL21(DE3) was used for expression of the *cry2Ab25* gene. Plasmids pGEM-T (Promega) and pET-28a(+) (Novagen) were used for cloning and expression of the *cry2Ab25* gene as described by the manufacturer.

Amplification and Sequencing of *cry2Ab25* Gene

To amplify the *cry2Ab25* gene, genomic DNA was extracted using standard phenol/chloroform extraction procedures [34]. DNA pellets were dissolved in 10 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until use.

The presence of the *cry2* gene in *B. thuringiensis* subsp. *kurstaki* strain MnD was previously determined by PCR [20]. For subtyping of the *cry2* gene, we used degenerate primers designated to conserved regions of *cry2A* genes that belong to different *B. thuringiensis* strains from the GenBank database. The following primers were used to amplify the *cry2A* gene: forward, Cry2A-Fw-U, 5'-GGCATATGAATAVTTTGAATARYSGAARAAM-3'; reverse, Cry2A-Rv-U, 5'-GGAAGCTTTCATTAATAAAGTGGTGRAAKWTTAGTTGGH-3' (underlined sequences indicate the *Nde*I and *Hind*III restriction sites, respectively). PCR amplifications were performed in a total volume of 50 μ l, which included 5 μ l of 5 \times *Taq*-DNA polymerase reaction buffer (10 mM Tris-HCl, pH 8.3), 3 μ l of 2.5 mM MgCl₂, 1 μ l of dNTP-mix (10 mM), 1.5 μ l each of the opposing amplification primers (10 mM), 2.5 units of *Taq*-DNA polymerase (Promega), and 50 ng of genomic DNA, and the final volume was completed with ddH₂O. PCR amplifications were initiated with a 3 min denaturation at 94 $^{\circ}\text{C}$. Following this, 10 cycles were conducted as 94 $^{\circ}\text{C}$ for 1 min, 45 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 3 min. Finally, 25 cycles were of 94 $^{\circ}\text{C}$ for 1 min, 48 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ 1 min; extension was at 72 $^{\circ}\text{C}$ for 10 min. The PCR product was separated on 1.0% agarose gels, stained with ethidium bromide, and viewed under UV light. After confirming the PCR product, the amplified fragment was excised from the gel and directly cloned into a pGEM-T-easy cloning vector (Promega). The ligation mixture was transformed into the *E. coli* DH5 α strain. After amplification, plasmid DNA samples of each isolate were digested by restriction

Table 1. Nucleotide sequence homology of *cry2Ab25* and the other known *cry2Ab* genes.

Other <i>cry2Ab</i> genes		Percent similarity
<i>cry2Ab</i> genes	Accession number	
<i>cry2Ab1</i>	AAA22342	99%
<i>cry2Ab2</i>	CAA39075	99%
<i>cry2Ab3</i>	AAG36762	99%
<i>cry2Ab4</i>	AAO13296	99%
<i>cry2Ab5</i>	AAQ04609	99%
<i>cry2Ab6</i>	AAP59457	99%
<i>cry2Ab7</i>	AAZ66347	99%
<i>cry2Ab8</i>	ABC95996	99%
<i>cry2Ab9</i>	ABC74968	99%
<i>cry2Ab10</i>	EF157306	97%
<i>cry2Ab11</i>	CAM84575	99%
<i>cry2Ab12</i>	ABM21764	97%
<i>cry2Ab13</i>	ACG76120	99%
<i>cry2Ab14</i>	ACG76121	99%
<i>cry2Ab15</i>	HM037126	99%

enzymes and sequenced. The sequences obtained were used to perform BLAST searches using the NCBI GenBank database [4].

Phylogenetic Analysis

A total of 15 CryAb protein sequences (Cry2Ab1–Cry2Ab15) were downloaded from GenBank and used for phylogenetic analysis (Table 1). Sequences of the ORF of each Cry2Ab were aligned with BioEdit [17]. Cluster analyses were performed using Clustal W (BioEdit version 7.09). Phylogenetic analyses using the neighbor-joining method were performed with MEGA 4.1 software [21, 40]. The reliability of the dendrogram was tested by bootstrap analysis with 1,000 replicates by using MEGA 4.1.

