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(54) Titre : PROTEINE DELTA ENDOTOXINE CHIMERIQUE CRY1EA ET CRY1CA
(54) Title: CHIMERIC DELTA ENDOTOXIN PROTEIN OF CRY1EA AND CRY1CA

(57) Abrégé/Abstract:
The present invention relates to a chimeric d endotoxin protein Cry 1E of SEQ ID No. 1 with extraordinarily high insecticidal property and a chimera gene of SEQ ID No. 2 encoding the said chimeric protein from d endotoxins cry1Ea and cry1Ca of Bacillus thuringiensis and also a method of treating insect infested plants using said chimera protein.

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(54) Title: CHIMERIC DELTA ENDOTOXIN PROTEIN OF CRY1EA AND CRY1CA

(57) Abstract: The present invention relates to a chimeric d endotoxin protein Cry 1E of SEQ ID No. 1 with extraordinarily high insecticidal property and a chimera gene of SEQ ID No. 2 encoding the said chimeric protein from d endotoxins cry1Ea and cry1Ca of Bacillus thuringiensis and also a method of treating insect infested plants using said chimera protein.

CHIMERIC DELTA ENDOTOXIN PROTEIN OF CRY1EA AND CRY1CA

Technical Field

The present invention relates to a chimeric δ endotoxin protein Cry 1E of SEQ ID No. 1 with extraordinarily high insecticidal property and a chimera gene of SEQ ID No. 2 encoding the said chimeric protein, and also a method of treating insect infested plants using said chimera protein.

Background Art

Damage due to insects costs billions of dollars annually in form of crop losses and in the expense of keeping these pests under control. The losses caused by pests in agricultural production environments include decrease in crop yield, poor crop quality, increased harvesting costs, and loss to health and environment.

Reference may be made to Hofte H. and Whiteley H.R., 1989, "Insecticidal crystal protein of *Bacillus thuringiensis*", Microbiol. Rev. 53: 242-255, wherein *Bacillus thuringiensis* (B.t.) is a ubiquitous gram-positive spore-forming soil bacterium, known for its ability to produce parasporal crystalline inclusions during sporulation. These inclusions consist of proteins known as crystal proteins or Cry proteins or δ-endotoxins, which exhibit insecticidal activity, particularly against larvae of insect species in orders lepidoptera, diptera and coleoptera. Proteins with toxicity to insects of orders hymenoptera, homoptera, orthoptera, mallophaga; nematodes; mites and protozoans have also been mentioned in literature (Feitelson J.S., 1993, "The *Bacillus thuringiensis* family tree", 63-71, In L. kim ed. Advanced engineered pesticides. Marcel Dekker. Inc., New York., N.Y. and Feitelson *et al.*, 1992, "*Bacillus thuringiensis*: insects and beyond", Bio/Tech. 10: 271-275; may be sited for this). Several strains of *Bacillus thuringiensis* (B.t.) have been identified with different host spectra and classified into different subspecies or serotypes on the basis of flagellar antigens. Pasteur Institute, France has catalogued 55 different flagellar serotypes and 8 non-flagellated biotypes. The reference may be made to Schnepf *et al.*, 1998, "*Bacillus thuringiensis* and its pesticidal crystal proteins", Microbiol. Mol. Biol. Riv. 62: 775-806, wherein several B.t. toxin-coding genes have been cloned, sequenced, characterised and recombinant DNA-based products have been produced and approved for commercial use. Through the employment of genetic engineering techniques, new

approaches have been developed for delivering these B.t. toxins to agricultural environments, including the use of the genetically engineered crops and the stabilised intact microbial cells as δ-endotoxin delivery vehicles (Gaertner, F.H., Kim L., 1988, TIBTECH 6: 54-57). Thus, δ-endotoxin genes coding for proteins targeted to kill hosts, especially pests and insects that cause economic losses are becoming commercially valuable.

Commercial use of B.t. pesticides in a given crop environment is limited because a given δ-endotoxin shows toxicity to a narrow range of target pests. Preparations of the spores and crystals of *B. thuringiensis* subsp. *kurstaki* have been used for many years as commercial insecticides against lepidopteran pests. For example, *B. thuringiensis* var. *kurstaki* HD-1 produces several δ-endotoxins, and is therefore toxic to a relatively broader range of lepidopteran insects. However, formulations based on the known δ-endotoxins, including B.t.k. HD-1 are not effective against some of the important crop pests, like *Spodoptera* sp. that also belong to order lepidoptera. Other species of B.t., namely *israelensis* and *tenebrionis* have been used commercially to control certain insects of the orders diptera and coleoptera, respectively (Gaertner, F.H., 1989, "Cellular Delivery Systems for Insecticidal Proteins: Living and Non-Living Microorganisms," in Controlled Delivery of Crop Protection Agents, R.M. Wilkins, ed., Taylor and Francis, New York and London, 1990, pp. 245-255; Couch T.L., 1980, "Mosquito Pathogenicity of *Bacillus thuringiensis* var. *israelensis*", Development in Industrial Microbiology 22: 61-76 and Beegle C.C., 1978, "Use of Entomogenous Bacteria in Agroecosystems", "Developments in Industrial Microbiology 20: 97-104; may be sited for this). Kreig *et. al.* (1983) in Z. ang. Ent. 96: 500-508, describe *Bacillus thuringiensis* var. *tenebrionis*, which is reportedly active against two beetles in the order Coleoptera i.e., Colorado potato beetles, *Leptinotarsa decemlineata* and *Agelastica alni*.

Reference may be made to Crickmore *et. al.*, 1998, "Revision in the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins", Microbiol. Mol. Biol. Rev. 62: 807-813, wherein crystal protein genes are classified into 22 classes, primarily on the basis of amino acid sequence homology. The cloning and expression of a B.t. crystal protein gene in *Escherichia coli* has been described in several cases in the published literature (Schnepf *et al.*, 1981, "Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*" may be cited for this). U.S. Pat. Nos. 4,448,885 and 4,467,036 disclose

the expression of B.t. crystal protein in *E. coli*. U.S. Pat. Nos. 4,797,276 and 4,853,331 disclose *B. thuringiensis* strain *tenebrionis*, which can be used to control coleopteran pests in various environments. U.S. Pat. No. 4,918,006 discloses B.t. toxins having activity against dipterans. U.S. Pat No. 4,849,217 discloses B.t. isolates, which have activity against the alfalfa weevil. U.S. Pat. No. 5,208,017 discloses coelopteran-active *Bacillus thuringiensis* isolates. U.S. Pat. Nos. 5,151,363 and 4,948,734 disclose certain isolates of B.t., which have activity against nematodes. Extensive research and resources are being spent to discover new B.t. isolates and their uses. As of now, the discovery of new B.t. isolates and new uses of the known B.t. isolates remains an empirical, unpredictable art. Several laboratories all over the world are trying to isolate new δ-endotoxin genes from *B. thuringiensis* for different host range and mechanism of action.

Bulla *et al.*, 1980, "Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*". CRC Crit. Rev. Microbiol. 8: 147-204 and Grochulski *et al.*, 1995, "*Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation", J. Mol. Biol, 254: 447-464; have reported that majority of B.t. insecticidal crystal proteins are synthesised in natural form as protoxins (molecular weight 130-140 kDa), which form parasporal inclusions by virtue of hydrophobic interactions, hydrogen bondings and disulfide bridges. The protoxins, which are not toxic to insect larvae, are composed of two segments the - N-terminal half and C-terminal half. The protoxins are converted into functionally active toxins (60-70 kDa) in insect mid gut following their site-specific cleavage by proteases at alkaline pH. Such proteolytically processed, truncated δ-endotoxins bind to specific receptors in insect mid-gut and cause mortality by making pores in the epithelial membrane (the references, Bietlot *et al.*, 1989, "Facile preparation and characterization of the toxin from *Bacillus thuringiensis* var. *kurstaki*", Biochem. J., 260: 87-91; Choma *et al.*, 1990, "Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*: structural implications", Eur. J. Biochem. 189: 523-27; and Hofte *et al.*, 1986, "Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis berliner* 1715", Eur. J. Biochem. 161: 273-280; may be cited for this). The protease-resistant active toxin corresponds to N-terminal half of the protoxin molecule. The other segment corresponding to C-terminal half is believed to be required for the formation of highly stable crystals. During the proteolytic processing, a small polypeptide comprising about 25-30 amino acid residues is removed from N-terminal of the protoxin.

The crystal structure of the core toxic segment of Cry1Aa and Cry3Aa δ-endotoxins are known (Grochulsky *et al.*, 1995, "*Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation", J. Mol. Biol. 254: 447-464 and Li *et al.*, 1991, "Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution", Nature. 353: 815-821) and their three-dimensional structures are superimposable. Reasonably conserved polypeptide domains suggest that related toxins have similar topological structure. These are globular molecules composed of 3 distinct structural domains connected by small peptide linkers. There are no crossovers of the polypeptide chains between the domains. Domain I consists of 7 α helical structures. Domain II consists of three anti-parallel β-sheets and two short α-helices. Domain III is a β-sandwich of two anti-parallel highly twisted β-sheets. Domains II and III are located on the side where they face helix α7 of domain I. These domains are closely packed by virtue of numerous van der Wall forces, hydrogen bonds and electrostatic interactions (salt bridges) between the domains.

