(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2007 (26.04.2007)

PCT

(10) International Publication Number
WO 2007/045160 A1

(51) International Patent Classification:
C12N 15/32 (2006.01) *C07K 14/325* (2006.01)
C12N 15/63 (2006.01) *A01N 63/02* (2006.01)

(21) International Application Number:
PCT/CN2006/002724

(22) International Filing Date: 17 October 2006 (17.10.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
200510019596.0 17 October 2005 (17.10.2005) CN

(71) Applicant *(for all designated States except US)*: **HUAZHONG AGRICULTURAL UNIVERSITY** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SUN, Ming** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **YU, Ziniu** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **CHEN, Shouwen** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **ZHANG, Zhenyu** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **RUAN, Lifang** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **GUO, Suxia** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **DAI, Jingyuan** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **LI, Lin** [CN/CN]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (CN). **LIU, Ziduo** [CH/SG]; SHIZISHAN STREET_1, Hongshan District, Wuhan, Bubei 430070 (SG).

(74) Agent: **CHINA PATENT AGENT (H.K.) LTD.**; 22/F, Great Eagle Centre, 23 Harbour Road, Wanchai, Hong Kong (CN).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** GENE CRY7BAL ENCODING AN INSECTICIDAL CRYSTAL PROTEIN OF BACILLUS THURINGIENSIS

(57) **Abstract:** The present invention discloses the isolation, cloning and use of genes of insecticidal crystal proteins from Bacillus thuringiensis. The present invention isolates a novel insecticidal crystal protein gene cry7Bal from B. thuringiensis subsp. huazhongensis YBT-978 strain, and said gene encodes a novel insecticidal crystal protein Cry 7BaI, which shows insecticidal activity against Lepidopteran insects. The present invention also discloses the gene sequence of the novel insecticidal crystal protein, uses suitable expression vectors to transform micro-organisms so as to express the product Cry7Bal encoded by the gene and make it exert the insecticidal activity of the protein against Lepidopteran pests.

WO 2007/045160 A1

Gene *cry7Ba1* Encoding An Insecticidal Crystal Protein of *Bacillus thuringiensis*

Field of the Invention

The present invention relates to micro-organism genetic engineering. In particular, the present invention relates to the separation and cloning of insecticidal crystal proteins from *Bacillus thuringiensis*. The present invention relates to the genetic engineering of biological pesticides.

Background of the Invention

The *Bacillus thuringiensis* is a rod-shaped, Gram-positive, endospore-forming bacterium which is widely existing in various ecological environments. During the spore-forming phase, *B. thuringiensis* forms parasporal crystals consisting of Insecticidal Crystal Proteins (ICPs) which have specific toxicity against insects and specific biological activities to more than 500 species of insects in 10 orders belonging to the class Insecta, including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, etc, as well as to some harmful varieties in Protozoa, Nematomorpha, Platyhelminthes (Schnepf, H E., Crickmore, N., Rie, J. V., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. & Dean, D. H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev* 62, 775-806).

Wildtype strains of *B. thuringiensis* typically have genes encoding insecticidal crystal proteins and each strain usually has multiple copies of said genes. Schnepf et al. cloned the first gene encoding insecticidal crystal protein in 1981 from *B. thuringiensis* subsp. *kurstaki* Strain HD1 and deduced the amino acid sequence of the first *B. thuringiensis* insecticidal crystal protein based on the DNA base sequence (Schnepf HE,

Wong HC, Whiteley HR. The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J Biol Chem.* 1985 May 25;260(10):6264 - 6272.). Afterwards, new genes encoding insecticidal crystal proteins have been actively pursued in connection with studies of *B. thuringiensis*. Many new genes encoding different insecticidal crystal proteins have been identified, cloned and sequenced. Therefore, in 1995, the *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee was founded by scholars including Crickmore in the Annual Meeting of the Society for Invertebrate Pathology. In 1996, a new classification system for the *B. thuringiensis* insecticidal crystal proteins based on the homology of amino acid sequences was formally proposed, and nomenclature rules and the principle for classification were set, wherein it stipulates that: *cry* gene is an insecticidal gene from *B. thuringiensis* encoding parasporal crystal protein, or any gene that has obvious sequence similarity to a known *cry* gene; *cyt* gene is a gene encoding a parasporal crystal protein from *B. thuringiensis* that exhibits hemolytic activity, or any gene encoding a protein that has obvious sequence similarity to a known Cyt protein. They are classified in 4 ranks based on the homology of the amino acid sequence deduced from the full length gene. The boundaries between each rank represent approximately 95%, 78% and 45% sequence identity. The genes of the insecticidal crystal protein are classified into 4 ranks. By August, 2005, the number of genes of *Bacillus thuringiensis* insecticidal crystal proteins has reached 319, representing varieties from 48 types (Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum, and D. H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62:807-813; see for example the internet site at lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/index.html on the world wide web).

In the beginning, pesticides based on *B. thuringiensis* are produced with screened wildtype strains. With the advancement of molecular biology, people have gradually altered the wildtype strains by genetic engineering means. At the same time, people have been keeping on transforming genes of *B. thuringiensis* insecticidal crystal proteins into plants and have been producing transgenic plants that are resistant to agricultural pests.

However, with the development of B.t. pesticides and the increasing usage of these pesticides,, resistance in targeted pests has been continuously discovered by the scientists. The scholars have extensively studied the resistance against B.t pesticides in targeted pests. *Bacillus thuringiensis* insecticidal crystal proteins have to go through the following process in order to give the insecticidal effects: the solubilization of the crystals and activation of the protoxin crystals, binding of the toxin fragments to receptors on the epithelial linings in the midgut, and the insertion into the membrane to create pores, wherein the activity spectrum and toxicity mainly depend on the recognition and interaction of the toxin fragments with the specific receptors on the epithelial linings in the midgut of the insects. Further, the development of resistance against B.t. pesticides in insects is closely related to the recognition and binding to the pesticide receptor. Therefore, cloning and application of new, especially novel genes of insecticidal crystal proteins have become the key to prevent and control the resistance against B.t pesticides in targeted pests and the core issue in the various insect control strategies. In recent years, searching and cloning novel genes of insecticidal crystal proteins have been the most active area in the study for *B. thuringiensis*. The significance of the present invention lies in this. In China, a new subspecies, YBT-978 strain, which is a subspecies of *B. thuringiensis* has been separated and characterized in 1996, which belongs to subspecies *huazhongensis*, serotype H40 (for the source of the strain, please refer to Dai J et al. 1996. *Bacillus thuringiensis* subsp. *huazhongensis*, serotype H40, isolated from soils in the People' s Republic of China. Letters in Applied Microbiology. 22(1): 42-45). It is found the parasporal crystal proteins has a highly efficient insecticidal activity to insects including *Plutella xylostella* through extracting said parasporal crystal proteins and subjecting to bioassays.

Contents of the Invention

The object of the present invention is to isolate and clone the genes of insecticidal crystal proteins having high toxic potency from *B. thuringiensis* and to provide a use for

the same.

The technical scheme of the present invention is as shown in Figure 1.

In order to achieve the aforesaid object, the present invention isolates from *Bacillus thuringiensis* subsp. huazhongensis, serotype H40, strain YBT-978 (for the source of the strain, please refer to Dai J et al. 1996. *Bacillus thuringiensis* subsp. huazhongensis, serotype H40, isolated from soils in the People's Republic of China. *Letters in Applied Microbiology*. 22(1): 42-45) a novel insecticidal crystal protein gene, the coding region of which consists of 3465 bases and has a nucleotide sequence shown in SEQ ID NO:1. The *cry7Ba1* gene of the present invention encodes Cry7Ba1 protein, which consists of 1154 amino acid residues and has an amino acid sequence as shown in SEQ ID NO:2. The gene *cry7Ba1* of the present invention is expressed in microorganisms as Cry7Ba1 protein, which possesses insecticidal activity against insects of the order Lepidoptera.

The highest homology between the Cry7Ba1 protein of the present invention and any one of the proteins which has been disclosed is 58.2%. Moreover, the highest homology between the sequence of the N-terminal half of the Cry7Ba1 protein of the present invention (amino acid 1 to 658 of the amino acid sequence shown in SEQ ID NO:2) and any one of the proteins that has been disclosed is 37.1%.

The present invention provides the aforesaid gene sequence, which is a novel insecticidal crystal protein gene having insecticidal activity. The said gene can be used in transforming microorganisms and/or plants to impart insecticidal activity against pests of the Lepidoptera, as well as to overcome and delay the resistance in pests against genetically engineered insecticides and transgenic plants.

The following descriptions and examples disclose the technical solution in more detail:

It should be noted that the standard procedures and the reagents used on DNA are according to "Guide for Molecular Cloning Experiments" (refer to J. Sambrook et al (2002). Molecular Cloning: A Laboratory Manual. 3rd Edition. Translated by Jin Dong-yan et al. Science Press. Beijing).

1. The sequencing of the N-terminal amino acids of crystal protein Cry7Ba1 in *B. thuringiensis* YBT-978

The present invention uses *B. thuringiensis* YBT-978 as source strain for insecticidal crystal protein Cry7Ba1. *B. thuringiensis* YBT-978 is a standard strain of *B. thuringiensis* serotype H40, the source of which was recited in Dai J et al. 1996. The strain can form the typical rhombus-shaped crystals, and the molecular weight of the main component of the crystal protein Cry7Ba1 is about 130 kDa. The specific steps for sequencing the N-terminal amino acid sequence of Cry7Ba1 are as follows:

(1) The culture of bacterial strains and the manipulation of culture

B. thuringiensis YBT-978 is inoculated on 5 ml LB (1% NaCl, 1% peptone, 0.5% yeast extraction, pH 7.0) using an inoculation loop under sterile conditions and is incubated overnight in 30°C on a shaker at 200 rpm. Then, 350 μl of the culture is transferred under sterile conditions to 35 ml ICPM culture medium (0.6% peptone, 0.5% glucose, 0.1% $CaCO_3$, 0.1% $MgSO_4.7H_2O$, 0.05% KH_2PO_4, pH 7.0), and is incubated in 30°C on a shaker at 200 rpm until gemma and parasporal crystals completely fall off. The culture is collected and is centrifuged for 1 minute at 8000 rpm. Discard the supernatant. Use 0.5% NaCl solution and sterilized de-ionized water to rinse the precipitates for 3 times separately.