Expression of *cry2Ab25* Gene in *Escherichia coli* BL21(DE3) and SDS-PAGE Analysis

In order to investigate the expression of the *cry2Ab25* gene, an expression vector was constructed as follows: pGEM-T easy vector carrying the *cry2Ab25* gene was digested with *Nde*I and *Hind*III restriction enzymes. After that, *E. coli* BL21/pET-28a(+) cells were grown in LB medium including kanamycin (50 $\mu\text{g}/\text{ml}$). Plasmid DNA isolation was done from these cells and the obtained pET-28a(+) plasmids were digested with the same enzymes and ligated to the *cry2Ab25* fragment. The final vector pET-28a(+)/*cry2Ab25* was mobilized into *E. coli* strain BL21(DE3) according to the standard protocol [34].

E. coli BL21(DE3) transformants were grown in 3 ml of LB medium containing kanamycin (50 $\mu\text{g}/\text{ml}$) at 37 $^{\circ}\text{C}$ overnight, subcultured into fresh medium (the volume ratio of 1:100) for 4 h, and then induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4–6 h. Cells were harvested by centrifugation and washed once in 20 mM Tris-HCl (pH 7.5). The cultures were resuspended in 5 ml of 20 mM Tris-HCl (pH 7.5), sonicated, and centrifuged. The final cultures were resuspended in 2 ml of 20 mM Tris-HCl (pH 7.5) and used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was determined by utilizing the Bradford assay [9]. Eighty μg protein of each sample was loaded on the gel.