One of the major reasons for narrow host range of δ-endotoxins is that these proteins need specific receptors in the insect gut in order to make pores and cause toxicity. Since a given δ-endotoxin exhibits toxicity to a very narrow range of insects, it is desirable to engineer these proteins for modifying their receptor recognition in larval midgut, to widen host range and to improve toxicity. Two approaches have been followed for this purpose - first, the development of chimeric (or hybrid) genes by exchanging functional domains of the proteins and secondly, the development of improved δ-endotoxin proteins by site directed mutagenesis. References may be made to U.S. Pat. Nos. 5,128,130 and 5,055,294 wherein hybrid B.t. crystal proteins have been constructed, which exhibit increased toxicity and display an expanded host range to the target pests.

The reference may be made to Honee *et al.*, 1990, "A translation fusion product of two different insecticidal crystal protein gene of *Bacillus thuringiensis* exhibits an enlarged insecticidal spectrum" Appl. Environ. Microbiol. 56: 823-825, wherein translational fusion of two *cry* genes (*cry1Ab* and *cry1Ca*) has been made. The resulting hybrid protein had wider toxicity spectrum that overlapped those of the two contributing parental crystal proteins. However, the drawback is that the activity of the chimeric toxin did not increased over any of the parental toxins towards the target insect pests. In spite of poor toxicity, fusion gene was expressed in tobacco after partial modification, which conferred only

partial protection to transgenic plants against a broader range of insects, including *Spodoptera exigua*, *Heliothis virescens* and *Manduca sexta* (the reference, van der Salm *et al.*, 1994, "Insect resistance of transgenic plants that express modified *Bacillus thuringiensis cryIAb* and *cryIC* genes: a resistance management strategy", Plant Mol. Biol. 26: 51-59, may be cited for this).

The reference may be made to Honee *et al.*, 1991, "The C-terminal domain of the toxic fragment of *Bacillus thuringiensis* crystal protein determines receptor binding", Mol. Microbiol. 5: 2799-2806, wherein 11 chimeric genes have been constructed using *cry1Ab* and *cry1Ca* as parent genes by exchanging functional domains. The draw back is that only two chimeric proteins, in which pore-forming domains had been exchanged, exhibited insecticidal activity. However, the efficacy of the toxin chimeric proteins was lower than the parental proteins. Other hybrid proteins were non-toxic.

Masson *et al.*, 1992, "Insecticidal properties of a crystal protein gene product isolated from *Bacillus thuringiensis* subsp. *kenyae*", Appl. Environ. Microbiol. 58: 2, 642-646, reported that one of the Cry1 δ-endotoxins, namely Cry1Ea does not exhibit toxicity against *Spodoptera* larvae. Further, the reference may be made to Bosch *et al.*, 1994, "Recombinant *Bacillus thuringiensis* Crystal protein with new properties: possibilities for resistance management", Bio/Tech 12: 915-918, wherein many chimeric genes have been developed following *in vivo* recombination of *cry1Ca* and *cry1Ea* genes. The δ-endotoxin expressed from one of the chimeric genes, which consisted of domain I and II of Cry1Ea and domain III of Cry1Ca protein exhibited larvicidal activity. The transfer of domain III of Cry1Ca to Cry1Ea protein gave an insecticidal protein. However, the chimeric toxin was not an improved toxin over the Cry1Ca, which is best-reported toxin to *Spodoptera* sp. Another chimeric toxin exhibited very poor toxicity. The remaining chimeric toxins were either unstable or non-toxic.

Reference may be cited as Rang *et al.*, 1999, "Interaction between functional domain of *Bacillus thuringiensis* insecticidal crystal protein", Appl Environ Microbiol, 65, 7: 2918-25, wherein many chimeric genes have been developed by exchanging the regions coding for either domain I or domain III among Cry1Ab, Cry1Ac, Cry1Ca and Cry1Ea δ-endotoxins and checked their stability in *E. coli* and plasma membrane permeability of *Sf*9 cells. A chimeric toxin (consisting of domains I and II of Cry1Ca and domain III of Cry1Ab) was more toxic than the parental toxins. Exchange of domain III of Cry1Ab with

that of Cry1Ca made the chimeric protein more active than the Cry1Ca protein. Proteins with the exchange of other domains were either unstable or less toxic than the parent proteins. However, the toxicity of the chimeric protein to insect larvae was not tested. Pore formation in insect cell line was compared but that cannot be correlated with the insecticidal activity of the δ-endotoxin.

Reference may be made to Chandra *et al.*, 1999, "Amino acid substitution in alpha-helix 7 of Cry1Ac δ-endotoxin of *Bacillus thuringiensis* leads to enhanced toxicity to *Helicoverpa armigera* Hubner", FEBS Lett. 458: 175-179; wherein a hydrophobic motif in the C-terminal end of the fragment B of diphtheria toxin was found to be homologous to helix α7 of δ-endotoxins. Upon substitution of helix α7 of Cry1Ac protein by this polypeptide, the chimeric protein exhibited 7-8 fold enhancement in toxicity towards *Helicoverpa armigera*. The increased toxicity was due to higher pore forming ability.

These examples establish the potential of protein engineering for the improvement of native toxins, to develop commercially useful δ-endotoxins.

Most of the lepidopteran pests are polyphagous in nature. *Spodoptera* is a common lepidopteran insect and its 5 species (*litura*, *littoralis*, *exigua*, *frugiperda* and *exempta*) are found worldwide. *Spodoptera littoralis* (the Egyptian cotton leaf worm, CLW) is a major pest of cotton and other crops of agronomical importance in Europe (the reference Mazier *et al.*, 1997, "The *cryIC* gene from *Bacillus thuringiensis* provides protection against *Spodoptera littoralis* in young transgenic plants", Plant Sci. 127: 179-190, may be cited for this). It is a notorious pest of cotton, groundnut, chilli, pulses and several vegetable crops, especially in warm and humid regions, as the southern parts of India. High fecundity, short life cycle, destructive feeding habits and often-reported emergence of resistance to chemical insecticides have made the control of *Spodoptera* an increasing agricultural problem. Reference may be made to Bai *et al.*, 1993, "Activity of insecticidal proteins and strains of *Bacillus thuringiensis* against *Spodoptera exempta* (Walker)" J. Inverteb. Pathol. 62: 211-215, wherein it is discussed that the young larvae are susceptible to certain δ-endotoxins, but the larvae beyond 2nd instar display considerable tolerance. This has been attributed to the high content of alkaline proteases in the gut juice (the reference Keller *et al.*, 1996, "Digestion of δ-endotoxin by gut proteases may explain reduced sensitivity of advanced instar larvae of *Spodoptera littoralis* to CryIC", Insect Biochem. Mol. Biol. 26: 365-373, may be cites for this).

Four different δ-endotoxins have been reported to cause low level of mortality to the *Spodoptera* sp.. Of these, Cry1Ca is the most effective toxin. The plants expressing Cry1Ca at a high level caused mortality and hence conferred protection against early instar larvae (the references Mazier *et al.*, 1997, "The *cryIC* gene from *Bacillus thuringiensis* provides protection against *Spodoptera littoralis* in young transgenic plants". Plant Sci. 127: 179-190 and Strizhov *et al.*, 1996, "A synthetic *cry1C* gene, encoding a *Bacillus thuringiensis* δ-endotoxin, confers *Spodoptera* resistance in alfalfa and tobacco" Proc. Natl. Acad. Sci. USA. 93: 15012-15017 may be cited for this). However, complete protection against *Spodoptera* has not been reported in any case. The larvae in advanced developmental stages are not killed at moderate levels of the known δ-endotoxins. Hence, transgenic plants expressing Cry1Ca are not as effective as desirable in protection against *Spodoptera*. Other genes, like *cry1Cb*, *cry1Ea* and *cry1F* have not been employed for the development of transgenic plants against *Spodoptera* because of their comparatively low toxicity. Cry1Cb δ-endotoxin is 5-fold less toxic than Cry1Ca. The toxicity of Cry1Ea δ-endotoxin is very low and is disputed in certain reports (Masson *et al.*, 1992 and Bosch *et al.*, 1994 may be cited for this). Cry1F exhibits mild toxicity to *Spodoptera* larvae (Chambers *et al.*, 1991 may be cited for this).