(2) The SDS-PAGE electrophoresis of crystal proteins and the transfer of PVDF blotting membrane

Resuspend the rinsed precipitates in de-ionized water at a ratio of precipitates:de-ionized water = 1:5 v/v, and add in an equal volume of 2x loading buffer. Mix, and then incubate in a boiling bath for 3 to 5 minutes, centrifuge at 12000 rpm for 5 minutes, and perform SDS-PAGE electrophoresis on the supernatant. For the formulation of 2x loading buffer and the procedure for performing SDS-PAGE electrophoresis, please refer to the method recited by Laemmli (Laemmli, UK. 1970. Digestion of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) 227:680-685).

Then, the protein samples on the gel are transferred to PVDF membrane using Trans-Blot SD Semi-Dry Transfer Cell (BIO-RAD) using standard method (see Cook RG. 1994. Amino acid sequence analysis of blotted proteins, p.207-220. In. B.S. DunBar (ed.), Protein Blotting: A practical approach. Oxford University Press, New York).

3) Determination of the N-terminal amino acid sequence of the crystal protein

Use a protein N-terminal amino acid sequencer (model number:ABI 491 Protein Sequence, ABI Procise) to sequence the N-terminal amino acid sequence. The sequencing result is N-terminal-MDIRNQNKYEVVYPA-C-terminal (SEQ ID NO: 3).

2. Acquisition of the sequence of 5'end DNA fragment of the insecticidal crystal protein gene *cry7Ba1*

Perform amplification using adaptor-ligation PCR. The principles and steps of adaptor-ligation PCR was described in Slebert P. D. et al. (Slebert P D et al. 1995. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Research* 23:1087-1088). Specific steps are as follows:

1) Extraction of total DNA from *B. thuringiensis* YBT-978

B. thuringiensis YBT-978 is inoculated on 5 ml LB culture medium using an

inoculation loop under sterile conditions and is cultured overnight in 30°C on a shaker at 200 rpm. Then, 50 μl of the culture is transferred under sterile conditions to 5 ml fresh LB culture medium, and incubate under same conditions till logarithmic growth phase. Collect the bacteria after centrifugation at 5000 rpm for 3 minutes. Rinse once with 1 ml STE. Add 100 μl solution I and 10 μl lysozyme (50 mg/ml) and allow for action for 30 minutes or above. Add 200 μl 2% SDS, and incubate with a water bath at 50-60°C for 30 minutes. Add 200 μl 5 mol/L NaCl and mix. Add 500 μl phenol/chloroform/isopentyl alcohol (25:24:1 v/v) and centrifuge for 5 minutes at 12000 rpm. Collect the supernatant and repeat the extraction once or twice. Transfer the upper layer DNA solution to Eppendorf tubes and then add equal volumes of 95% ethanol. Set in room temperature for 5 minutes, and then centrifuge for 5 minutes at 12000 rpm. Use 200 μl 70% ethanol to rinse the precipitates once and freeze-dry the precipitates, then dissolve the freeze-dried precipitates in 50 μl TE solution.

2) Enzymatic digestion of the total DNA with restriction endonuclease and ligation with adaptor

(1) Allow the prepared total DNA of *Bacillus thuringiensis* to be enzymatically digested by *HpaI* enzyme at 37°C overnight (the enzymatic digestion system is 50 μl comprising about 1 μg total DNA); (2) Add 50 μl enzymatic digestion product into 250 μl phenol:chloroform:isopentyl alcohol (25:24:1 v/v), centrifuge at 12000 rpm for 5 minutes, transfer the upper layer DNA solution to Eppendorf tubes, add equal volumes of 95% ethanol, set at room temperature for 5 minutes, then centrifuge at 12000 rpm for 5 minutes. The precipitate is rinsed with 200 μl 70% ethanol once and then freeze-dried and dissolved in 50 μl sterilized water; (3) Mix equal volume of long and short adaptors (50 μM) (the sequence of long adaptor is: 5 GCTCGAGTCGACCGTGGTACGCGTGTGTGAGCTCCCGGGATCCGGT-3' (SEQ ID NO: 4); the sequence of short adaptor is 5 ACCGGATC-NH_2-3') to give a 4 μl solution, which is incubated in 70°C for 10 minutes; (4) 4 μl adaptor solution (25 μM), 10 μl the solution of

the total DNA enzymatic digestion product (contains about 0.5 μg DNA), 8U T4 ligase, 2 μl T4 ligase buffer; and add water to make a 20 μl system. Ligate at 16°C for 12 hours; (5) After heating at 60°C for 5 minutes, the ligation system used as a template for PCR amplification;

3) Nested PCR and sequencing

Two rounds of PCR amplification are carried out with the aforesaid ligation product.

Based on the sequenced N-terminal amino acid sequence, synthetic gene-specific primers which are abbreviated as 130P1 and 130P2 are designed. Moreover, synthetic adaptor primers abbreviated as AP1 and AP2 are designed based on the long adaptor sequence. The sequences of the primers are as follows:

130P1: 5-ATGGATATHMGNAATCARAAYAARTAYG-3 (SEQ ID NO:5)

130P2: 5-AATAAATATGARGTWGTNTAYCCNGC-3 (SEQ ID NO:6)

AP1: 5-GCTCGAGTCGACCGTGGTA-3 (SEQ ID NO:7)

AP2: 5-GTACGCGTGTGTGAGCTCC-3 (SEQ ID NO:8)

(H=A, C, or T; M-A or C; N=A, G, C, or T; R=A or G; Y=C or T; W=A or T)

(A) First round PCR. 25 μl reaction system contains: 2.5 μl 10 x PCR reaction buffer, 1 μl dNTP (2.5 mM each), 0.5 μl adaptor primer AP1 (20 mM), 0.5 μl specific primer 130P1 (20 mM), 1 μl template (the above ligation product), 0.5 μl ExTaq enzyme. Add sterilized de-ionized water to make it to 25 μl. The PCR conditions are: 94°C, 1 minute for 1 cycle; 94°C, 1 minute, 52°C, 1 minute, 72°C, 1.5 minute for 25 cycles; 72°C, 5 minutes for 1 cycle. The first round PCR product is diluted 50-fold to give the amplification template for the second round of PCR.

(B) Second round PCR. 50 μl reaction system contains: 5 μl 10 x PCR reaction buffer, 2 μl dNTP (2.5 mM each), 1 μl adaptor primer AP2 (20 mM), 1 μl specific primer 130P2 (20 mM), 1 μl template (the product of the first round of PCR), 1 μl ExTaq enzyme. Add

sterilized de-ionized water to make it to 50 μl. The PCR reaction conditions are the same as aforesaid, except that the annealing temperature is 56°C.

(C) The products of the second round of PCR are recovered by PCR product recovering kit (purchased from Veta-Gene Company) and are then ligated to T-vector. The ligation product is used to transform E. coli *E.*DH5α. Perform PCR amplification to screen the transformants by using AP2 and 130P2 as primers and using single colony bacteria as the template. And sequence the exogenous fragments in positive transformant T-vector. The sequencing result is the sequence between position 19 to 491 of the nucleotide in SEQ ID NO:1.

Specific primers abbreviated as 130S1 and 130A1-2 are designed based on the above sequencing result. They are used to screen BAC genomic library containing the gene for crystal protein and various positive transformants, and size of the amplification product is 434 bp. The sequence of the primers are:

130S1-2: TTCTAATACAACATCAAAGTATCCACTC (SEQ ID NO:9)

130A1-2: GGTATCGTTCCAATACTAATTCTAAAC (SEQ ID NO:10)

3. The cloning of insecticidal crystal protein gene *cry7Ba1* of *B. thuringiensis* YBT-978

1) Construction of genomic BAC library of *B. thuringiensis* YBT-978 bacterial strain

Use BAC library vector pBeloBAC11 (as shown in Figure 2) to construct the genomic BAC library of *B. thuringiensis* YBT-978 according to the method disclosed by Luo and Wing (2003) (refer to Luo and Wing. 2003. An improved method for plant BAC library construction, p.3-19. In Grotewold, Erich, Plant functional genomic: methods and protocols. Scientific and medical publishers,/Humana Press, Totowa, USA). That is, *B. thuringiensis* YBT-978 is allowed to grow in LB culture medium till mid-logarithmic growth phase, and then the bacteria is collected by centrifugation at 10000 rpm for 1

minute in clean and sterilized 50 ml centrifugation tubes. Add 1 ml TE buffer (1 mM EDTA, 10 mM Tris base, pH 8.0) to the collected bacteria, which is then lightly dispersed on an vibrator. Add about 40 ml TE buffer, set for 5 minutes and collect the bacteria by centrifugation at 10000 rpm for 1 minute. Set a 50 ml centrifugation tube containing the bacteria in warm water at 50°C and add 1.5 ml TE25S (0.3 M sucrose, 25 mM EDTA, 25 mM Tris Base, pH 8.0), then mix well and add 1.5 ml 2% warmed gel (which is a low melting point gel and specialized for making embedding block, 0.1 g of such gel is dissolved in 5 ml TE25S). Mix well using an autopipette and add the mixture into molds for making embedding blocks, which are then cooled in a refrigerator at 4°C to set the embedding blocks. The remaining steps, which include the enzymatic digestion of YBT-978 in the embedding blocks by *Hin*dIII and the recovery of enzymatic digestion products, the preparation of BAC library vector pBeloBAC11, the ligation of the enzymatic digestion products to the vectors and the qualification of the transformed DH10B and BAC libraries, are identical to the methods reported by Luo and Wing (2003).

2) Screening of BAC genomic library and cloning of the insecticidal crystal protein gene *cry7Ba1*

(A) Screening of BAC genomic library

PCR amplification is carried out by using 130S1 and 130A1 as primers and using BAC library single colony bacteria as the template. The reaction system is 25 μl, the formulation of which is identical to aforesaid. The reaction conditions are the same with aforesaid, except that the annealing temperature is 55°C. Positive clone EMB0491 is selected. Extract recombinant plasmid pBMB0491 according to the method disclosed by J. Sambrook *et al.* (J. Sambrook et al (2002). Molecular Cloning: A Laboratory Manual. 3rd Edition. Translated by Jin Dong-yan et al. Science Press. Beijing). Enzymatic digestion result indicates that pBMB0491 contains an about 60 kb fragment of YBT-978 genome.