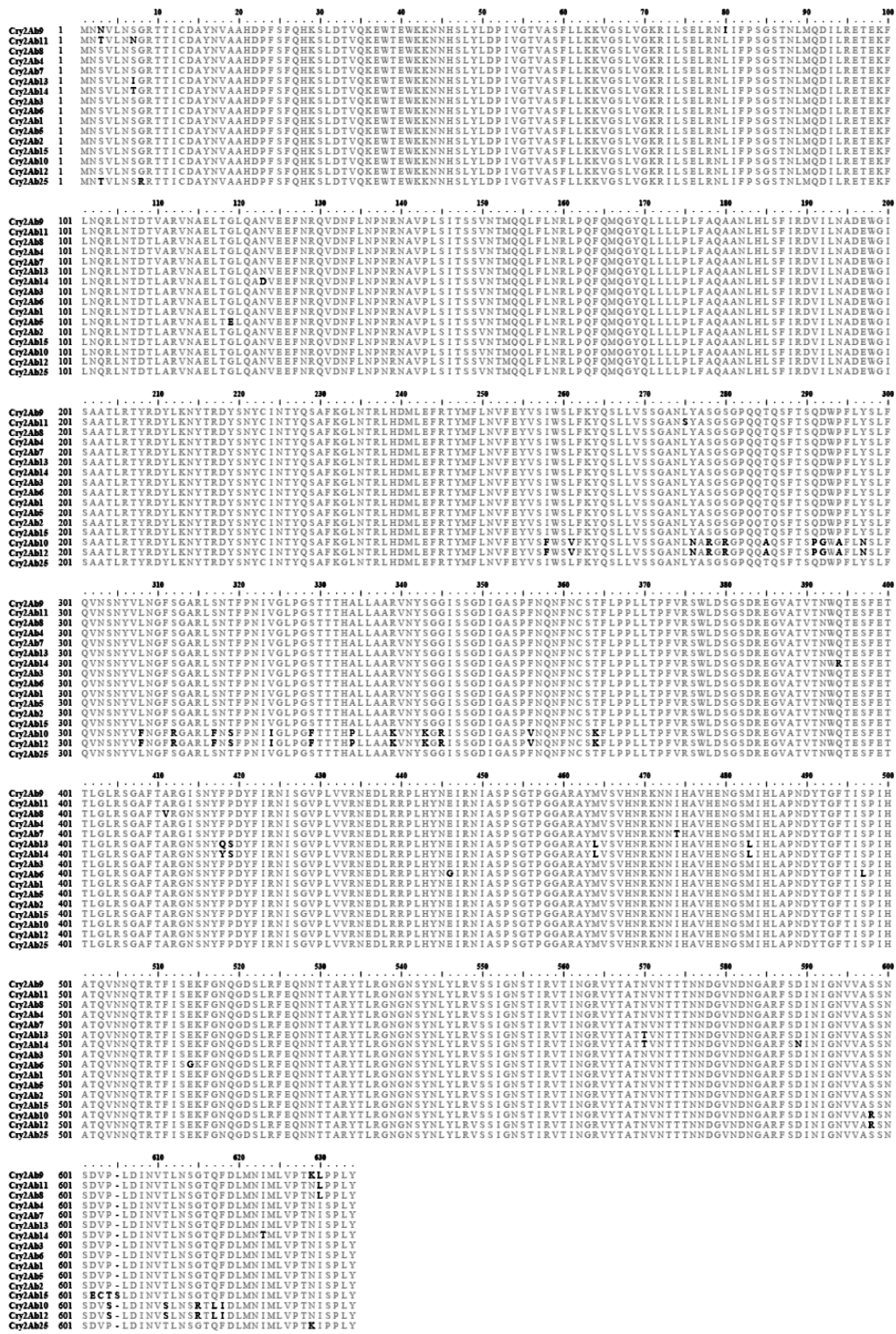


Fig. 1. Alignment of the deduced amino acids of Cry2Ab proteins. Amino acid differences among Cry2Ab proteins are indicated in bold type.

Bioassay

Bioassay studies were conducted against the third instar larvae of *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae) and *Rhagoletis cerasi* (Diptera: Tephritidae). The diets were prepared from fresh berberis leaves for *M. neustria* and freshly collected cherry for *R. cerasi*. In bioassay experiments, the Cry2Ab25 protein expressed in *E. coli* BL21(DE3), and proteins from *E. coli* BL21(DE3)/pET-28a(+) and *E. coli* BL21(DE3) were used. Additionally, the spore-crystal mixture (SCM) of the isolate MnD was used. Water was used as the negative control. Concentrations of proteins were adjusted to 100 µg/ml and the MnD suspension contained approximately 10⁹ SCM/ml. The diets were contaminated with proteins and the SCM of the isolate MnD. Ten third instar larvae of *M. neustria* and *R. cerasi* were placed into 300 ml plastic boxes with suitable diet. Mortality was recorded 5 days after initiation of the treatment, with all dead larvae removed from the containers. All bioassays were repeated three times on different occasions. Percent mortality was corrected according to Abbott's formula [1]. The data were subjected to ANOVA and subsequently to LSD multiple comparison test to compare isolates and the control group in terms of mortality using SPSS 15.0 statistical software.

Nucleotide Sequence Accession Number

The *cry2Ab25* nucleotide sequence accession number at the GenBank nucleotide sequence database is JN415485.

RESULTS AND DISCUSSION

Sequence of the *cry2Ab25* Gene and Phylogeny

In a previous study, a *cry2* gene was identified in the MnD strain of *B. thuringiensis* subsp. *kurstaki* by PCR [20]. In this paper, primers prepared based on the conserved regions of *cry2Ab* genes in *B. thuringiensis* strains were used to amplify the DNA fragment of *B. thuringiensis* subsp. *kurstaki*. An approximately 1,900 bp *cry2Ab* gene was amplified *via* PCR. Cloning and sequencing of the expected product revealed that the 1,902 bp DNA fragment encoding 633 amino acids residue with predicted molecular mass of 70 kDa and pI value of 8.98, and an ORF of the *cry2Ab25* gene in *B. thuringiensis* subsp. *kurstaki*. The mean G+C content of the gene is 53.55%. The comparison of homology of the nucleotide sequence and deduced amino acid sequence at the Website of NCBI using the BLAST program revealed that they shared the highest sequence homology (97–99%) with the other known *cry2Ab* genes. This suggested that the cloned fragment from *B. thuringiensis* subsp. *kurstaki* was a real *cry2Ab* gene, which was named as *cry2Ab25* by the *Bacillus thuringiensis* Delta-Endotoxin Nomenclature Committee, which suggests the use of the quaternary rank that distinguishes between toxins that are more than 95% identical [12].