Reference may be made to Kalman *et al.*, 1993, "Cloning of a novel *cryIC*-type gene from a strain of *Bacillus thuringiensis* subsp. *Galleriae*", Appl. Environ. Microbiol. 59: 4: 1131-1137, wherein Cry1Cb δ-endotoxin is reported to be 5-fold less toxic than Cry1Ca. First two domains of these proteins are highly homologous (92% identical). A major difference is observed in domain III that exhibits only 48% homology. Higher toxicity (5-fold) of Cry1Ca over Cry1Cb δ-endotoxin suggested us that domain III of Cry1Ca might have an important role in its efficacy. The toxicity of Cry1Ea δ-endotoxin is very poor as it binds to the receptor very weakly in the midgut of *Spodoptera exigua* but the exchange of Domain III of Cry1Ca with Cry1Ea, made the latter toxic. This suggests the role of Domain III of Cry1C protein in receptor binding in the midgut of *Spodoptera* (the reference Bosch *et. al.*, 1994, "Recombinant *Bacillus thuringiensis* Crystal protein with new properties: possibilities for resistance management". Bio/Tech 12: 915-918, may be cited for this). In this publication, Bosch *et al.* (1994) established the advantage of hybrid toxin as it binds to a receptor where Cry1Ca does not bind. However, the toxicity of both, the native Cry1Ca and the hybrid Cry1Ea was comparable. They filed a patent (US 5736131) in which 1.9-fold improvement in the toxicity towards *Spodoptera exigua* was claimed. The difference

in results in the publication that reports no enhancement in toxicity and in the patent that claims 1.9 fold improved toxicity, makes the overall picture unclear.

Plant genetic engineering technology has made significant progress during the last 10 years. It has become possible to stably introduce foreign genes into plants. This has provided exciting opportunities for modern agriculture. Derivatives of the Ti-plasmid of the plant pathogen, *Agrobacterium tumefaciens*, have proven to be efficient and highly versatile vehicles for the introduction of foreign genes into plant tissue. In addition, a variety of methods to deliver DNA, such as electroporation, microinjection, pollen-mediated gene transfer and particle gun technology, have been developed for the same purpose.

The major aim of plant transformation by genetic engineering has been crop improvement. A substantial effort has been made for engineering the plants for useful traits such as insect-resistance. In this respect, progress in engineering insect resistance in transgenic plants has been achieved through the use of genes, encoding δ-endotoxins, from *B. thuringiensis* strains. Since δ-endotoxins possess a specific insecticidal spectrum and display no toxicity towards other non-host animals and humans, these are highly suited for developing commercially useful plants. No other protein is known which shows as high toxicity as (at ppm levels) and is still as safe to non-target organisms as the δ-endotoxins.

The feasibility of generating insect-resistant transgenic crops expressing δ-endotoxins and their success in commercial agriculture has been demonstrated. (References may be made to Vaeck *et al.*, 1987, "Transgenic plants protected from insect attack", Nature. 328: 33-37; Fischoff *et al.*, 1987, "Insect tolerant transgenic tomato plants", Bio/Tech. 5: 807-813"; Barton *et al.*, 1987, "*Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabaccum* provides resistance to lepidopteran insects", Plant Physiol. 85: 1103-1109 may be made for this). Transgenic plants offer an attractive alternative to insect control in agriculture, which is at the same time safe, environment friendly and cost-effective. Successful insect control has been observed under field conditions (Reference may be made to Delannay *et al.*, 1989; Meeusen and Warren, 1989).

A reference may be cited to Von Tersch *et al.* 1991, "Insecticidal toxins from *Bacillus thuringiensis* subsp kanyae: Gene cloning and characterization and comparison with *B. thuringiensis* subsp *kurstaki* Cry1A(c) toxin" in Appl and Environ Microbial, 57: 2: 349-

58, wherein two variants of *cry1Ac* were isolated from two different strains. Their amino acid composition was different at 7 positions. Both the δ-endotoxins were expressed in *E. coli* and toxicity experiment was conducted. The two toxins did not exhibit any difference in efficacy towards target pests.

In another study (Schnepf *et al.*, 1998, "*Bacillus thuringiensis* and its pesticidal crystal proteins", 62: 3, 775-806), amino acid residues GYY of Cry1Ac δ-endotoxin at position 312 to 314 were altered to replace these with ASY, GSY and GFS. No difference in toxicity of the three proteins was noticed.

There are two natural variants of Cry1C δ-endotoxin namely Cry1Ca and Cry1Cb. These proteins show 81% (Schnepf *et al.*, 1998, "*Bacillus thuringiensis* and its pesticidal crystal proteins", 62: 3, 775-806) amino acid sequence identity. Despite this difference, both the toxins are toxic to their target pest, though there is some difference in the level of toxicity. Their host range is also same. (Kalman *et* a*l.*, 1993. "Cloning of a noval *cry1C* type gene from a strain of *Bacillus thuringiensis* subsp *galleriae* " Appl. Environ Microbial 59: 4: 1131-37)

Objects of the present invention

The main object of the present invention is to develop a chimeric δ endotoxin protein.

Another main object of the present invention is to develop a chimera gene coding for a chimeric δ endotoxin protein.

Yet another object of the present invention is to develop a method of developing said chimeric protein.

Still another object of the present invention is to develop a method of overexpressing said chimeric protein in a suitable microbe.

Still another object of the present invention is to develop a method of treating insect infested plants using said chimeric protein.

Further object of the present invention is to develop an insecticide for multidrug resistant insects.

Another object of the present invention is to develop an effective insecticide.

Yet another object of the present invention is to develop an insecticide having no adverse effect on the plants.

Still another object of the present invention is to develop an insecticide with about 100% insecticidal activity.

Summary of the present invention

The present invention relates to a chimeric δ endotoxin protein Cry 1E of SEQ ID No. 1 with extraordinarily high insecticidal property and a chimera gene of SEQ ID No. 2 encoding the said chimeric protein, and also a method of treating insect infested plants using said chimera protein.

Detailed description of the present invention

Accordingly, the present invention relates to a chimeric δ endotoxin protein Cry 1E of SEQ ID No. 1 with extraordinarily high insecticidal property and a chimera gene of SEQ ID No. 2 encoding the said chimeric protein, and also a method of treating insect infested plants using said chimera protein.

In one embodiment of the present invention, a chimeric δ endotoxin protein Cry1E of SEQ ID No. 1 and other proteins of 75% and above homology in the sequence.

In another embodiment of the present invention, a chimeric δ endotoxin protein Cry1E of SEQ ID No. 1.

In yet another embodiment of the present invention, wherein said protein is of length 641 amino acid residues.

In still another embodiment of the present invention, wherein said chimeric protein is designed from δ endotoxins Cry1Ea and Cry1Ca of *Bacillus thuringiensis*.

In still another embodiment of the present invention, wherein said chimeric protein of length of 641 residues is consisting of residues 1 to 529 from endotoxin Cry1Ea of same position, residues 530 to 599 from Cry1Ca of position 533 to 602, residues 600 to 616 from Cry1Ea of position 588 to 604 and residues 617 to 641 of a synthetic polypeptide.

In still another embodiment of the present invention, wherein peptide domain from 530 to 587 of Cry1Ea can be replaced with that of any other δ endotoxin.

In still another embodiment of the present invention, wherein last 25 amino acid residues improve the stability against the proteases of plants.

In still another embodiment of the present invention, wherein said chimeric protein is stable at temperature ranging between 4-35^{0}C.

In further embodiment of the present invention, A chimera gene of SEQ ID No. 2.

In another embodiment of the present invention, wherein said chimera encodes chimeric protein of SEQ ID No. 1.

In still another embodiment of the present invention, wherein said chimera is of length 1990 base pairs (bp).

In still another embodiment of the present invention, wherein said chimera is a 1.99-kb double stranded DNA.

In still another embodiment of the present invention, wherein said chimera contains plant preferred codon distributed evenly to facilitate efficient translation.

In still another embodiment of the present invention, wherein said chimera contains plant preferred translation initiation codon of ATGGCT at 5' extreme.

In still another embodiment of the present invention, wherein said chimera contains plant preferred translation termination codon of TAATGA.

In still another embodiment of the present invention, wherein said chimera contains 33 restriction sites distributed uniformly throughout the length of the gene at a distance of about 40-80 bp.

In still another embodiment of the present invention, wherein restriction sites are enzymes selected from a group comprising *Hind* III, *Eco*RI, and *Bam*HI.

In still another embodiment of the present invention, wherein said chimera is divided into 58 overlapping oligonucleotides of length 40 to 65 bp each located at a distance of 6 to 26 base pairs. (bp)

In still another embodiment of the present invention, wherein said chimera contains said overlapping nucleotides with an overlap of 13 to 18 nucleotides with the immediately adjacent oligonucleotides on the complementary strain.

In still another embodiment of the present invention, wherein said chimera has T_m value ranging between 44 to 55^0C.

In further embodiment of the present invention, a method of overexpressing insecticidal chimeric protein Cry1E in microbes.

In another embodiment of the present invention, cloning gene Cry1E of SEQ ID No. 2 encoding said chimeric protein in a vector.

In still another embodiment of the present invention, transforming microbe with said cloned vector.

In still another embodiment of the present invention, overexpressing said chimeric protein into said microbe.

In still another embodiment of the present invention, wherein said chimera is expressed into a microbe selected from a group comprising bacteria, algae, and fungi.

In still another embodiment of the present invention, wherein restriction enzymes for said cloning are selected from a group comprising *Hind* III, *Eco*RI, N*col*, *Mfe* I and *Bam*HI.

In still another embodiment of the present invention, wherein inducing overexpression of said protein by using isopropylthiogalactoside (IPTG).

In still another embodiment of the present invention, wherein overexpressing said protein at 15^0C to avoid mis-folding of said proteins.

In still another embodiment of the present invention, wherein said vectors are selected from a group comprising Plasmids, viral DNA, and cosmids.