(B) Acquisition of insecticidal crystal protein gene *cry7Ba1*

Use *Hind*III to complete enzymatically digest the YBT-978 genomic fragment in the positive clones. The enzymatic digestion product is ligated with cloning vector pUC18 (see Figure 3) which is also complete enzymatically digested by *Hind*III. And the ligation product is used to transform *E. coli.* DH5α. Primers numbered as 130S1-2 and 130A1-2 are again used as primers and transformant single colony bacteria is used as template to perform PCR amplification to screen positive transformant EMB0493. Extract recombinant plasmid pBMB0492 from EMB0493. Enzymatic digestion result indicates that pBMB0492 contains an about 16 kb fragment of YBT-978 genome. Further analysis showed the insecticidal crystal protein gene *cry7Ba1* locates in the 5kb *Xho*I fragment.

(C) Sequence analysis on insecticidal crystal protein gene *cry7Ba1* and insecticidal crystal protein Cry7Ba1

Analyze the nucleotide sequence of 5kb *Xho*I fragment in which the insecticidal crystal protein gene *cry7Ba1* locates and obtain a sequence comprising 5235 bp, wherein 3465 bp are coding region. The coding sequence is shown as SEQ ID NO:1.

The said coding sequence may encode a polypeptide consisting of 1154 amino acids, i.e. insecticidal crystal protein Cry7Ba1, the molecular weight of which is deduced to be 130558 Da.

Searching and comparing this polypeptide with amino acid sequences of other known Cry and Cyt proteins in the GenBank gene database on the web at ncbi.nlm.nih.gov, it is discovered that, among all proteins publicly disclosed, the one which is closest to insecticidal crystal protein Cry7Ba1 is insecticidal crystal protein Cry7Ab2 (GenBank registration number: U04368), Cry7Ab1 (GenBank registration number: U04367) and CryAa1 (GenBank registration number: M64478), with the homology is 58.2%, 57.9% and 57.1% respectively. The homology of the sequence at the N-terminal half (position 1 to 658 amino acid in SEQ ID NO:2) is very low, which is the homology of 37.1%, 37.0%

and 36.4% respectively. Said half is the part responsible for insecticidal activity and is essential and adequate for exerting insecticidal activity (Schnepf H E, Crickmore N, Rie J V, Lereclus D, Baum J, Feitelson J, Zeigler D R, Dean D H. 1998. *B. thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev*, 62:775-806). From this it can be seen the insecticidal gene is a novel insecticidal gene. Therefore, International *B. thuringiensis* Gene Nomenclature Committee named the said insecticidal crystal protein gene as *cry7Ba1* which is determined to be a model gene of *cry7B* gene. Moreover, the sequence is highly similar to the known proteins at the C-terminal half (i.e. position 659 to 1154 amino acids of SEQ ID NO:2), with the homology reaching 87.9%, 86.9% and 87.9% respectively. The sequence of this part is the functional structural domain for forming the parasporal crystal, which is not relevant to insecticidal activity.

It can be seen from the above sequence analysis that, among all disclosed proteins, the homology of the one which is closest to the insecticidal crystal protein Cry7Ba1 of the present invention is 58.2%, which does not exceed 80%. When the homology of the amino acid sequence of a certain protein to the insecticidal crystal protein Cry7Ba1 exceeds 80%, it is deemed as falling within the scope of insecticidal crystal protein Cry7Ba1 of the present invention. Similarly, among all disclosed proteins, the homology of the one which is closest to the N-terminal half (position 1 to 658 amino acids of SEQ ID NO:2) of the insecticidal crystal protein Cry7Ba1 of the present invention is 37.1%, which does not exceed 50%. When the homology of amino acid sequence of a protein to the N-terminal half (position 1 to 658 amino acids of SEQ ID NO:2) of insecticidal crystal protein Cry7Ba1 exceeds 50%, it is deemed as falling within the scope of insecticidal crystal protein Cry7Ba1 of the present invention.

4. Insecticidal activity of insecticidal crystal protein Cry7Ba1 of *B. thuringiensis* YBT-978

5 kb *Xho*I fragment in which *cry7Ba1* locates is transferred to *E. coli-B. thuringiensis* shuttle vector pHT304 (see Figure 4, for origin of the vector, please refer to Arantes O and Lereclus D. 1991. Construction of cloning vectors for *B. thuringiensis*. Gene 108:115-119)

to give a recombinant plasmid pBMB0495 (see Figure 5). Then, pBMB0495 is transformed to an acrystalliferous mutant of *B. thuringiensis* CryB strain (for the origin of the strain, please refer to Wu D, Federici BA. 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis. J. Bacteroil.*, 175:5276-5280) using well established electroporation method (Wu Lan, Sun Ming, Yu Ziniu. A New Resolution Vector with *cry1Ac10* Gene Based on *Bacillus thuringiensis* Transposon Tn4430. Acta Microbiologica Sinica. 2000, 40:264-269) to give recombinant bacteria BMB-0502.

(1) Expression of insecticidal crystal protein Cry7Ba1

Activate BMB0502 by incubating it in LB culture medium (erythromycin can be added at a final concentration of 20 μg/ml) overnight. Then, it is transferred to ICPM culture medium (erythromycin can be added at a final concentration of 20 μg/ml), incubate until gemma completely mature and fall off. Collect the bacteria and rinse them with 0.5% NaCl and sterilized de-ionised water separately for 3 times. Re-mix the bacteria with water at the ratio bacteria: sterilized de-ionised water = 1:5, then add an equal volume of 2x loading buffer and mix well. Incubate in a boiling water bath for 3 to 5 minutes, then centrifuge at 12000 rpm for 5 minutes. Perform SDS-PAGE electrophoresis with the supernatants. For the formulation of 2x loading buffer and the procedure for performing SDS-PAGE electrophoresis, please refer to the method recited by Laemmli (Laemmli, UK. 1970. Digestion of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) 227:680-685). As shown in Figure 6, BMB0502 can form a 130 kDa crystal protein, the size of which is identical to YBT-978 of the starting strain, whilst negative control BMB0503 (which only contains the shuttle vector pHT304) does not form such a corresponding band.

(2) Crystal morphology of insecticidal crystal protein Cry7Ba1

Activate BMB0502 by incubating it in LB culture medium (erythromycin can be

added at a final concentration of 20 μg/ml) overnight. Then, it is transferred to ICPM culture medium (erythromycin can be added at a final concentration of 20 μg/ml), incubate until gemma almost mature and fall off. Collect the bacteria and rinse them with sterilized de-ionised water once. Re-mix the bacteria with water at the ratio bacteria: sterilized de-ionised water = 1:5. Prepare samples for transmission electron microscopy (TEM) and embed with Epon812. Make ultra-thin slices, perform transmission observation and photo taking at 80 kv. MBM0502 can form bi-pyramid shaped parasporal crystal, which is shown in Figure 7.

(3) Evaluation of insecticidal effect of insecticidal crystal protein Cry7Ba1

Insecticidal effect of insecticidal crystal protein Cry7Ba1 is evaluated using third instar larvae of *Plutella xylostella*. Activate BMB0502 by incubating it in LB culture medium (erythromycin can be added at a final concentration of 20 μg/ml) overnight. Then, it is transferred to ICPM culture medium (erythromycin can be added at a final concentration of 20 μg/ml), incubate till gemma almost mature and fall off. Collect the bacteria and rinse them with sterilized de-ionised water once to give a parasporal crystal mixture. Perform bioassay using standard assay procedure (please refer to Shen Ju-qun, Wang Mian-ju, Yu Zi-niu (1990). Standard Procedure and Method for *Bacillus thuringiensis* formulations. Chinese Journal of Biological Control (Suppl.): 12-16). The bioassay is repeated once for each of the samples. Calculate LC_{50}. The results showed the insecticidal crystal protein Cry7Ba1 exerted very high toxicity against *Plutella xylostella* (as shown in Table 1). This means the Cry7Ba1 protein cloned in the present invention shows very high insecticidal activity against insects of the Lepidoptera Order.

Table 1 Experimental results from bioassay of the Cry7Ba1 protein cloned in the present invention against *Plutella xylostella*

Time (Day)	Material	Regression equation	Regression coefficient	LC_{50} value (95% confidence limit) (ng/ml)
3	BMB0502	y=0.9928x + 3.0334	0.9946	95.7 (93.9-97.5)
5	BMB0502	y=1.0554x+3.3103	0.9901	39.9 (38.2-41.6)

Description of the drawings

Figure 1: Technical flowchart of the present invention

Figure 2: Construction chart of BAC vector pBleoBAC11 of the example of the present invention

Figure 3: Construction chart of cloning vector pUC18 constructed in the present invention

Figure 4: Construction chart of the shuttle vector constructed in the example of the present invention

Figure 5: Recombinant plasmid pBMB0495 constructed in the example of the present invention

Figure 6: SDS-PAGE electrophoresis analysis of *Bacillus thuringiensis* Cry7Ba1 parasporal crystal protein cloned in the present invention

M: molecular weight markers

1: parasporal crystal protein of YBT-978 strain

2: parasporal crystal protein of BMB0502 strain

3: control BMB0503 strain

Figure 7: Morphology of parasporal crystal produced by *Bacillus thuringiensis* YBT-978 and BMB0502 strains involved in the present invention (the line standard represents 1 μm)

A, B: parasporal crystal morphology of YBT-978 strain;

C, D: parasporal crystal morphology of BMB0502 strain.

Specific Embodiments of the Present Invention

The followings are examples showing the present invention. It should be noted that the examples only demonstrate the present invention but by no means limit the present invention. The various experimental procedures involved are standard techniques in the field of the invention. For the contents that are not specifically explained, skilled persons in the art can refer to various reference books, scientific articles, relevant manuals and handbooks that are available prior to the application date of the present invention to carry out the said contents.