The alignment of the deduced amino acid sequence of Cry2Ab25 with other Cry2Ab proteins showed that the sequence was 99% identical to the other published Cry2Ab proteins, except for Cry2Ab10 and Cry2Ab12 with 97%

homology (Table 1). Based on the alignment of all of the Cry2Ab proteins, one amino acid of Cry2Ab25, corresponding to the 8th amino acid (Arginine), was different from the other Cry2Ab proteins (Fig. 1). The other many amino acid differences between Cry2Ab25 and Cry2Ab proteins reported so far are indicated in Fig. 1.

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ATGAATACTGTATGAAATAGTCGAAGAAC TACTAITTTGTGATCGGTATAATGTAGCGGCT
M N T V L N S R R T T I C D A Y N V A A
CATGATCCATTTAGTTTT CAACACAAATCATTAGATACC GTACAAAAGGAATGGACGGAG
H D P F S F Q H K S L D T V Q K E W T E
TGGAAAAAATAATCATAGTTTATACCTAGATCC TATTGTTGGAAGCTGIGGCTAGTTTT
W K K N N H S L Y L D P I V G T V A S F
CTGTTAAAGAAAGTGGGGAGTCTT GTTGGAAAAAGGATACTAAGTGAGTACGGAATTTA
L L K K V G S L V G K R I L S E L R N L
ATATTTCCTAGTGGTACAAATCTAATCGAAGATATTTAAGAGAGACAGAAAAATTC
I F P S G S T N L M Q D I L R E T E K F
CTGAATCAAAGACTTAATACAGACACTCTTGC CCGTAAATGCGGAAATGACAGGGCTG
L N Q R L N T D T L A R V N A E L T G L
CAAGCAAATGTAGAGAGTTTAAATCGACAAGTAGAATATTTTT GAACCCTAACCGAAAC
Q A N V E E F N R Q V D N F L N P N R N
GCTGTTCCCTTTTCAATAACTTCTTCAGT TAATACAATGCAACAATTAATTTCTAAATAGA
A V P L S I T S S V N T M Q Q L F L N R
TTCCCGAGTTCAGATGCAAGGATACCACTGT TATTAATACCTTTTATGACAGGCA
L P F Q M Q G Y Q L L L P L F A Q A
GCCAATTTACATCTTTCTTTTATAGAGATGTTA TTTCTAAATGCGAGATGAATGGGGAAT
A N H L S F I R D V I L N A D E W G I
TCAGCAGCAACATTCAGTACGTATCGAGATTA CTGAAAAATATACAGAGATTACTCT
S A A T L R T Y R D Y L K N Y T R D Y S
AACTATTGTATAAATACGTATCAAAGTGC GTTTAAAGGT TTAACACACTCGTTTACAGCAT
N Y C I N T Y Q S A F K G L N T R L H D
ATGTTAGAATTTGAACATAATATGTTTTT AAATGTAITTTGAGTATGTAICTAICTGGICG
M L E F R T Y M F L N V F E Y V S I W S
TTGTTTAAATATCAAAGTCTTCTAGTATCTT CCGGIGCTAATTTATATGCAAGTGGTAGT
L F K Y Q S L L V S S G A N L Y A S G S
GGACCAAGCAGACCCAAATCATTACTTCA CAAAGACTGGCCCAITTTTATATCTCTTTTC
G P Q Q T Q S F T S Q D W P F L Y S L F
CAAGTTAATCAAATATGTTTAAATGGATTTA GIGGTTGCTAGGCTTCTAATACCTTC
Q V N S N Y V L N G F S G A R L S N T F
CCTAATAGTGTGTTTACCTGGTTCTTACTA CAACTCAGCAATGCTTGTCTGCAAGGGT
P N I V G L P G S T T T H A L L A A R V
AATACAGTGGAGGAATTCGCTGGTATATAG GTGCACTCCGTTTAAATCAAATTTT
N Y S G G I S S G D I G A S P F N Q N F
AATGTAGCACATTTCTCCCCCAATGTTT AACGCCAATTTGTTAGGAGTTGGCTAGATICA
N C S T F L P P L L T P F V R S W L D S
GGTTCAGATCGGGAGGGCGTTGCCACCGT TACAATTTGGCAAACAGAATCCTTTGAGACA
G S D R E G V A T V T N W Q T E S F E T
ACTTTAGGGTTAAGGAGTGGTGTCTTTTAC AGCTCAGCGTAATTCAACTAATTTCCAGAT
T L G L R S G A F T A R G N S N Y F P D
TATTTTTCGTAATATTTCTGGAGTTCCTTTA GTTGTAGAAATGAGATTTAAGAAGA
Y F I R N I S G V P L V V R N E D L R R
CCGTTACATATAAATAAGAAATATAGCAA GCTTCAGGAACACCTGGTGGAGCA
P L H Y N E I R N I A A S P S G T P G G A
CGAGCTTATATGGTATCTGTGCATACAGAA AATAATCCATGCTGTTCCATGAAT
R A Y M V S V H N R K N N I H A V H E N
GGTTCATGATTCATTTAGCGCCAAATGACTA TA CAGGATTTACTAATTTCCGCGATACAT
G S M I H L A P N D Y T G F T I S P I H
GCAACTCAAGTGAATAATCAAACA CGAACATTTTCTGAAAAATTTGGAAATCAAGGT
A T Q V N N N Q T R T F I S E K F G N Q G
GATTCITTAAGGT TTGAACAAAACAACAC GACAGCTCGTTATAGCCTTAGAGGGAATGGA
D S L R F E Q N N T T A R Y T L R G N
AATAGTTACAATCTTTATTTAAGAGTTCCTT CCAATAGGAAATTCCTATTCGAGTTACT
N S Y N L Y L R V S S I G N S T I R V T
ATAAACGGTAGGGTATATCTGCTACAAATG TTTAATCTACTACTACAAAATAACGATGGAGT
I N G R V Y T A T N V N T I T N N D G V
AATGATAATGGAGCTCGT TTTTCAGATAT TAATACTGGTAAATGTAGTAGCAAGTATGTAAT
N D N G A R F S D I N I G N V V A S S N
TCTGATGATACCATAGATATAAATGTAACAT TAACTCCGGTACTCAAATTTGATCTTATG
S D V P L D I N V T L N S G T Q F D L M
AATATTATGCTGTACCAACTAAAATTCACCA CTTTATTA
N I M L V P T K I P P L Y *
    