In still another embodiment of the present invention, wherein expression of chimera in the microbe is confirmed by RT-PCR, western Analysis, and ELISA.

In still another embodiment of the present invention, wherein presence of chimera in the microbe is confirmed by PCR and southern Analysis.

In further embodiment of the present invention, a method of treating plants infected with insects using said insecticidal chimeric protein.

In another embodiment of the present invention, incorporating gene encoding chimera protein Cry1E into plant infected with insects.

In yet another embodiment of the present invention, exposing transgenic plant to insects.

In still another embodiment of the present invention, determining insecticidal activity of said transgenic plants.

In still another embodiment of the present invention, wherein insect pests are selected from a group comprising *spodoptera sp.*, and *Helicoverpa sp.*

In still another embodiment of the present invention, wherein plants are selected from a group comprising tobacco, cotton, chickpea, pegeonpea, groundnut, cauliflower, cabbage, chilli, and capsicum.

In still another embodiment of the present invention, wherein restriction enzymes for said cloning are selected from a group comprising *Hind* III, *Eco*RI, N*col*, and *Bam*HI.

In still another embodiment of the present invention, wherein chimeric protein shows high degree of expression in plants by having about 0.5% of total soluble protein of plants.

In still another embodiment of the present invention, wherein said chimeric protein is stable in said transgenic plant.

In still another embodiment of the present invention, wherein insects exposed to said chimeric protein show weight loss before death.

In still another embodiment of the present invention, wherein said chimeric protein shows insecticidal property against insect at all developmental stages.

In still another embodiment of the present invention, wherein said chimeric protein is multifold more potent insecticide as compared to parental proteins.

In still another embodiment of the present invention, wherein insecticidal activity of said chimeric protein shows mortality of insect pests ranging between 80-100% within about 4 hours of exposure.

In still another embodiment of the present invention, wherein insects exposed to said chimeric protein for about 1 hour shows delayed development, infertility and disrupted metamorphosis.

In still another embodiment of the present invention, wherein EC_{50} for *Helicoverpa sp.* is ranging between 250-350 ng/ml of artificial diet of insects.

In still another embodiment of the present invention, wherein EC_{50} of *Spodoptera sp.* is ranging between 25-50ng/ml of artificial diet.

In still another embodiment of the present invention, the said method shows no adverse effect on the normal growth of the transformed plants.

In further embodiment of the present invention, a novel chimeric *Bacillus thuringiensis* δ-endotoxin is strategically developed by replacing a polypeptide domain of a δ-endotoxin, herein said Cry1Ea with the corresponding domain of other δ-endotoxins and a novel polypeptide at the C-terminus extreme. A gene is theoretically designed and chemically synthesized to encode the novel chimeric toxin and express it at high level in plant tissue. The gene was expressed in a microbe (*E. coli*) and two dicot plants (tobacco and cotton). Efficacy of the strategically designed δ-endotoxin was established in both the systems. The toxicity of the chimeric protein to lepidopteran insect larvae (*Spodoptera* and *Helicoverpa*) was improved as compared to the parent proteins. The chimeric synthetic gene is commercially valuable as it can be used to develop agronomically improved crop plants for resistance to insect pests.

In another embodiment of the present invention, accordingly, the present invention provides "a novel δ-endotoxin improved for insecticidal activity and a gene for its high level expression in plants" which comprises strategic designing of a novel chimeric δ-endotoxin, herein said chimeric Cry1E (616 amino acid residues), by replacing a polypeptide domain (from position 530 to 587) of Cry1Ea protein by that of Cry1Ca (from position 533 to 602), incorporation of a novel polypeptide of 25 amino acid residues at the C-terminus extreme, theoretical designing of a gene to code 641 amino acid residue long chimeric δ-endotoxin at a high level in plants, designing and chemical synthesis of oligonucleotides representing theoretically designed gene, assembly of oligonucleotides into double stranded DNA, cloning and sequence analysis of cloned synthetic DNA, construction of vectors for the expression of chimeric gene in *E. coli* and plants, expression of synthetic chimeric gene in *E. coli*, comparison of the toxicity of the chimeric protein with the parental proteins against *Spodoptera litura* and *Helicoverpa armigera*, transformation of tobacco with the chimeric gene, high level expression of the engineered

protein in transgenic plants, purification of chimeric δ-endotoxin protein from transgenic plants and confirmation of its efficacy on larvae of *Spodoptera litura* and *Helicoverpa armigera*, evaluation of the potential of the chimeric toxin expressed in transgenic plants for protection against target insect pests.

In an embodiment of the present invention, a naturally occurring δ-endotoxin, namely Cry1Ea has been strategically designed and modified accordingly to make it biologically active against larvae of insect pests, like *Spodoptera* sp.

In another embodiment of the present invention, 616 amino acid residue long chimeric δ-endotoxin, herein said chimeric Cry1E, was theoretically designed by replacing a peptide domain of Cry1Ea from position 530 to 587 by that of Cry1Ca from position 533 to 602. Further, a novel polypeptide of 25 amino acid residues was introduced at the C-terminus extreme of δ-endotoxin.

In yet another embodiment of the present invention, two peptide domains from positions 530 to 545 and 578 to 587 of Cry1Ea can be replaced by those of Cry1Ca from 533 to 555 and 588 to 602. Such chimeric toxin may also perform similar (or equivalent) biological activity.

In still another embodiment of the present invention, the peptide domain from 530 to 587 of Cry1Ea can be replaced by that of other δ-endotoxins.

In still another embodiment of the present invention, a polypeptide of 25 amino acid residues is introduced at the C-terminus of δ-endotoxin. This polypeptide improved the stability of δ-endotoxin against the proteases of plant. This might have improved the stability of the δ-endotoxin insect mid gut.

In still another embodiment of the present invention, 25 amino acid residues long polypeptide may be included at the C-terminus of other δ-endotoxins, such toxins may become stable against different kind of proteases.

In still another embodiment of present invention, 25 amino acid residues long polypeptide may be incorporated at the C-terminus of any recombinant protein for their stability against proteases.

In still another embodiment of the present invention, a 1.99-kb double stranded DNA was theoretically designed to encode the chimeric protein. Plant preferred codons were used to encode amino acids of the chimeric toxin protein to facilitate high-level expression of the gene in plants.

There are 6 variants of Cry1Aa δ-endotoxin namely Cry1Aa1 to Cry1Aa6 given in EMBL database. Clustal analysis for amino acid sequence of these δ-endotoxin proteins is shown below. At three positions (77, 148 and 302), the amino acid residues are different. The variant positions are shown below in bold letters. However, all six genes have been deployed in toxicity experiments by different laboratories. The efficacy of all six variants in their toxicity towards insects is comparable.

CRY1AA1
MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF 50

CRY1AA4 MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
50

CRY1AA5 MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
50

CRY1AA6 MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
50

CRY1AA2 MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
50

CRY1AA3 MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEF
50

CRY1AA1
VPGAGFVLGLVDIIWGIFGPSQWDAF**P**VQIEQLINQRIEEFARNQAISRL 100

CRY1AA4
VPGAGFVLGLVDIIWGIFGPSQWDAF**P**VQIEQLINQRIEEFARNQAISRL 100

CRY1AA5
VPGAGFVLGLVDIIWGIFGPSQWDAF**L**VQIEQLINQRIEEFARNQAISRL 100

CRY1AA6
VPGAGFVLGLVDIIWGIFGPSQWDAF**L**VQIEQLINQRIEEFARNQAISRL 100

CRY1AA2
VPGAGFVLGLVDIIWGIFGPSQWDAF**L**VQIEQLINQRIEEFARNQAISRL 100

CRY1AA3
VPGAGFVLGLVDIIWGIFGPSQWDAF**L**VQIEQLINQRIEEFARNQAISRL 100

CRY1AA1
EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLLAV 150
CRY1AA4
EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLLAV 150

CRY1AA5
EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLLAV 150

CRY1AA6
EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLLAV 150

CRY1AA2
EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV 150

CRY1AA3
EGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLFAV 150

CRY1AA1
QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI 200

CRY1AA4
QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI 200

CRY1AA5
QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI 200

CRY1AA6
QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI 200

CRY1AA2
QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI 200

CRY1AA3
QNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLI 200

CRY1AA1
GNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTVLDIVALFSNY 250

CRY1AA4
GNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTVLDIVALFSNY 250

CRY1AA5
GNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTVLDIVALFSNY 250

CRY1AA6
GNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTVLDIVALFSNY 250

CRY1AA2
GNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTVLDIVALFSNY 250

CRY1AA3
GNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRRELTLTVLDIVALFSNY 250

CRY1AA1
DSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300

CRY1AA4
DSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300

CRY1AA5
DSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300

CRY1AA6
DSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300

CRY1AA2
DSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300

CRY1AA3
DSRRYPIRTVSQLTREIYTNPVLENFDGSFRGMAQRIEQNIRQPHLMDIL 300

CRY1AA1
NSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLV 350

CRY1AA4
NSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLV 350

CRY1AA5
NSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLV 350

CRY1AA6
NSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLV 350

CRY1AA2
NRITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLV 350
CRY1AA3
NSITIYTDVHRGFNYWSGHQITASPVGFSGPEFAFPLFGNAGNAAPPVLV 350