Example 1 The cloning of insecticidal crystal protein gene *cry7Ba1* of *Bacillus thuringiensis* YBT-978

According to the method disclosed by Luo and Wing (2003) (refer to Luo and Wing. 2003. An improved method for plant BAC library construction, p.3-19. In Grotewold, Erich, Plant functional genomic: methods and protocols. Scientific and medical publishers,/Humana Press, Totowa, USA), BAC library vector pBeloBAC11 was used to construct genomic BAC library of *Bacillus thuringiensis* YBT-978 strain (for the source of the bacterial strain, please refer to Dai J et al. 1996. *Bacillus thuringiensis* subsp. huazhongensis, serotype H40, isolated from soils in the People's Republic of China. *Letters in Applied Microbiology*. 22(1): 42-45). Primers numbered as 130S1-2 and 130A1-2 were used as primers and BAC library single colony bacteria is used as the template to perform PCR amplification. Positive clone EMB0491 was selected. *Hin*dIII was used to complete enzymatically digest the YBT-978 genomic fragment in the positive clones. The enzymatic digestion product was ligated with cloning vector pUC18 which was also completely enzymatically digested by *Hin*dIII to transform *E. coli.* DH5α. Primers numbered as 130S1-2 and 130A1-2 were again used as primers and transformant single colony bacteria is used as template to perform PCR amplification to screen positive transformant EMB0493. Recombinant plasmid pBMB0492 was extracted from EMB0493.

Enzymatic digestion result revealed that pBMB0492 contains a fragment of about 16 kb of YBT-978 genome. Further analysis showed the insecticidal crystal protein gene *cry7Ba1* locates in the 5kb *Xho*I fragment.

Example 2 Sequence Analysis on insecticidal crystal protein gene *cry7Ba1* of *Bacillus thuringiensis* YBT-978

The nucleotide sequence of the 5kb *Xho*I fragment, in which the insecticidal crystal protein gene *cry7Ba1* locates, was analyzed. The result was that it was a sequence comprising 5235 bp, wherein 3465 bp were the coding sequence. The coding sequence is shown as SEQ ID NO:1.

The said coding sequence might encode a polypeptide consisting of 1154 amino acids, the molecular weight of which was deduced to be 130558 Da.

Comparing this polypeptide with amino acid sequences of other known Cry and Cyt proteins, it was discovered that it was closest to Cry7Ab2, Cry7Ab1 and CryAa1, with the homology being 58.2%, 57.9% and 57.1% respectively. The homology of the sequence at the N-terminal half (position 1 to 658 amino acid in SEQ ID NO:1) was very low, with the homology being 37.1%, 37.0% and 36.4% respectively. As the polypeptide was similar to Cry7A insecticidal crystal protein, it was named by the International *Bacillus thuringiensis* Gene Nomenclature Committee as *cry7Ba1*.

Example 3 Expression of insecticidal crystal protein gene *cry7Ba1*

5 kb *Xho*I fragment, in which *cry7Ba1* locates, was transferred to *E. coli-B. thuringiensis* shuttle vector pHT304 (for origin of the vector, please refer to Arantes O and Lereclus D. 1991. Construction of cloning vectors for *Bacillus thuringiensis*. Gene 108:115-119) to give recombinant plasmid pBMB0495. Then, pBMB0495 was transferred to an acrystalliferous mutant of *Bacillus thuringiensis* CryB strain (for the

origin of the strain, please refer to Wu D, Federici BA. 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J. Bacteroil.*, 175:5276-5280) using well established electroporation method (Wu Lan, Sun Ming, Yu Ziniu. A New Resolution Vector with *cry1Ac10* Gene Based on *Bacillus thuringiensis* Transposon Tn4430. Acta Microbiologica Sinica. 2000, 40:264-269) to give recombinant bacteria BMB-0502.

The above recombinant bacteria named as BMB0502 was activated by incubating it in LB culture medium (erythromycin could be added at a final concentration of 20 μg/ml) overnight. Then, it was transferred to ICPM culture medium (erythromycin could be added at a final concentration of 20 μg/ml), incubation was carried out until gemma completely matured and fell off. The bacteria was collected and rinsed with 0.5% NaCl and sterilized de-ionised water separately for 3 times. The bacteria was re-mixed with water at the ratio bacteria: sterilized de-ionised water = 1:5, then an equal volume of 2x loading buffer was added and mixed well. The mixture was treated in a boiling water bath for 3 to 5 minutes, then centrifuged at 12000 rpm for 5 minutes. SDS-PAGE electrophoresis was performed for the supernatants. For the formulation of 2x loading buffer and the procedure for performing SDS-PAGE electrophoresis, please refer to the method recited by Laemmli (Laemmli, UK. 1970. Digestion of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) 227:680-685). As shown in Figure 6, BMB0502 forms a 130 kDa crystal protein, the size of which was identical to YBT-978 of the starting strain, whilst the negative control BMB0503 (which only contained the shuttle vector pHT304) did not form such a corresponding band.

Example 4: Insecticidal effect of insecticidal crystal protein Cry7Ba1

Insecticidal effect of insecticidal crystal protein Cry7Ba1 was evaluated using third instar larvae of *Plutella xylostella*. BMB0502 was activated by incubating it in LB culture medium (erythromycin could be added at a final concentration of 20 μg/ml) overnight. Then, it was transferred to ICPM culture medium (erythromycin could be added at a final

concentration of 20 μg/ml), incubation was carried out until gemma almost matured and fell off. The bacteria was collected and rinsed with sterilized de-ionised water once to give a parasporal crystal mixture. Bioassay was performed using standard assay procedure (please refer to Shen Ju-qun, Wang Mian-ju, Yu Zi-niu (1990). Standard Procedure and Method for *Bacillus thuringiensis* formulations. Chinese Journal of Biological Control (Suppl.): 12-16). The bioassay was repeated once for each of the samples. LC_{50} was calculated. The results showed the insecticidal crystal protein Cry7Ba1 exerted very high toxicity against *Plutella xylostella*. This means the Cry7Ba1 protein cloned in the present invention showed very high insecticidal activity against insects of the Lepidoptera. The effects achieved by carrying out the example was shown in Table 1.

Claims

1. An isolated insecticidal crystal protein gene from *Bacillus thuringiensis*, wherein said gene encodes a Cry7Ba1 protein having at least 80% homology to the amino acid sequence shown in SEQ ID NO:2.

2. The isolated insecticidal crystal protein gene from *Bacillus thuringiensis* of claim 1, wherein said gene comprises a nucleotide sequence shown in SEQ ID NO:1.

3. An isolated insecticidal crystal protein gene from *Bacillus thuringiensis*, wherein said gene encodes a Cry7Ba1 protein comprising an amino acid sequence having at least 50% homology to the partial sequence numbered 1 to 658 of SEQ ID NO:2.

4. A recombinant DNA construct comprising the insecticidal crystal protein gene of claim 1 or 3.

5. A micro-organism comprising the recombinant DNA construct of claim 4.

6. A *Bacillus thuringiensis* insecticidal crystal protein comprising an amino acid sequence having at least 80% homology to the amino acid sequence shown in SEQ ID NO:2.

7. The *Bacillus thuringiensis* insecticidal crystal protein of claim 5, wherein said protein comprises an amino acid sequence shown in SEQ ID NO: 2.

8. A *Bacillus thuringiensis* insecticidal crystal protein comprising an amino acid sequence having at least 50% homology to the partial sequence numbered 1 to 658 of SEQ ID NO:2.

9. An insecticidal fragment of the amino acid sequence as shown in SEQ ID NO:2.

10. A plant cell transformed with a nucleotide sequence encoding the amino acid sequence of claim 9.

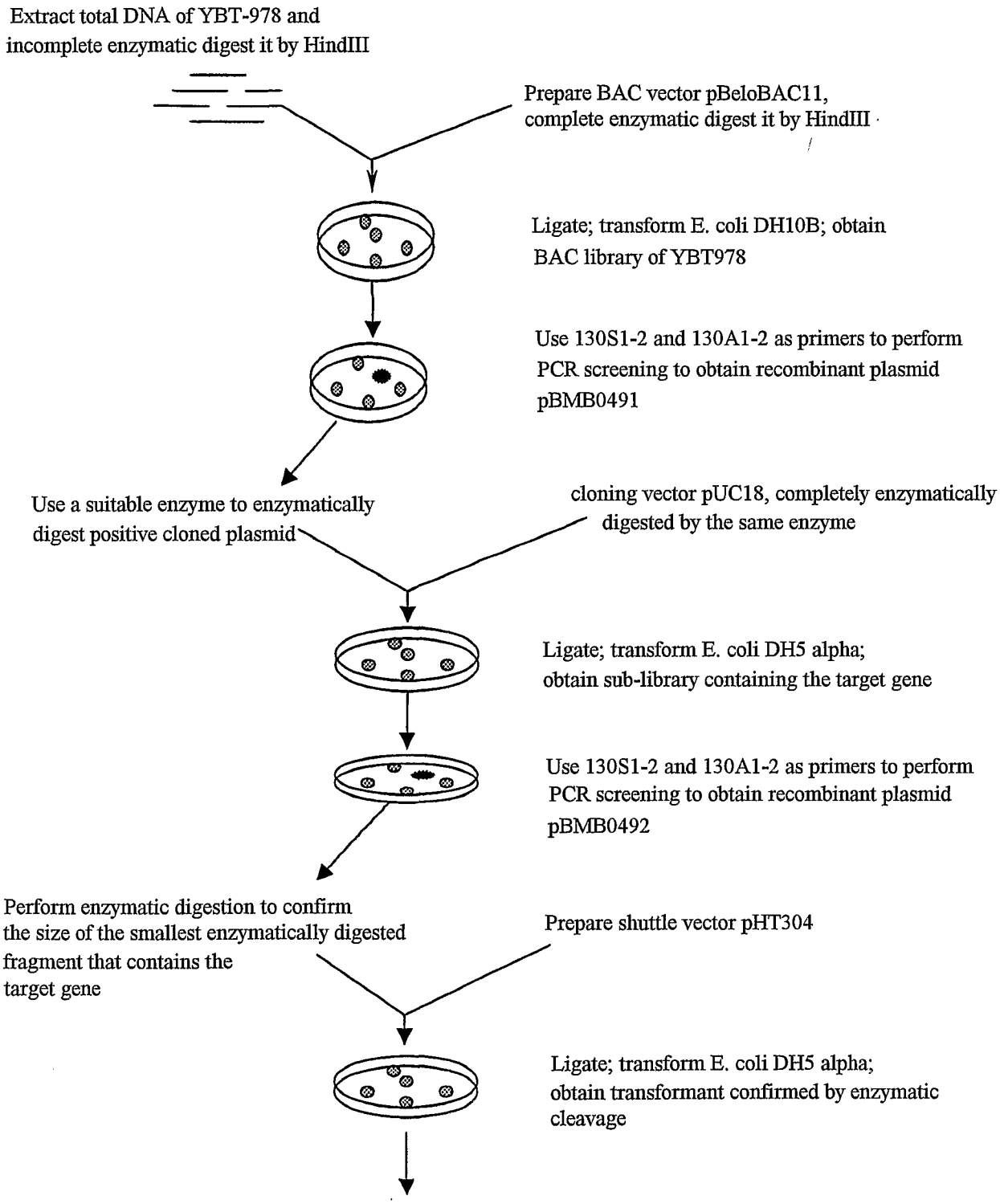
Extract total DNA of YBT-978 and incomplete enzymatic digest it by HindIII
Prepare BAC vector pBeloBAC11, complete enzymatic digest it by HindIII
Ligate; transform E. coli DH10B; obtain BAC library of YBT978
Use 130S1-2 and 130A1-2 as primers to perform PCR screening to obtain recombinant plasmid pBMB0491
Use a suitable enzyme to enzymatically digest positive cloned plasmid
cloning vector pUC18, completely enzymatically digested by the same enzyme
Ligate; transform E. coli DH5 alpha; obtain sub-library containing the target gene
Use 130S1-2 and 130A1-2 as primers to perform PCR screening to obtain recombinant plasmid pBMB0492
Perform enzymatic digestion to confirm the size of the smallest enzymatically digested fragment that contains the target gene
Prepare shuttle vector pHT304
Ligate; transform E. coli DH5 alpha; obtain transformant confirmed by enzymatic cleavage
Extract correct transformant plasmid pBMB0495, transform CryB, culture the crystal, perform bioassay