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Fig. 2. Nucleotide and deduced amino acid sequence of the Cry2Ab25 toxin of *B. thuringiensis* subsp. *kurstaki* strain MnD. Conserved amino acid blocks are underlined. Start and stop codons are in bold face. Leucine amino acid, which is indicated as shaded, was different from the first block defined by Schnepf *et al.* [35].

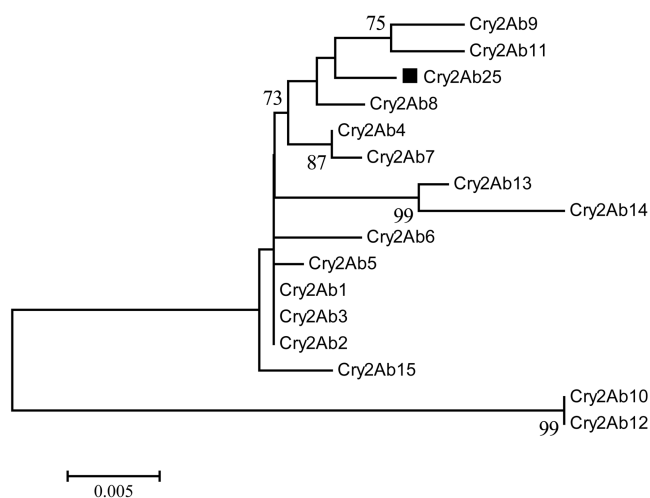


Fig. 3. Evolutionary relationship of Cry2Ab proteins.

Neighbor-joining analysis with the p-distance method was used to construct the dendrogram. Bootstrap values shown next to nodes are based on 1,000 replicates using MEGA 4.1. Bootstrap values of $C \geq 70\%$ are labeled. Cry2Ab25 is indicated by the black square. The scale on the bottom of the dendrogram shows the degree of dissimilarity.