CRY1AA1 SLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPST
400

CRY1AA4 SLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPST
400

CRY1AA5 SLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPST
400

CRY1AA6 SLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPST
400

CRY1AA2 SLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPST
400

CRY1AA3 SLTGLGIFRTLSSPLYRRIILGSGPNNQELFVLDGTEFSFASLTTNLPST 400

CRY1AA1
IYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAP 450

CRY1AA4
IYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAP 450

CRY1AA5
IYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAP 450

CRY1AA6
IYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAP 450

CRY1AA2
IYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAP 450

CRY1AA3
IYRQRGTVDSLDVIPPQDNSVPPRAGFSHRLSHVTMLSQAAGAVYTLRAP 450

CRY1AA1
TFSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILR 500

CRY1AA4
TFSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILR 500

CRY1AA5
TFSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILR 500

CRY1AA6
TFSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILR 500

CRY1AA2
TFSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILR 500

CRY1AA3
TFSWQHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILR 500

CRY1AA1
RTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF 550

CRY1AA4
RTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF 550

CRY1AA5
RTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF 550

CRY1AA6
RTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF 550

CRY1AA2
RTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF 550

CRY1AA3
RTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF 550

CRY1AA1
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR 600

CRY1AA4
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR 600

CRY1AA5
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR 600

CRY1AA6
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR 600

CRY1AA2
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR 600

CRY1AA3
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR 600

CRY1AA1 IEFVPAEVT 609

CRY1AA4 IEFVPAEVT 609

CRY1AA5 IEFVPAEVT 609

CRY1AA6 IEFVPAEVT 609

CRY1AA2 IEFVPAEVT 609

CRY1AA3 IEFVPAEVT 609

A reference may be cited to Von Tersch *et al.* 1991, "Insecticidal toxins from *Bacillus thuringiensis* subsp kanyae: Gene cloning and characterization and comparison with *B. thuringiensis* subsp *kurstaki* Cry1A(c) toxin" in Appl and Environ Microbial, 57: 2: 349-58, wherein two variants of *cry1Ac* were isolated from two different strains. Their amino acid composition was different at 7 positions. Both the δ-endotoxins were expressed in *E. coli* and toxicity experiment was conducted. The two toxins did not exhibit any difference in efficacy towards target pests.

Further, this clearly states that in proteins, more particularly in the field of endotoxins, the high homology of the sequence is not found to make any significant difference in activity. The above-referred example of endotoxins Cry1Aa1 to Cry1Aa6 clearly reflect the essence of this work. In the instant Application, the applicant has observed extraordinarily high insecticidal activity. Further, the homology of 70% and above in the sequence of chimeric protein Cry1E of the instant Application is also found to show no significant change in the activity. This means that the proteins with sequence homology of 70% and above for chimeric protein Cry 1E are used as insecticidal agents.

In another study (Schnepf *et al.*, 1998, "*Bacillus thuringiensis* and its pesticidal crystal proteins", 62: 3, 775-806), amino acid residues GYY of Cry1Ac δ-endotoxin at position 312 to 314 were altered to replace these with ASY, GSY and GFS. No difference in toxicity of the three proteins was noticed.

In addition, there are two natural variants of Cry1C δ-endotoxin namely Cry1Ca and Cry1Cb. These proteins show 81% (Schnepf *et al.*, 1998, "*Bacillus thuringiensis* and its pesticidal crystal proteins", 62: 3, 775-806) amino acid sequence identity. Despite this difference, both the toxins are toxic to their target pest, though there is some difference in the level of toxicity. Their host range is also same. (Kalman *et al.*, 1993. "Cloning of a noval *cry1C* type gene from a strain of *Bacillus thuringiensis* subsp *galleriae* " Appl. Environ Microbial 59: 4: 1131-37)

Brief description of the accompanying drawings

Figure 1 shows represents PCR based assembly of 58 overlapping oligonucleotides into 1.99 kb novel chimeric *cry1E* DNA. Lanes 2-4 represent gene assembly in different PCR conditions, the desired DNA fragment is shown with arrow Lane 1 showing λDNA/HindIII-EcoRI molecular weight marker.

Figure 2 represents the restriction analysis of plasmid pPK59 having E-35-S promoter at the upstream of the novel chimeric gene. The plasmid was digested with SalI restriction enzyme (40 bp downstream of gene). The linear plasmid was further digested with NcoI (lane 2), NheI (lane 3), BstXI (lane 4), NruI (Lane 5) and SacI (lane 6). Lane 1 and 7 represent λDNA/HindIII-EcoRI and λDNA/HindIII molecular weight markers, respectively.

Figure 3 represent map of plasmids pPK202, pPK141, pPK135 and pPk206.

Figure 4 represents insect bioassay with transgneic tobacco plants. 1st instar larvae of *Spodoptera litura* showing 100% mortality after 2 days of feeding on transgenic leaf expressing the novel chimeric *cry1E* gene (left). Control leaf was eaten by larvae voraciously (right).

Figure 5 represents 3rd instar larvae of *Spodoptera litura* showing 100% mortality after 2 days of feeding (left). Transgenic plant leaf exhibited high level of protection against larvae in contrast to the control plant leaf (right).

Figure 6 represents 100% mortality of 5th instar larvae of *Spodoptera* following 48 h of feeding on transgenic tobacco leaf (left). During same period, the larvae fed on control plant leaf ingested 6 leaves (right).

In still another embodiment of the present invention, 58 oligonucleotides were designed to represent 1.99-kb chimeric *cry1E* DNA. All oligonucleotides were synthesised chemically and fused enzymatically to obtain desired double stranded DNA. (Please refer figure 1)

In still another embodiment of the present invention, two independent constructs were made, each for *E. coli* and plant expression. The parental genes (*cry1Ea* and *cry1Ca*) were also introduced in other expression vectors for their expression in *E. coli*.

In still another embodiment of the present invention, all three genes were expressed in *E. coli*. The efficiency of the chimeric Cry1E against *Spodoptera litura* was compared to the parental toxins (Cry1Ea and Cry1Ca). The toxicity experiments established that the engineered toxin is several fold more toxic as compared to the parental proteins.

In still another embodiment of the present invention, the chimeric protein expressed in plants was shown to be also toxic to *Helicoverpa armigera*, another serious insect pest. This established the improvement in the host range of the novel chimeric toxin designed in this study.

In still another embodiment of the present invention, the synthetic gene was introduced in tobacco for expression of the chimeric toxin. The transgenic plants expressed chimeric toxin and accumulated up to 0.5% of total soluble protein. The transgenic plants exhibited excellent protection against larvae of *Spodoptera litura* and caused 100% mortality at all the developmental stages.

In still another embodiment of the present invention, the novel chimeric toxin was purified from total soluble protein from the leaf of the transgenic tobacco plant and mixed in semi synthetic diet. The toxicity experiments again established the efficacy of the hybrid toxin.

The subject invention concerns the discovery of highly active chimeric δ-endotoxins. A novel δ-endotoxin, 641 amino acid residues long, herein said chimeric Cry1E was strategically designed by replacing a polypeptide domain (from position 530 to 587) of Cry1Ea protein by that of Cry1Ca (from position 533 to 602). In this way chimeric toxin comprises amino acid residues 1 - 529 of Cry1Ea, 530 to 599 of Cry1Ca and 600 to 616 of Cry1Ea. A novel polypeptide of 25 amino acid residues was included as the C-terminus extreme of the δ-endotoxin. In other words, this polypeptide constituted amino acid residue 617 – 641 of the chimeric toxin. Several chimeric toxins can be created by replacing different parts of Cry1Ea toxin with strategically designed amino acid sequences or parts of the other toxins. A 1.99 kb nucleotide sequence was theoretically designed to code for the above-mentioned chimeric δ-endotoxin. The gene encoding toxin protein, herein said chimeric *cry1E* was designed for high-level expression in plants, by introducing plant-preferred codons. The plant preferred codons for each amino acid were distributed evenly to facilitate efficient translation. A translation initiation context appropriate to gene expression in plants (TAAACC**ATGGCT**) was included at 5' extreme and two translation stop codons (signals) were introduced at the end of the reading frame of the chimeric toxin. A total of 33 unique restriction sites were introduced uniformly throughout the length of the gene at a distance of 40-80 bp. *Bam*HI and *Hind*III restriction sites were created at the upstream and *Bam*HI and *Eco*RI at the downstream of the gene to facilitate its cloning. (Please refer figure 2).

The translation initiation context automatically created an *Nco*I site at the immediate start of the gene. The gene was divided into 58 overlapping oligonucleotides (40 to 65 nucleotides long) with 6 to 26 base long gaps in between (Please refer figure 1). Each oligonucleotide had 13 -18 nucleotide long overlap with the immediately adjacent oligonucleotides on the complementary strand. The complementary overlaps were designed to keep T_m value between 48 - 50°C. The oligonucleotides were synthesised on a DNA synthesiser (Gene Assembler Special, Pharmacia, Sweden) at 200 nmole scale and purified on denaturing urea-PAGE. All 58 oligonucleotides were assembled into 1.99 kb double-stranded DNA, herein said chimeric *cry1E* gene following the ligation-free gene

synthesis method of Singh *et al.* (1996) and as shown in figure 1. The DNA was digested with *Hind*III and *Eco*RI restriction enzymes and cloned in pBluescriptII SK(+) (Stratagene, La Jolla, CA). The plasmid was named as pPK200. The nucleotide sequence of the synthetic DNA was confirmed by sequencing the cloned synthetic DNA on automated DNA sequencing system (Applied Biosystems model 373A).