Figure 1

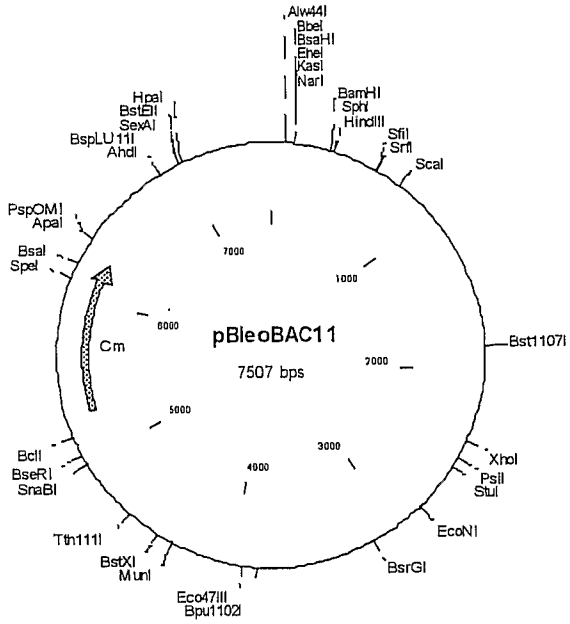

Figure 2

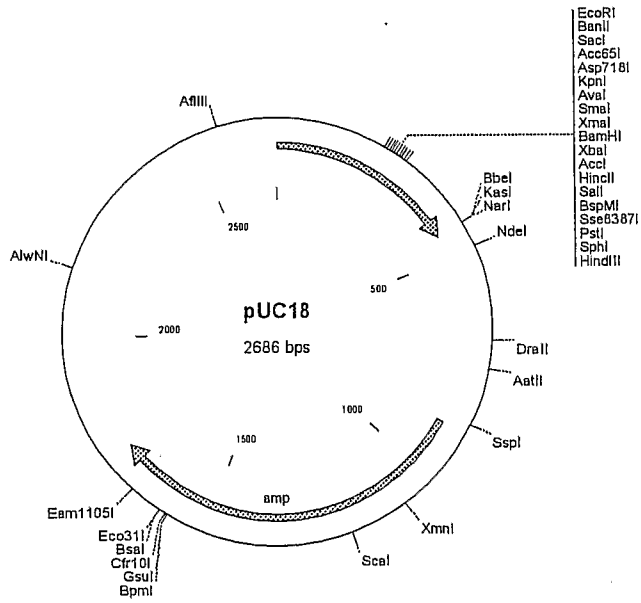

Figure 3

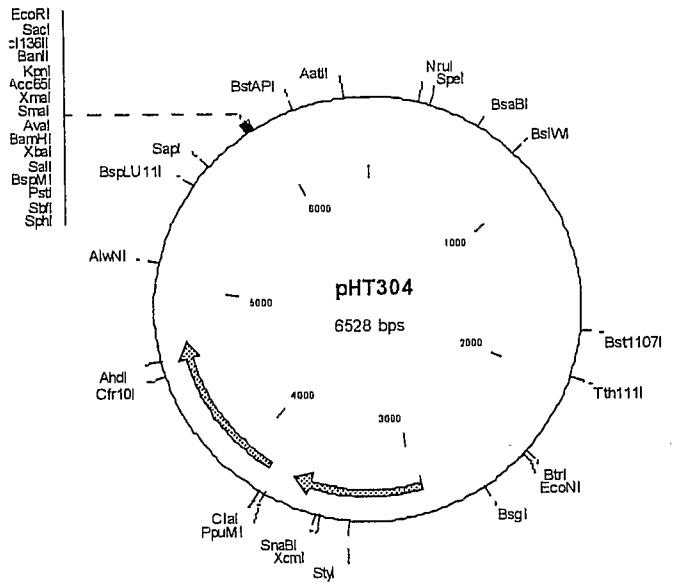

Figure 4

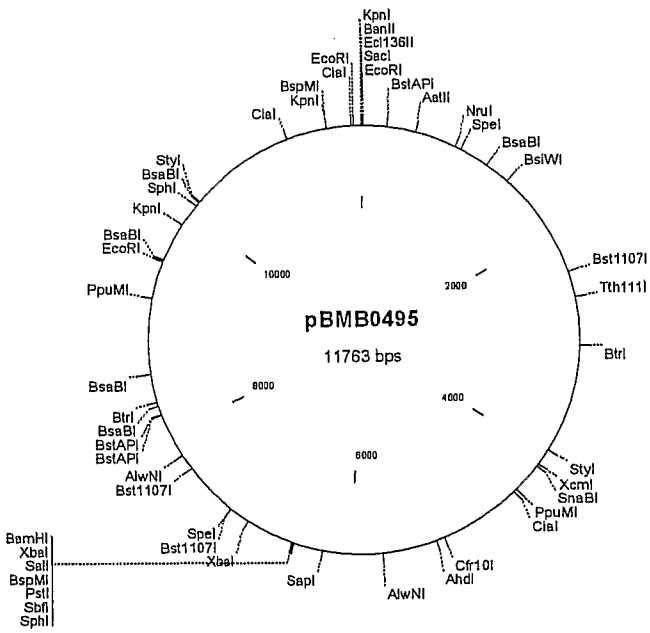

Figure 5

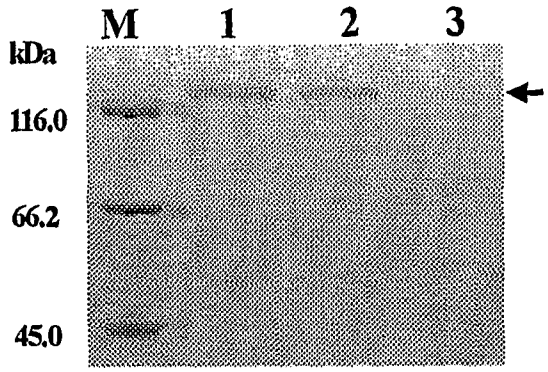

Figure 6

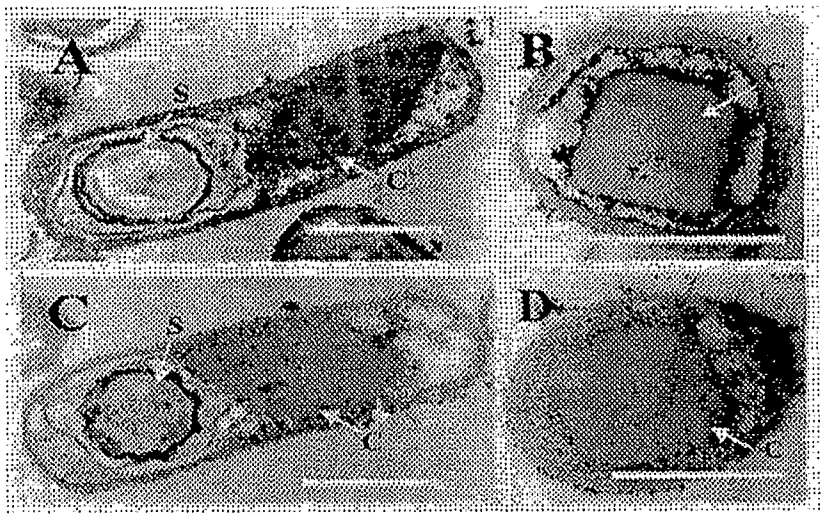

Figure 7

SEQUENCE LISTING

<110> Huazhong Agricultural University

<120> Gene cry7Ba1 Encoding An Insecticidal Crystal Protein of Bacillus thuringiensis

<130>

<141> 2005-10-13

<160> 2

<170> PatentIn version 3.1

<210> 1

<211> 3465

<212> DNA

<213> Bacillus thuringiensis

<220>

<221> gene

<222> (1)..(3465)

<223>

<220>

<221> CDS

<222> (1)..(3465)