Predictably, the Cry2Ab25 protein contained two conserved amino acid residue blocks that are present in almost all Cry proteins as described by Schnepf *et al.* [35]. However, one amino acid variation (15th aa within the first block of Cry2Ab25) was determined in the first block of Cry2Ab25. Whereas this amino acid is methionine in the described block of Schnepf *et al.* [35], it is leucine in Cry2Ab25 (Fig. 2).

The phylogenetic relationship of Cry2Ab proteins belonging to different *Bt* isolates is shown in Fig. 3. According to the dendrogram, the Cry2Ab25 protein is closely related to the Cry2Ab9 and Cry2Ab11 proteins. Based on fully sequenced *cry2Ab*-type genes and phylogenetic analysis of Cry2Ab proteins, it is possible to say that *cry2Ab25* is a novel *cry2Ab* gene. In fact, its deduced amino acid sequence is different at one site from those of all the other published *cry2Ab* genes (Fig. 1).

Expression of *cry2Ab25* Gene in *E. coli* BL21(DE3)

To date, different *cry* genes encoding the insecticidal crystal proteins have been cloned and expressed in various hosts such as *E. coli* [19], *B. thuringiensis* [22], *Pseudomonas cepacia* [38], *Cyanobacteria* [27], and *Rhizobium* [37], and *B. subtilis* and *B. licheniformis* [41].

In this study, we aimed to express the *cry2Ab25* gene in *E. coli* cells to determine molecular mass of the toxin and insecticidal activity of the toxin against a number of pest species. To this end, the ORF of *cry2Ab25* was ligated to pET-28a(+) (5.369 bp) at the *Nde*I and *Hind*III cutting sites and used to transform the *E. coli* by the described protocol of Sambrook *et al.* [34]. Recombinant clones were selected based on the restriction digestion pattern with *Nde*I and

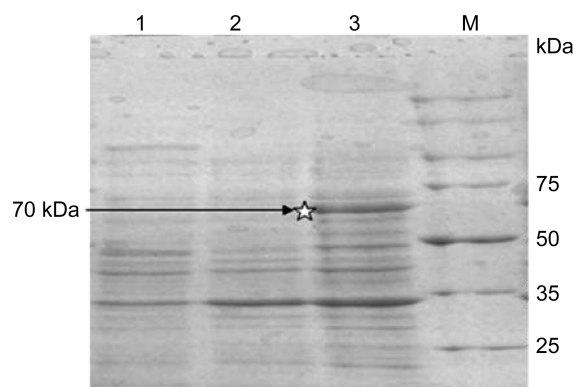


Fig. 4. SDS-PAGE analysis of the *cry2Ab25* gene expressed in *E. coli* BL21(DE3) cells.

M, ProSieve® protein marker; 1, *E. coli* BL21(DE3); 2, *E. coli* BL21(DE3)/pET-28a vector; 3, *E. coli* BL21(DE3)/pET-28a-*cry2Ab25* vector.

*Hind*III that releases the approximately 1,902 bp ORF of *cry2Ab25* and 5.369 bp vector. For controlling, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)/pET-28a(+) vector were used. SDS-PAGE analysis revealed that Cry2Ab25 toxin was expressed as a 70 kDa protein in *E. coli* BL21(DE3) strain induced by IPTG as the molecular mass predicted from the sequence (Fig. 4). There was no prominent band of approximately 70 kDa in the case of the control, BL21(DE3).

Toxicity Assay

The Cry2Ab25 protein showed high toxicity against the third instar larvae of *M. neustria* and *R. cerasi*. In the case of *M. neustria*, the Cry2Ab25 protein (73%) and the SCM of the isolate MnD (76%) showed higher mortality in comparison with the other treatments ($F=96.57$, $df=4$, $p<0.05$). There was no significant difference between the Cry2Ab25 protein and the SCM of the isolate MnD in terms of mortality ($F=96.57$, $df=4$, $p<0.05$) (Fig. 5A).