A cassette was constructed also for the expression of the chimeric toxin in *E.coli* under the control of T7*lac* promoter. The plasmid pPK200 was digested with the restriction enzymes *Nco*I and *Bam*HI and cloned in expression vector pET-19b (Novagen, Madison WI). The plasmid was named as pPK206. DNA encoding Cry1Ca and Cry1Ea toxins were amplified with polymerase chain reaction, using suitable primers, which created *Nco*I and *Bam*HI restriction sites at the upstream and the downstream of the amplicon. The amplified products were cloned in the pET-19b vector. The constructs having Cry1Ca and Cry1Ea toxin coding DNA were named as pPK135 and pPK141, respectively. *E. coli* BL21DE3 strain was transformed with the constructs pPK206, pPK135 and pPK141. (Please refer figure 3). The toxin proteins were expressed by induction with appropriate concentrations of IPTG. The expression was carried out at 15°C to avoid possible mis-folding. *E. coli* cells were lysed with lysozyme and sonicated to release the δ-endotoxins. The toxin proteins were found in inclusion bodies. These were sloubilised in 50 mM Bicarbonate buffer (pH 9.5) at 28 °C. The toxin proteins were quantified densitometrically. Serial dilutions of the toxins were mixed in semi-synthetic diet and the mixture was poured in the petri dishes. Total *E. coli* protein served as control diet. Fifteen neonatal larvae of *Spodoptera litura* were released onto the cakes of the diet mixture in a 100-ml beaker and the mouth was covered with muslin cloth to allow gas exchange. Each experiment was conducted with 6 replicates. The diet was changed after every alternate day. Bio-assay was conducted with 16/8 h photoperiod at 25±0.2°C. Toxicity data was recorded after 7 days of the feeding. EC_{50} was determined by standard log-probit analysis. All three proteins were tested simultaneously. The representative results are as shown in Table 1 here below.

Table 1

δ-endotoxins(S)	**EC_{50} (μg / ml semisynthetic diet)**
Cry1Ea	**>108**
Cry1Ca	**29.48±1.77**
Chimeric Cry1E	**6.27±0.59**

The result showed that the *E. coli* strain expressing the chimeric toxin was several fold more toxic over Cry1Ea and more than four fold toxic over Cry1Ca protein. The Cry1Ea toxin protein failed to cause any effect on the *Spodoptera* larvae. The result established the successful engineering of the Cry1Ea toxin to develop a novel protein chimeric Cry1E, which is biologically active and an improved toxin. The engineered protein was four fold more toxic than Cry1Ca protein, which is the best-known δ-endotoxin against *Spodoptera* sp.

All three δ-endotoxins were also tested against 72-h old larvae of *Helicoverpa sp*. Each larva was released on diet in a separate box. 40 larvae were challenged with each concentration of the toxin. The toxicity results show that the chimeric toxin is also toxic to *Helicoverpa*. A representative result is shown in Table 2 as here below.

Table 2

δ-endotoxins(S)	**EC_{50} (µg / ml semisynthetic diet)**
Cry1Ea	**>176**
Cry1Ca	**136.22±8.77**
Chimeric Cry1E	**26.71±1.39**

As used here, reference to the "toxin" means, the N-terminal segment, which is responsible for the insect pesticidal activity. A person skilled in this art can convert this toxin into a protoxin by including a part or complete C-terminus fragment of a homologous or hetrologous δ-endotoxin. Such protoxin will be a very stable molecule inside a microbial cells for example, in *E. coli*, *Pseudomonas* etc. and can be used in developing microbial formulations. Such formulations can be used as pesticides. Development of such protoxins and formulations using the novel toxin developed by us is also within the scope of the invention claimed by us.

The gene and toxin useful according to the subject invention include not only 641 amino acid long toxin but also fragments of the novel sequence, variants and mutants, which retain the characteristic pesticidal activity of the toxin specifically exemplified herein. As used here, the terms "variants" or "variations" of genes refer to nucleotide sequences, which encode the same toxins or which encode toxins having lower or equivalent pesticidal activity. As used here, the term "equivalent pesticidal activity" refers to toxins having similar or essentially the same biological activity against the target pests as the claimed toxins.

It is well within the skill of a person trained in the art to create alterative DNA sequences encoding the same or essentially the same, toxin. These variant DNA sequences are within the scope of the subject invention. As used herein, reference to "essentially the same" sequence refers to sequences, which have amino acid substitutions, deletions, additions or insertions, which do not materially affect pesticidal activity. Fragments retaining pesticidal activity are also included in this definition.

A novel chimeric toxin of the subject invention has been specifically exemplified herein. It should be readily apparent that the subject invention comprises variants or equivalent toxins (and nucleotide sequences encoding equivalent toxins) having the same or similar pesticidal activity of the exemplified toxin. Equivalent toxins will have amino acid homology with the exemplified toxin. This amino acid homology will typically be greater than 75%, preferably be greater than 90% and most preferably be greater than 95%. The amino acid homology will be highest in critical regions of the toxin, which account for the biological activity or are involved in the determination of three-dimensional configuration, which ultimately is responsible for the biological activity. In this regard, certain amino acid substitutions are acceptable and can be expected if these substitutions are in regions, which are not critical in biological activity or are conservative amino acid substitutions, which do not affect the three-dimensional configuration of the molecule. For example, amino acids may be placed in the following classes: non-polar, uncharged polar, basic and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the δ-endotoxin. Table 3 as given here below provides a listing of examples of amino acids belonging to each class.

TABLE 3

Class of Amino Acid	Examples of Amino Acids
Non-polar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. The critical factor is that these substitutions must not significantly detract from the biological activity of the toxin. It is well within the skill of a person trained in the art of protein engineering to substitute any amino acid of the chimeric toxin with alanine. The substitution of any amino

acid is safest, as alanine is a typical amino acid. Such substitution is also well within the scope of the invention.

A gene encoding the chimeric toxin of the subject invention can be introduced into a wide variety of microbial or plant hosts. Expression of the toxin gene results, directly or indirectly, in the intracellular production and maintenance of the pesticidal chimeric toxin. With suitable microbial hosts, e.g., *Pseudomonas*, the microbes can be applied to the sites where the pests proliferate. This will result into the control of the pest. Alternatively, the microbe hosting the toxin gene can be treated under conditions that prolong the activity of the toxin and stabilize the cell. The treated cell, which retains the toxic activity, then can be applied to the environment of the target pest. Where the gene encoding the chimeric toxin is introduced via a suitable vector into a microbial host, and said host is applied to the environment in a living state, it is essential that certain host microbes be used. Microorganism hosts are selected which are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplane) of one or more crops of interest. These microorganisms are selected so as to be capable of successfully competing in the particular environment (crop and other insect habitats) with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the polypeptide pesticide, and, desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

A large number of microorganisms are known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilius*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, *Arthrobacter*, *Azotobacter*, *Leuconostoc*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula* and *Aureobasidium*. Of the particular interest are such phytosphere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*, *Agrobacterium tumefaciens*, *Rhodopseudomonas spheroids*, *Xanthomonas campestris*, *Rhozobium melioti*, *Alcaligenes entrophus* and *Azotobacter vinlandii* and phytosphere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantiaca*, *Cryptococcus albidus*, *C.*

diffluens, *C. laurentii*, *Saccharomyces rosei*, *S. pretoriensis*, *S. cerevisiae*, *Sporobolomyces roseus*, *S. odorus*, *Kluyveromyces veronae* and *Aureobasidium pollulans*. of particular interest are the pigmented microorganisms. A wide variety of ways are available for introducing a gene encoding a chimeric toxin into a microorganism host under conditions, which allow for the stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Pat. No. 5,135,867.

A plant transformation vector was constructed for the development of transgenic plants. A plasmid pPK58 (having CaMV35S promoter with duplicated enhancer) was digested with *Bam*HI and *Hind*III and pPK200 with *Hind*III and *Eco*RI to excise out CaMV35S promoter with the duplicated enhancer and chimeric *cry1E* gene, respectively. A triple ligation was carried for cloning of the two fragments in pLITMUS38 cloning vector (New England Biolabs). The plasmid, namely pPK59 had CaMV35S promoter with the duplicated enhancer at the upstream of the chimeric gene. Restriction analysis of pPK59 is shown in figure 2. The *nos* transcription terminator was cloned at the downstream of the chimeric gene. DNA of *nos* polyadenylation element was amplified using pBI101.1 as template with suitable primers, which created *Mfe*I and *Eco*RI restriction sites at the upstream and downstream, respectively. The plasmid pPK59 was digested with *Eco*RI and the PCR product was cloned following digestion with the *Mfe*I and *Eco*RI restriction enzymes. The clone in which *Eco*RI restriction site of the synthetic gene was ligated to *Mfe*I site of *nos* terminator (as they have compatible ends), selected and named as pPK201. The correct orientation of *nos* terminator was confirmed by restriction analysis and also by sequencing. The expression cassette (the synthetic *cry* gene with E-35S promoter and *nos* terminator) was cloned in Ti binary vector. *Bam*HI-*Eco*RI fragment of plasmid pPK201 was cloned in pBI101.1 replacing *Bam*HI-*Eco*RI fragment (*uidA* gene and *nos* terminator) of the plasmid. This binary vector was named as pPK202. The map of the *E. coli* and plant expression vectors are shown in figure 3.