<223>

<400> 1

```
atg gat ata aga aat caa aat aaa tat gaa gta gta tac cca gca ata       48
Met Asp Ile Arg Asn Gln Asn Lys Tyr Glu Val Val Tyr Pro Ala Ile
1               5                  10                  15

aat gaa aca act tct aat aca aca tca aag tat cca ctc gca agt gat       96
Asn Glu Thr Thr Ser Asn Thr Thr Ser Lys Tyr Pro Leu Ala Ser Asp
            20                  25                  30

cca atc aaa caa tat caa aat atg aat tat aaa gat agt ttg aat ata      144
Pro Ile Lys Gln Tyr Gln Asn Met Asn Tyr Lys Asp Ser Leu Asn Ile
        35                  40                  45

att gag ggg aat aac gta atc act cca gta tct gga act gct gtt ttg      192
Ile Glu Gly Asn Asn Val Ile Thr Pro Val Ser Gly Thr Ala Val Leu
    50                  55                  60
```

```
gca act gca agg aaa att ggt ggt aag atc gtt aag gct ata ggg gaa      240
Ala Thr Ala Arg Lys Ile Gly Gly Lys Ile Val Lys Ala Ile Gly Glu
65                  70                  75                  80
caa atc ttg tct aaa atc ttg aaa gag att ctt gat tat tta tgg ccg      288
Gln Ile Leu Ser Lys Ile Leu Lys Glu Ile Leu Asp Tyr Leu Trp Pro
                85                  90                  95
tct tca agc tcg tcc aat tca tgg gaa gag atg atg aag gaa gta gag      336
Ser Ser Ser Ser Ser Asn Ser Trp Glu Glu Met Met Lys Glu Val Glu
            100                 105                 110
tat ctt att gat aaa aaa ata gag gaa tat gca aga aat aag gca ctt      384
Tyr Leu Ile Asp Lys Lys Ile Glu Glu Tyr Ala Arg Asn Lys Ala Leu
        115                 120                 125
gcg gtt ttg gaa gga ata gga aat gct gta gaa tcc tat tat agt gca      432
Ala Val Leu Glu Gly Ile Gly Asn Ala Val Glu Ser Tyr Tyr Ser Ala
    130                 135                 140
tta gaa gcg tgg gaa tta gaa tct agt gaa cgt agt tta gaa tta gta      480
Leu Glu Ala Trp Glu Leu Glu Ser Ser Glu Arg Ser Leu Glu Leu Val
145                 150                 155                 160
ttg gaa cga tat cag ttt gcg gtg cag ttt gca aga agt tca atg cca      528
Leu Glu Arg Tyr Gln Phe Ala Val Gln Phe Ala Arg Ser Ser Met Pro
                165                 170                 175
tca ttt gcg att ata aat tat gaa att ccc tta tta gca aca tat gca      576
Ser Phe Ala Ile Ile Asn Tyr Glu Ile Pro Leu Leu Ala Thr Tyr Ala
            180                 185                 190
aat gct gca aat gtt cat tta ctt tta atg aga gat ata caa ata tac      624
Asn Ala Ala Asn Val His Leu Leu Leu Met Arg Asp Ile Gln Ile Tyr
        195                 200                 205
ggg gat aga tgg gga ata tct caa aat gat atg aat ctc ttc tta aaa      672
Gly Asp Arg Trp Gly Ile Ser Gln Asn Asp Met Asn Leu Phe Leu Lys
    210                 215                 220
gaa caa gaa ata tac acg tct gaa tat tcg gaa cat tgc gta aag tgg      720
Glu Gln Glu Ile Tyr Thr Ser Glu Tyr Ser Glu His Cys Val Lys Trp
225                 230                 235                 240
tat aat gag gga tta aat caa ttg aaa act aaa ggt ggc gca agt ggt      768
Tyr Asn Glu Gly Leu Asn Gln Leu Lys Thr Lys Gly Gly Ala Ser Gly
                245                 250                 255
```

```
tta gtt tgg gag aat tat aac agt ttc cgt aca gaa atg aca att atg      816
Leu Val Trp Glu Asn Tyr Asn Ser Phe Arg Thr Glu Met Thr Ile Met
            260                 265                 270
gta tta gat ctt gta gct ata ttt cca gcc tac aat atg agc aaa tat      864
Val Leu Asp Leu Val Ala Ile Phe Pro Ala Tyr Asn Met Ser Lys Tyr
        275                 280                 285
cct ata gaa tca aca gta gaa tta aca aga aca att tat aca gat cca      912
Pro Ile Glu Ser Thr Val Glu Leu Thr Arg Thr Ile Tyr Thr Asp Pro
    290                 295                 300
ctt ggt tac aca ggg tat agc aat gat gaa cat ccc aca tat tat tct      960
Leu Gly Tyr Thr Gly Tyr Ser Asn Asp Glu His Pro Thr Tyr Tyr Ser
305                 310                 315                 320
tct gca aaa cca ttt tca tca ata gag agt aga gcc gta cta gca ccc     1008
Ser Ala Lys Pro Phe Ser Ser Ile Glu Ser Arg Ala Val Leu Ala Pro
                325                 330                 335
tca tta ttc aaa tgg atc act caa ctt gaa gta tat aca aaa aaa tac     1056
Ser Leu Phe Lys Trp Ile Thr Gln Leu Glu Val Tyr Thr Lys Lys Tyr
            340                 345                 350
agc tac tct tcc caa tat act acg ttg tgg act gga cta aga gtg att     1104
Ser Tyr Ser Ser Gln Tyr Thr Thr Leu Trp Thr Gly Leu Arg Val Ile
        355                 360                 365
gct cag cct act aaa gat ttt act gat act gta tat gat tac gga agt     1152
Ala Gln Pro Thr Lys Asp Phe Thr Asp Thr Val Tyr Asp Tyr Gly Ser
    370                 375                 380
tct tcg ggt tct gag aac aag gat gtc ttt gac ctt tat ggc aat gat     1200
Ser Ser Gly Ser Glu Asn Lys Asp Val Phe Asp Leu Tyr Gly Asn Asp
385                 390                 395                 400
gta tat gac aca caa agt gtt gtt tca tcg tat aag cct aca ggt ggt     1248
Val Tyr Asp Thr Gln Ser Val Val Ser Ser Tyr Lys Pro Thr Gly Gly
                405                 410                 415
ggc cat ttt ggg gtt cct cag ttt aga tta ttc tgg att act aaa tct     1296
Gly His Phe Gly Val Pro Gln Phe Arg Leu Phe Trp Ile Thr Lys Ser
            420                 425                 430
aat ggg cta aga gaa caa att ttt aat tat gcg aat aat atg ggt tct     1344
Asn Gly Leu Arg Glu Gln Ile Phe Asn Tyr Ala Asn Asn Met Gly Ser
        435                 440                 445
```

```
tac agt gcg tat agg ttt agt aag gac gaa tta cca ata gaa ttg ttg     1392
Tyr Ser Ala Tyr Arg Phe Ser Lys Asp Glu Leu Pro Ile Glu Leu Leu
    450                 455                 460
cag cca cct ctt ttt gga gat ata gag gaa tac agt cat agg tta agt     1440
Gln Pro Pro Leu Phe Gly Asp Ile Glu Glu Tyr Ser His Arg Leu Ser
465                 470                 475                 480
cac gtt tca gag gta att aaa gat tat ggt gaa gga atc att cct gta     1488
His Val Ser Glu Val Ile Lys Asp Tyr Gly Glu Gly Ile Ile Pro Val
                485                 490                 495
tta ggt tgg aca cat gta agt gta act cgt gac aat aga att tat cca     1536
Leu Gly Trp Thr His Val Ser Val Thr Arg Asp Asn Arg Ile Tyr Pro
            500                 505                 510
gat aag att aca caa ctt cca gcg gta aaa atg tat gag tta cta agc     1584
Asp Lys Ile Thr Gln Leu Pro Ala Val Lys Met Tyr Glu Leu Leu Ser
        515                 520                 525
tca gcc gtt gtt gta aaa gga cct gga ttt aca ggt gga gat tta gtt     1632
Ser Ala Val Val Val Lys Gly Pro Gly Phe Thr Gly Gly Asp Leu Val
    530                 535                 540
aag aga acg ggc aat ggt ggc att gga cat ttt aat gtt agt gta gag     1680
Lys Arg Thr Gly Asn Gly Gly Ile Gly His Phe Asn Val Ser Val Glu
545                 550                 555                 560
tcc cct ggt act cag agg tat cgc ctg aga ata cgt tat agt tca gag     1728
Ser Pro Gly Thr Gln Arg Tyr Arg Leu Arg Ile Arg Tyr Ser Ser Glu
                565                 570                 575
gtt agt gga gta ttt cat atg caa att aac gat ata gaa act att cag     1776
Val Ser Gly Val Phe His Met Gln Ile Asn Asp Ile Glu Thr Ile Gln
            580                 585                 590
gga gaa ttt agt agt act gct gat tca aca agt act ctg tca agc gaa     1824
Gly Glu Phe Ser Ser Thr Ala Asp Ser Thr Ser Thr Leu Ser Ser Glu
        595                 600                 605
gca ttt caa ctt aga gaa tac tcc act acc ttc acg ttt cca aca aat     1872
Ala Phe Gln Leu Arg Glu Tyr Ser Thr Thr Phe Thr Phe Pro Thr Asn
    610                 615                 620
atg aca aag ata aag gta tct tta ggt gct att gaa ggt gca gga gga     1920
Met Thr Lys Ile Lys Val Ser Leu Gly Ala Ile Glu Gly Ala Gly Gly
625                 630                 635                 640
```

```
ttc tat tta gat aga att gaa ttc att cca gta gat gaa aat cac gat     1968
Phe Tyr Leu Asp Arg Ile Glu Phe Ile Pro Val Asp Glu Asn His Asp
                645                 650                 655
aac aga gta aca cta gaa aaa gca cag aaa gcc gtg aat gcc ttg ttt     2016
Asn Arg Val Thr Leu Glu Lys Ala Gln Lys Ala Val Asn Ala Leu Phe
            660                 665                 670
aca gcg gga aga aac gca cta caa aca gat gtg aca gat tac aaa gta     2064
Thr Ala Gly Arg Asn Ala Leu Gln Thr Asp Val Thr Asp Tyr Lys Val
        675                 680                 685
gat cag gtt tcc att tta gtg gat tgt gta tca ggg gag tta tat cca     2112
Asp Gln Val Ser Ile Leu Val Asp Cys Val Ser Gly Glu Leu Tyr Pro
    690                 695                 700
aat gag aaa cgc gaa cta ctc agt tta gtc aaa tac gca aaa cgt ttg     2160
Asn Glu Lys Arg Glu Leu Leu Ser Leu Val Lys Tyr Ala Lys Arg Leu
705                 710                 715                 720
agc tat tct cgt aat tta ctc cta gat cca aca ttc gat tct att aat     2208
Ser Tyr Ser Arg Asn Leu Leu Leu Asp Pro Thr Phe Asp Ser Ile Asn
                725                 730                 735
tcg tca gat gag aat ggc tgg tac gga agt aat ggt att gca att gga     2256
Ser Ser Asp Glu Asn Gly Trp Tyr Gly Ser Asn Gly Ile Ala Ile Gly
            740                 745                 750
aat ggg aac ttt gta ttc aaa gga aac tat tta att ttc tca ggt acc     2304
Asn Gly Asn Phe Val Phe Lys Gly Asn Tyr Leu Ile Phe Ser Gly Thr
        755                 760                 765
aat gat aca caa tac cca acg tat ctc tat caa aaa att gat gaa tcc     2352
Asn Asp Thr Gln Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu Ser
    770                 775                 780
aag ctc aaa gaa tat aca cgc tat aaa ctg aga gga ttt atc gaa aat     2400
Lys Leu Lys Glu Tyr Thr Arg Tyr Lys Leu Arg Gly Phe Ile Glu Asn
785                 790                 795                 800
agt caa gat tta gaa gca tat gtg att cgc tat gat gca aaa cat gaa     2448
Ser Gln Asp Leu Glu Ala Tyr Val Ile Arg Tyr Asp Ala Lys His Glu
                805                 810                 815
aca ttg gat gta tcc aat aat cta ttg ccg gat att tct cct gta aat     2496
Thr Leu Asp Val Ser Asn Asn Leu Leu Pro Asp Ile Ser Pro Val Asn
            820                 825                 830
```

```
gca tgc gga gaa cca aat cgt tgt gct gca tta caa tac ctg gat gaa     2544
Ala Cys Gly Glu Pro Asn Arg Cys Ala Ala Leu Gln Tyr Leu Asp Glu
        835                 840                 845
aat cca agg tta gaa tgt agt tcg ata caa gac ggt att tta tct gat     2592
Asn Pro Arg Leu Glu Cys Ser Ser Ile Gln Asp Gly Ile Leu Ser Asp
    850                 855                 860
tcg cat tcg ttc tct ctc aat ata gat aca ggt tct att gat ttc aat     2640
Ser His Ser Phe Ser Leu Asn Ile Asp Thr Gly Ser Ile Asp Phe Asn
865                 870                 875                 880
gag aac gta gga att tgg gtg ttg ttt aaa att tcc aca ccg gaa ggg     2688
Glu Asn Val Gly Ile Trp Val Leu Phe Lys Ile Ser Thr Pro Glu Gly
                885                 890                 895
tat gcg aaa ttt gga aac cta gaa gtg att gaa gat agc cca gtc att     2736
Tyr Ala Lys Phe Gly Asn Leu Glu Val Ile Glu Asp Ser Pro Val Ile
            900                 905                 910
gga gaa gca tta gcc cgt gta aaa cgc caa gaa acg aag tgg cga aac     2784
Gly Glu Ala Leu Ala Arg Val Lys Arg Gln Glu Thr Lys Trp Arg Asn
        915                 920                 925
aag ttg aca caa ctg cga acg gaa aca caa gcg att tat aca cga gca     2832
Lys Leu Thr Gln Leu Arg Thr Glu Thr Gln Ala Ile Tyr Thr Arg Ala
    930                 935                 940
aaa caa gcc att gat aat gta ttc aca aat gca cag gac tct cac tta     2880
Lys Gln Ala Ile Asp Asn Val Phe Thr Asn Ala Gln Asp Ser His Leu
945                 950                 955                 960
aaa ata ggt acg aca ttt gcg gca att gtg gct gcg cga aag att gtc     2928
Lys Ile Gly Thr Thr Phe Ala Ala Ile Val Ala Ala Arg Lys Ile Val
                965                 970                 975
caa tcc ata cgc gaa gcg tat atg tca tgg tta tca atc gtt cca ggt     2976
Gln Ser Ile Arg Glu Ala Tyr Met Ser Trp Leu Ser Ile Val Pro Gly
            980                 985                 990
gta aat tat cct att ttt aca gag  ttg aat gag aga gta  cag cga gca   3024
Val Asn Tyr Pro Ile Phe Thr Glu  Leu Asn Glu Arg Val  Gln Arg Ala
        995                 1000                1005
ttt caa  tta tat gat gta cgg  aat gtc gtg cgt aat  ggc cga ttc      3069
Phe Gln  Leu Tyr Asp Val Arg  Asn Val Val Arg Asn  Gly Arg Phe
    1010                1015                1020
```