For *R. cerasi*, the highest mortality values were obtained from the Cry2Ab25 protein and the SCM of the isolate MnD (86%) compared with other treatments ($F=40.69$, $df=4$, $p<0.05$), and there was no significant difference between the Cry2Ab25 protein and the SCM of the isolate MnD ($F=40.69$, $df=4$, $p<0.05$) (Fig. 5B).

Up to now, many scientists have cloned and characterized *cry2Ab*-type genes from different *B. thuringiensis* isolates and showed their insecticidal activity against Lepidopteran pests [3, 13, 18, 25, 39]. However, there are a few studies suggesting the toxicity of Cry2Ab proteins against Dipteran pests [2, 24]. In both studies, authors immersed mosquito larvae in water containing the spore-crystal complex [24] or *B. megaterium* expressing the toxin [2]. However, in this study, we used a feed-based test of Cry2Ab25 toxin to determine the Dipteran activity of the toxin and observed a significant mortality value against *R. cerasi*. All these

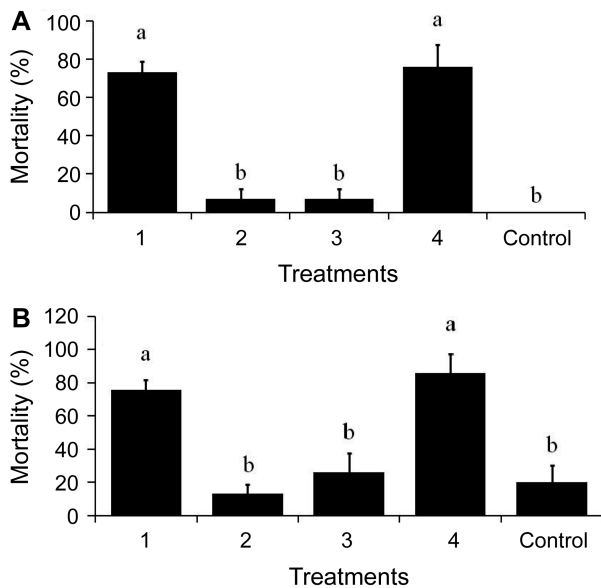


Fig. 5. Mortality of the larvae of *Malacosoma neustria* (A) and of *Rhagoletis cerasi* (B) within 5 days after application of Cry2Ab25 toxin.

Mortality data were corrected according to Abbott's Formula [1]. Different letters show the difference between strains according to the LSD multiple comparison test ($p < 0.05$). Bars show standard deviation. 1, Cry2Ab25; 2, proteins from *E. coli* BL21(DE3)/pET-28a(+); 3, proteins from *E. coli* BL21(DE3); 4, the spore-crystal mixture of the strain MnD.

studies suggest that Cry2Ab toxins might be toxic to the Dipteran pests under laboratory conditions. However, more detailed toxicity assays such as the determination of LT_{50} and LC_{50} should be done to ensure the toxicity of Cry2Ab25 protein against Dipteran insects.

To date, many *B. thuringiensis* toxins have been found and some of them have been used world-wide as bioinsecticides or in transgenic plants to control crop pests and mosquito populations [6, 10]. Unfortunately, some insects have developed resistance against several of the *Bt* toxins [26] and this is becoming a growing problem in agriculture and forestry. In addition, *cry1*-type gene products have mostly been used in transgenic plant development. In this manner, recently, problems of narrow insecticidal spectrum and insect resistance have been observed owing to lengthy use of high concentrations of a single *Bt* toxin [32, 48]. In this regard, Cry2A toxins seem to be a good alternative candidate for use in transgenic plant development to control crop pests instead of *cry1*-type toxins. Therefore, the isolation of novel Cry proteins is an important progress to solve this kind of problems.

In conclusion, we have described a new type of *cry2Ab* gene in the indigenous isolate *B. thuringiensis* subsp. *kurstaki* and it was assigned as *cry2Ab25*. We showed that the protein encoded by the *cry2Ab25* gene has an important toxicity against Lepidopteran and Dipteran insects. The newly cloned *cry2Ab25* gene could be a valuable tool for

transgenic technology to import insect resistance in crop plants and to minimize the use of hazardous chemical pesticides in agriculture and forestry. However, further studies are needed to fully elucidate the insecticidal activity and insecticidal spectrum of this protein.

Acknowledgment

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