In order to study the efficacy of the chimeric Cry1E toxin in plants, tobacco was selected for the expression. *Agrobacterium tumefaciens* strain LBA 4404 containing the helper plasmid pAL4404 was transformed with the binary vector pPK202 following the modified protocol of "electroporation of *Agrobacterium*" discussed by Cangelosi *et al.* (1991) and transformed colony was selected on antibiotics streptomycin, rifampicin and kanamycin.

Agrobacterium mediated transformation of *Nicotiana tobacum* cv. Patit Havana was carried out following the method of Horsch *et al.*, 1985 and the transgenic plant were selected on the antibiotic kanamycin. The presence of the gene encoding chimeric toxin was confirmed with PCR and Southern analysis and the expression of the transgene was established with the RT-PCR, Western analysis and ELISA. ELISA result displayed 0.5% expression of the toxin protein out of total soluble leaf protein in a selected transgenic line. This high level of the expression was the result of the designing of the gene in which plant-preferred codons were exclusively used. Plant preferred translation initiation context used in this study would also have played an important role in achieving enhanced expression.

Insect bioassay was performed with two months old transgenic plants and 1st and 5th instar larvae of *Spodoptera litura*. (Please refer figures 4-6). 15 cm^2 leaf-discs of transgenic and control plants were placed in cylindrical boxes containing wet blotting paper at the bottom and ten 1st instar larvae were released onto them. Mouths of the boxes were covered with wet muslin cloth to maintain sufficient humidity and exchange of air. The toxicity experiments were conducted in three replicates at 25±0.2°C and 16/8h photoperiod was maintained. In bioassay with 5th instar larvae, complete leaves of transgenic and control plants were used. The leaf petiole was held in cotton plug over a 250 ml flask containing ½ MS salt solution to overcome wilting of the leaf. Five insect larvae were allowed to feed on each leaf. The leaves of control plant were changed after every 8 h and they were consumed completely by the insect larvae.

The result (figures 4 and 6) established that 1st as well as 5th instar larvae died within 48-72 h. The amount of leaf eaten by the 1st instar larvae was negligible as compare to the control plant leaf discs, which were eaten voraciously. Ingestion of approximately 1 cm^2 of transgenic leaf was sufficient to kill 5th instar larvae. These larvae appeared moribund after 8-16 h of feeding on the transgenic leaf and finally died within 2-3 days. Green coloured excreta with high water content were noticed on leaf surface. Some of the larvae showed heavy weight loss before death. In a separate experiment, different instar larvae were allowed to feed on leaves of transgenic plant for 1 h, 2 h, 4 h, 8 h and 16 h and then shifted to control plant leaves. It was observed that 8 h of feeding on transgenic plants was sufficient to cause 100% mortality of larvae in all stages of development, even after feeding on non-transgenic plants. Ingestion of very small amounts of the toxin by young larvae delayed their pupation by 10-15 days from normal larval cycle of 15 days. The few larvae that escaped mortality developed into flies. 40% of the paired matings where such

flies were used, gave eggs. However, the eggs were sterile and failed to hatch. The total soluble protein from transgènic tobacco plant was extracted and loaded on SepharoseQ ion exchange column. The protein was eluted with increasing gradient of sodium chloride and the peak containing δ-endotoxin was pooled and desalted on G10 column. The eluted protein was mixed in semi-synthetic diet. Similar protein extraction and purification from the leaf of non-transgenic tobacco plants was also performed and such plant protein served as control. The toxicity experiment was conducted as discussed earlier. EC_{50} for *Spodoptera litura* and *Helicoverpa armigera* was 37 ng/ml and 285 ng/ml of artificial diet. The result again confirmed the efficacy of chimeric δ-endotoxin towards target insect pests.

A novel δ-endotoxin for the control of insect pests and a gene for its high level expression in plants, which comprises theoretical designing of a novel δ-endotoxin, herein named chimeric Cry1E, strategically designed by replacing a polypeptide domain (from position 530 to 587) of Cry1Ea protein by that of Cry1Ca (from position 533 to 602), a novel 25 amino acid residues long polypeptide at the C-terminus extreme of the protein for stability, theoretical designing of the gene to express the chimeric δ-endotoxin at a high level in plants, designing and chemical synthesis of the oligonucleotides representing the theoretically designed gene, assembly of oligonucleotides into double stranded DNA, cloning and sequence analyses of the cloned synthetic DNA, construction of vectors for the expression of chimeric gene in *E. coli* and plants, expression of the synthetic gene in *E. coli*, comparison of the toxicity of the chimeric protein with the parental proteins against *Spodoptera litura*, transformation of plant, for example tobacco with the chimeric gene, high level expression of the engineered protein in transgenic plants and evaluation of the potential of the chimeric toxin in transgenic plants for protection against *Spodoptera litura*.

In still another embodiment of the present invention, wherein amino acid no. 1 to 529 of Cry1Ea, 530 to 599 of Cry1Ca, 600 to 616 of Cry1Ea and 617 to 641 a novel polypeptide and its structurally and/or functionally equivalent variants or fragments thereof.

In still another embodiment of the present invention, wherein the said toxin has an amino acid sequence shown in SEQ ID No.1; shows several fold higher toxicity to target lepidopterin insects as compared to the parental toxins Cry1Ea and Cry1Ca.

In still another embodiment of the present invention, wherein chimeric *cry1E* gene which encodes a chimeric toxin having activity against lepidopteran insects, has nucleotide sequence shown in SEQ ID No 2 or its fragment thereof.

In still another embodiment of the present invention, wherein a process of controlling lepidopteran pests employing the protein, with an effective amount of the toxin used as such or as a component of a chemical or microbial formulation.

In still another embodiment of the present invention, wherein a recombinant DNA transfer vector comprising a polynucleotide sequence, which encodes a toxin having activity against lepidopteran insects, wherein said polynucleotide sequence has the nucleotide sequence of SEQ ID NO. 2 or fragments thereof.

In still another embodiment of the present invention, wherein a recombinant host transformed with the gene.

In still another embodiment of the present invention, wherein the said host is a microbe for example *Escherichia coli*, *Pseudomonas*, Yeast, Cyanobacteria and/or other microbes.

In still another embodiment of the present invention, wherein said transformed host is a plant for example tobacco, cotton, chickpea, pegeonpea, groundnut, cauliflower, cabbage, chilli, capsicum and/or other plants, which expresses the toxin, wherein said toxin has the amino acid sequence of SEQ ID NO. 1, or its variants with equivalent activity against lepidopteran insects.

In further embodiment of the present invention, instant Application clearly states that in proteins, more particularly in the field of endotoxins, the high homology of the sequence is not found to make any significant difference in activity. The above-referred work on of endotoxins Cry1Aa1 to Cry1Aa6 clearly reflect the essence of this work. In the instant Application, the applicant has observed extraordinarily high insecticidal activity. Further, the homology of 70% and above in the sequence of chimeric protein Cry1E of the instant Application is also found to show no significant change in the activity. This means that the proteins with sequence homology of 70% and above for chimeric protein Cry 1E are used as insecticidal agents.

The following examples are given by way of illustrations and therefore should not be construed to limit the scope of the present invention.

Example 1
Comparative toxicity of the novel chimeric Cry1E expressed in *E. coli* to larvae of lepidopteran insect pests

A chimeric δ-endotoxin, 616 amino acid residues long, herein said chimeric Cry1E was strategically designed by replacing a polypeptide domain (from position 530 to 587) of Cry1Ea protein by that of Cry1Ca (from position 533 to 602). A polypeptide of 25 amino acid residues was additionally included at the C-terminus extreme as described earlier. A 1.99 kb nucleotide sequence was theoretically designed to code for the above-mentioned chimeric δ-endotoxin. The codons for each amino acid were distributed evenly to avoid temporary deficiency of the tRNA during translation. Several 6-base cutter restriction enzyme sites were created in the designed gene. *Bam*HI, *Hind*III and *Nco*I restriction sites were created at 5'-end and *Eco*RI at the 3'-end of the designed gene. The gene was divided into 58 overlapping oligonucleotides (40 to 65 nucleotides long). Each oligonucleotide had 13 -18 nucleotide long overlap with the immediately adjacent oligonucleotides on the complementary strand (T_m between 48-50°C). Oligonucleotides were synthesised on a DNA synthesiser (Gene Assembler Special, Pharmacia, Sweden) at 200 nmole scale and purified on denaturing urea-PAGE. All 58 oligonucleotides were assembled into the double-stranded DNA, herein said chimeric *cry1E* gene following the ligation-free gene synthesis method of Singh *et al.* (1996) and as shown in figure 1. The DNA was digested with *Hind*III and *Eco*RI restriction enzyme and cloned in pBluescriptII SK(+) (Stratagene). The plasmid was named as pPK200. The nucleotide sequence of the synthetic DNA was confirmed by sequencing of the cloned synthetic DNA on automated DNA sequencing system (Applied Biosystems model 373).