ctg aat gga gta tcg gat tgg att gtg aca tct gat gta aag gta 3114
Leu Asn Gly Val Ser Asp Trp Ile Val Thr Ser Asp Val Lys Val
1025 1030 1035

caa gaa gaa aat ggg aac aat gta tta gtt ctt tcc aat tgg gat 3159
Gln Glu Glu Asn Gly Asn Asn Val Leu Val Leu Ser Asn Trp Asp
1040 1045 1050

gcg caa gta tta caa tgt ctg aag ctc tat caa gat cgc gga tat 3204
Ala Gln Val Leu Gln Cys Leu Lys Leu Tyr Gln Asp Arg Gly Tyr
1055 1060 1065

atc ttg cgt gta acg gca cgt aaa gaa gga ttg gga gaa gga tat 3249
Ile Leu Arg Val Thr Ala Arg Lys Glu Gly Leu Gly Glu Gly Tyr
1070 1075 1080

att aca att acg gat gaa gaa ggg cat aca gat caa ttg aca ttt 3294
Ile Thr Ile Thr Asp Glu Glu Gly His Thr Asp Gln Leu Thr Phe
1085 1090 1095

ggc aca tgt gag gaa ata gat gca tct aac acg ttc gta acc aca 3339
Gly Thr Cys Glu Glu Ile Asp Ala Ser Asn Thr Phe Val Thr Thr
1100 1105 1110

ggt tat att aca aaa gaa cta gaa ttt ttc cca gat aca gag aaa 3384
Gly Tyr Ile Thr Lys Glu Leu Glu Phe Phe Pro Asp Thr Glu Lys
1115 1120 1125

gtg cgt ata gaa att ggg gaa aca gaa gga acc ttc cag gta gaa 3429
Val Arg Ile Glu Ile Gly Glu Thr Glu Gly Thr Phe Gln Val Glu
1130 1135 1140

agt ata gag tta ttt ttg atg gaa gat cta tgt taa 3465
Ser Ile Glu Leu Phe Leu Met Glu Asp Leu Cys
1145 1150

<210> 2
<211> 1154
<212> PRT
<213> Bacillus thuringiensis
<400> 2

Met Asp Ile Arg Asn Gln Asn Lys Tyr Glu Val Val Tyr Pro Ala Ile
1 5 10 15
Asn Glu Thr Thr Ser Asn Thr Thr Ser Lys Tyr Pro Leu Ala Ser Asp

SUBSTITUTE SHEET (RULE 26)

20 25 30

Pro Ile Lys Gln Tyr Gln Asn Met Asn Tyr Lys Asp Ser Leu Asn Ile

35 40 45

Ile Glu Gly Asn Asn Val Ile Thr Pro Val Ser Gly Thr Ala Val Leu

50 55 60

Ala Thr Ala Arg Lys Ile Gly Gly Lys Ile Val Lys Ala Ile Gly Glu

65 70 75 80

Gln Ile Leu Ser Lys Ile Leu Lys Glu Ile Leu Asp Tyr Leu Trp Pro

85 90 95

Ser Ser Ser Ser Ser Asn Ser Trp Glu Glu Met Met Lys Glu Val Glu

100 105 110

Tyr Leu Ile Asp Lys Lys Ile Glu Glu Tyr Ala Arg Asn Lys Ala Leu

115 120 125

Ala Val Leu Glu Gly Ile Gly Asn Ala Val Glu Ser Tyr Tyr Ser Ala

130 135 140

Leu Glu Ala Trp Glu Leu Glu Ser Ser Glu Arg Ser Leu Glu Leu Val

145 150 155 160

Leu Glu Arg Tyr Gln Phe Ala Val Gln Phe Ala Arg Ser Ser Met Pro

165 170 175

Ser Phe Ala Ile Ile Asn Tyr Glu Ile Pro Leu Leu Ala Thr Tyr Ala

180 185 190

Asn Ala Ala Asn Val His Leu Leu Leu Met Arg Asp Ile Gln Ile Tyr

195 200 205

Gly Asp Arg Trp Gly Ile Ser Gln Asn Asp Met Asn Leu Phe Leu Lys

210 215 220

Glu Gln Glu Ile Tyr Thr Ser Glu Tyr Ser Glu His Cys Val Lys Trp

225 230 235 240

Tyr Asn Glu Gly Leu Asn Gln Leu Lys Thr Lys Gly Gly Ala Ser Gly

245 250 255

Leu Val Trp Glu Asn Tyr Asn Ser Phe Arg Thr Glu Met Thr Ile Met

260 265 270

Val Leu Asp Leu Val Ala Ile Phe Pro Ala Tyr Asn Met Ser Lys Tyr

275 280 285

Pro Ile Glu Ser Thr Val Glu Leu Thr Arg Thr Ile Tyr Thr Asp Pro

290 295 300

Leu Gly Tyr Thr Gly Tyr Ser Asn Asp Glu His Pro Thr Tyr Tyr Ser

305 310 315 320
Ser Ala Lys Pro Phe Ser Ser Ile Glu Ser Arg Ala Val Leu Ala Pro
325 330 335
Ser Leu Phe Lys Trp Ile Thr Gln Leu Glu Val Tyr Thr Lys Lys Tyr
340 345 350
Ser Tyr Ser Ser Gln Tyr Thr Thr Leu Trp Thr Gly Leu Arg Val Ile
355 360 365
Ala Gln Pro Thr Lys Asp Phe Thr Asp Thr Val Tyr Asp Tyr Gly Ser
370 375 380
Ser Ser Gly Ser Glu Asn Lys Asp Val Phe Asp Leu Tyr Gly Asn Asp
385 390 395 400
Val Tyr Asp Thr Gln Ser Val Val Ser Ser Tyr Lys Pro Thr Gly Gly
405 410 415
Gly His Phe Gly Val Pro Gln Phe Arg Leu Phe Trp Ile Thr Lys Ser
420 425 430
Asn Gly Leu Arg Glu Gln Ile Phe Asn Tyr Ala Asn Asn Met Gly Ser
435 440 445
Tyr Ser Ala Tyr Arg Phe Ser Lys Asp Glu Leu Pro Ile Glu Leu Leu
450 455 460
Gln Pro Pro Leu Phe Gly Asp Ile Glu Glu Tyr Ser His Arg Leu Ser
465 470 475 480
His Val Ser Glu Val Ile Lys Asp Tyr Gly Glu Gly Ile Ile Pro Val
485 490 495
Leu Gly Trp Thr His Val Ser Val Thr Arg Asp Asn Arg Ile Tyr Pro
500 505 510
Asp Lys Ile Thr Gln Leu Pro Ala Val Lys Met Tyr Glu Leu Leu Ser
515 520 525
Ser Ala Val Val Val Lys Gly Pro Gly Phe Thr Gly Gly Asp Leu Val
530 535 540
Lys Arg Thr Gly Asn Gly Gly Ile Gly His Phe Asn Val Ser Val Glu
545 550 555 560
Ser Pro Gly Thr Gln Arg Tyr Arg Leu Arg Ile Arg Tyr Ser Ser Glu
565 570 575
Val Ser Gly Val Phe His Met Gln Ile Asn Asp Ile Glu Thr Ile Gln
580 585 590
Gly Glu Phe Ser Ser Thr Ala Asp Ser Thr Ser Thr Leu Ser Ser Glu

595 600 605

Ala Phe Gln Leu Arg Glu Tyr Ser Thr Thr Phe Thr Phe Pro Thr Asn

610 615 620

Met Thr Lys Ile Lys Val Ser Leu Gly Ala Ile Glu Gly Ala Gly Gly

625 630 635 640

Phe Tyr Leu Asp Arg Ile Glu Phe Ile Pro Val Asp Glu Asn His Asp

645 650 655

Asn Arg Val Thr Leu Glu Lys Ala Gln Lys Ala Val Asn Ala Leu Phe

660 665 670

Thr Ala Gly Arg Asn Ala Leu Gln Thr Asp Val Thr Asp Tyr Lys Val

675 680 685

Asp Gln Val Ser Ile Leu Val Asp Cys Val Ser Gly Glu Leu Tyr Pro

690 695 700

Asn Glu Lys Arg Glu Leu Leu Ser Leu Val Lys Tyr Ala Lys Arg Leu

705 710 715 720

Ser Tyr Ser Arg Asn Leu Leu Leu Asp Pro Thr Phe Asp Ser Ile Asn

725 730 735

Ser Ser Asp Glu Asn Gly Trp Tyr Gly Ser Asn Gly Ile Ala Ile Gly

740 745 750

Asn Gly Asn Phe Val Phe Lys Gly Asn Tyr Leu Ile Phe Ser Gly Thr

755 760 765

Asn Asp Thr Gln Tyr Pro Thr Tyr Leu Tyr Gln Lys Ile Asp Glu Ser

770 775 780

Lys Leu Lys Glu Tyr Thr Arg Tyr Lys Leu Arg Gly Phe Ile Glu Asn

785 790 795 800

Ser Gln Asp Leu Glu Ala Tyr Val Ile Arg Tyr Asp Ala Lys His Glu

805 810 815

Thr Leu Asp Val Ser Asn Asn Leu Leu Pro Asp Ile Ser Pro Val Asn

820 825 830

Ala Cys Gly Glu Pro Asn Arg Cys Ala Ala Leu Gln Tyr Leu Asp Glu

835 840 845

Asn Pro Arg Leu Glu Cys Ser Ser Ile Gln Asp Gly Ile Leu Ser Asp

850 855 860

Ser His Ser Phe Ser Leu Asn Ile Asp Thr Gly Ser Ile Asp Phe Asn

865 870 875 880

Glu Asn Val Gly Ile Trp Val Leu Phe Lys Ile Ser Thr Pro Glu Gly

885 890 895

Tyr Ala Lys Phe Gly Asn Leu Glu Val Ile Glu Asp Ser Pro Val Ile

900 905 910

Gly Glu Ala Leu Ala Arg Val Lys Arg Gln Glu Thr Lys Trp Arg Asn

915 920 925

Lys Leu Thr Gln Leu Arg Thr Glu Thr Gln Ala Ile Tyr Thr Arg Ala

930 935 940

Lys Gln Ala Ile Asp Asn Val Phe Thr Asn Ala Gln Asp Ser His Leu

945 950 955 960

Lys Ile Gly Thr Thr Phe Ala Ala Ile Val Ala Ala Arg Lys Ile Val

965 970 975

Gln Ser Ile Arg Glu Ala Tyr Met Ser Trp Leu Ser Ile Val Pro Gly

980 985 990

Val Asn Tyr Pro Ile Phe Thr Glu Leu Asn Glu Arg Val Gln Arg Ala

995 1000 1005

Phe Gln Leu Tyr Asp Val Arg Asn Val Val Arg Asn Gly Arg Phe

1010 1015 1020

Leu Asn Gly Val Ser Asp Trp Ile Val Thr Ser Asp Val Lys Val

1025 1030 1035

Gln Glu Glu Asn Gly Asn Asn Val Leu Val Leu Ser Asn Trp Asp

1040 1045 1050

Ala Gln Val Leu Gln Cys Leu Lys Leu Tyr Gln Asp Arg Gly Tyr

1055 1060 1065

Ile Leu Arg Val Thr Ala Arg Lys Glu Gly Leu Gly Glu Gly Tyr

1070 1075 1080

Ile Thr Ile Thr Asp Glu Glu Gly His Thr Asp Gln Leu Thr Phe

1085 1090 1095

Gly Thr Cys Glu Glu Ile Asp Ala Ser Asn Thr Phe Val Thr Thr

1100 1105 1110

Gly Tyr Ile Thr Lys Glu Leu Glu Phe Phe Pro Asp Thr Glu Lys

1115 1120 1125

Val Arg Ile Glu Ile Gly Glu Thr Glu Gly Thr Phe Gln Val Glu

1130 1135 1140

Ser Ile Glu Leu Phe Leu Met Glu Asp Leu Cys

1145 1150

<210> 3
<211> 15
<212> PRT

<400> 3
MDIRNQNKYEVVYPA 15

<210> 4
<211> 46
<212> DNA

<400> 4
GCTCGAGTCGACCGTGGTACGCGTGTGTGAGCTCCCGGGATCCGGT 46

<210> 5
<211> 28
<212> DNA
<223> H=A, C, or T; M-A or C; N=A, G, C, or T; R=A or G; Y=C or T; W=A or T

<400> 5
ATGGATATHMGNAATCARAAYAARTAYG 28

<210> 6
<211> 26
<212> DNA
<223> H=A, C, or T; M-A or C; N=A, G, C, or T; R=A or G; Y=C or T; W=A or T

<400> 6
AATAAATATGARGTWGTNTAYCCNGC 26

<210> 7
<211> 19
<212> DNA

<400> 7
GCTCGAGTCGACCGTGGTA 19

<210> 8
<211> 19
<212> DNA

<400> 8
GTACGCGTGTGTGAGCTCC 19

<210> 9
<211> 28
<212> DNA

<400> 9
TTCTAATACAACATCAAAGTATCCACTC 28

<210> 10
<211> 27
<212> DNA

<400> 10
GGTATCGTTCCAATACTAATTCTAAAC 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2006/002724

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N C07K A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI EPODOC PAJ CNPAT CA BA CNKI cry7ba1 cry7 crystal cry bacillus thuringiensis insecticidal etc.
Genbank: sequence search of SEQ ID NO:1 and SEQ ID NO: 2 (or residues 1 to 658 of SEQ ID NO: 2)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO9506730A ((NOVS) NOVARTIS AG ET-AL) 09.Mar.1995(09.03.1995) See the whole document	1-10
A	CN1260397A((CHSC-N) CHINESE ACAD SCI HEREDITY INST) 19.Jul.2006(19.07.2006) See the whole document	1-10
A	ACTA MICROBIOLOGICA SINICA, Vol. 40, No.4, Aug. 2000, Sun Ming et al, "CHARACTERIZATION OF THE INSECTICIDAL CRYSTAL PROTEIN GENES OF BACILLUS THURINGIENSIS YBT-1520", pages 365-371	1-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09.Jan.2007(09.01.2007)	01 · FEB 2007 (01 · 02 · 2007)
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer DING, Huiping Telephone No. (86-10)62085300

Form PCT/ISA /210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2006/002724

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
WO9506730A	09. 03. 1995	AU7693294A	22. 03. 1995
		ZA9406770A	29. 05. 1996
		EP0719335A1	03. 07. 1996
		BRPI9407377A	16. 07. 1996
		JP9502343T	11. 03. 1997
		US5736131A	07. 04. 1998
		AU691522B	21. 05. 1998
		US6204246B1	20. 03. 2001
		TW430669A	21. 04. 2001
		RU2210593C2	20. 08. 2003
		US6780408B1	24. 08. 2004
		KR100360918B	15. 01. 2005
		EP0719335B1	07. 06. 2006
		DE69434758E	20. 07. 2006
CN1260397A	19. 07. 2006	CN1111600C	18. 06. 2003

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2006/002724

CLASSIFICATION OF SUBJECT MATTER

C12N 15/32(2006.01)i
C12N 15/63(2006.01)i
C07K 14/325(2006.01)i
A01N 63/02(2006.01)i