A cassette was constructed for the expression of the chimeric toxin in *E.coli* under control of T7 promoter. For this, plasmid pPK200 was digested with the restriction enzymes *Nco*I and *Bam*HI and cloned in expression vector pET-19b (Novagen). The plasmid was named as pPK206. DNA encoding toxin portion of Cry1Ea and Cry1Ca were amplified with polymerase chain reaction, using suitable primers, which created *Nco*I and *Bam*HI restriction sites at the upstream and the downstream of the amplicon, respectively in both the DNA. The amplified products were cloned at *Nco*I and *Bam*HI sites in the same vector (pET-19b). The constructs having Cry1Ea toxin DNA was named as pPK141 and Cry1Ca as pPK135 as described earlier. BL21DE3 strain of *E. coli* was transformed with the constructs pPK141, pPK135 and pPK206. The toxin proteins were expressed by induction with appropriate concentrations of IPTG. The expression was carried out at 15°C to avoid any possible mis-folding of the toxins. The toxin proteins were quantified densitometrically on the denaturing polyacrylamide gel. Serial dilutions of the toxins were mixed in semi-synthetic diet. Total *E. coli* protein served as control in the diet. Fifteen

neonatal larvae of *Spodoptera litura* were released onto the cakes of the diet mixture in a 100-ml beaker and the mouth of the beaker was covered with muslin cloth to allow gas exchange. Each experiment was conducted in 6 replicates. The diet was changed after every alternate day. Bio-assay was conducted with 16/8 h photoperiod at 25±0.2°C. Toxicity data was recorded after 7 days of the feeding. EC_{50} was determined by standard log-probit analysis. All three proteins were tested simultaneously. The representative results are presented in Table 4 as given here below.

Table 4

δ-endotoxins(S)	**EC_{50} (μg/ml semi-synthetic diet)**
Cry1Ea	**>108**
Cry1Ca	**29.48±1.77**
Chimeric Cry1E	**6.27±0.59**

The result showed that the chimeric toxin was several fold more toxic over Cry1Ea and more than four fold toxic over Cry1Ca protein. Cry1Ea toxin protein failed to cause any mortality or growth retardation of the *Spodoptera* larvae. The result established the successful engineering of the Cry1Ea toxin for converting it into a biologically active improved toxin. The engineered protein was more toxic than Cry1Ca protein, which is the best-known δ-endotoxoin against *Spodoptera* sp.

A similar toxicity experiment was conducted with the larvae of *Helicoverpa armigera*. In this case, 72 h old larvae were released on semi-synthetic diet containing one of the three proteins and only one larvae was released in each box. Weight loss was recorded after 7 days of feeding. The representative results are presented in Table 5 as shown here below.

Table 5

δ-endotoxins(S)	**EC_{50} (μg / ml semisynthetic diet)**
Cry1Ea	**>176**
Cry1Ca	**136.22±8.77**
Chimeric Cry1E	**26.71±1.39**

The result shows that the novel chimeric δ-endotoxoin designed by us is not only more toxic to *Spodoptera* but also effective against *Helicoverpa*. The designing has widened the host range of the toxin as well as substantially improved toxicity over the parental proteins.

Example 2

High larval toxicity of the transgenic plants expressing novel chimeric Cry1E protein

In order to establish efficacy of the novel chimeric toxin in plants, a plant transformation vector was constructed for the development of transgenic plants. A plasmid pPK58 (having CaMV35S promoter with duplicated enhancer) was digested with *Bam*HI and *Hind*III and pPK200 with *Hind*III and *Eco*RI to excise out CaMV35S promoter with the duplicated enhancer and chimeric *cry1E* gene, respectively. A triple ligation was carried for the cloning of the two fragments in pLITMUS38 cloning vector (New England Biolabs). The plasmid was named pPK59, which had CaMV35S promoter with the duplicated enhancer at the upstream of the chimeric gene. The *nos* transcription terminator was cloned at the downstream of the chimeric gene *nos* polyadenylation element was amplified using pBI101.1 as template with suitable primers, which created *Mfe*I and *Eco*RI restriction sites at the upstream and downstream, respectively. The plasmid pPK59 was digested with *Eco*RI and the PCR product was cloned following the digestion with the *Mfe*I and *Eco*RI restriction enzymes. The clone in which *Eco*RI restriction site of the synthetic gene ligated to *Mfe*I site of *nos* terminator (as they have compatible ends) was selected and named as pPK201. The correct orientation of *nos* terminator was confirmed by restriction analysis and also by DNA sequencing. The expression cassette (the synthetic *cry* gene with E-35S promoter and *nos* terminator) was cloned in Ti binary vector. *Bam*HI-*Eco*RI fragment of plasmid pPK201 was cloned in pBI101.1 replacing *Bam*HI-*Eco*RI fragment (*uidA* gene and *nos* terminator) of the plasmid. This binary vector was named as pPK202. The construction of *E. coli* and plant expression vector is schematically presented in figure 3. The construct had polynucleotide sequences TAAACCATG GCT as plant preferred translation initiation context, TAA TGA were introduced in synthetic gene for translational termination. *Agrobacterium tumefaciens* strain LBA 4404 containing helper plasmid pAL4404 was transformed with binary vector pPK202 following the modified protocol of "electroporation of *Agrobacterium*" discussed by Cangelosi *et al.* (1991) and transformed colony was selected on antibiotics streptomycin, rifampicin and kanamycin. *Agrobacterium* mediated transformation of *Nicotiana tabacum* cv. Patit Havana was carried out following the method of Horsch *et al.*, 1985 and the transgenic plant were selected on the antibiotic kanamycin. The presence of the gene encoding chimeric toxin was confirmed with the PCR and Southern Analysis and the expression of the transgene was established with the RT-PCR, Western analysis and ELISA. ELISA result established

0.5% expression of the toxin protein in total soluble leaf protein in the transgenic line selected for these experiments. This high level of the expression was the result of the designing of the gene in which plant-preferred codons were exclusively used. Plant preferred translation initiation context also would have played an important role in the expression. Insect bioassay was performed with the leaves of two-month-old transgenic plants and neonatal larvae of *Spodoptera litura*. 15 cm^2 leaf-discs of transgenic and control plants were placed in cylindrical boxes containing wet blotting paper at the bottom and ten 1^{st} instar larvae were released onto them. Mouths of the boxes were covered with wet muslin cloth to maintain sufficient humidity and to allow the exchange of air. The toxicity experiments were conducted in six replicates at 25±0.2°C and 16/8 h photoperiod was maintained. The result showed (figure 4) that the transgenic plants expressing novel chimeric protein were highly toxic to the neonatal larvae of *Spodoptera litura* and cause 100% mortality within 48 h of feeding. The damage of leaf-discs by the insect larvae was negligible as compared to control plant leaf-discs, which were almost completely eaten away. High level of the protection of the transgenic plant and mortality of *Spodoptera* larvae upon feeding on transgenic plants again established the efficacy of the chimeric toxin and the transgenic plants.

Example 3

High toxicity of the chimeric Cry1E protein to larvae of *Spodoptera sp.* in all the stages of their development

A bioassay was conducted on 1^{st} (3 days old), 3^{rd} (7 days old) and 5^{th} (12 days old) instar larvae to established the efficacy of the engineered protein expressed in the transgenic plants. Bioassay with 1^{st} instar larvae has been discussed in example 2. Complete leaves of transgenic and control plants were used for feeding the advanced stage larvae. The leaf petiole was held in cotton plug over a 250 ml flask containing ½ MS salt solution to overcome wilting of the leaf. 5 insect larvae were allowed to feed on each leaf. The leaves of control plant fed by 3^{rd} (figure 5) and 5^{th} (figure 6) instar larvae were changed after 16 h and 8 h, respectively, as they were consumed completely by the insect larvae. The result established that feeding on transgenic leaf causes mortality of larvae in all the developmental stages within 48 h. Ingestion of approximately 1 cm^2 of transgenic leaf was sufficient to kill 5^{th} instar larvae. These larvae appeared moribund after 8-16 h of feeding on the transgenic leaf and finally died within 2 days. Green coloured excreta with high water content were noticed on leaf surface. Some of the larvae showed heavy weight loss before death.

In a separate experiment, different instar larvae were fed on leaves of transgenic plants for 1 h, 2 h, 4 h, 8 h and 16 h and then shifted to control plant leaves. It was observed that 4 h of feeding on transgenic plants was sufficient to cause 100% mortality of larvae in all stages of development, even when they were subsequently fed on non-transgenic plants. Ingestion of very small amounts of the toxin by young larvae delayed their pupation by 10-15 days beyond the normal larval cycle of 15 days. The few larvae that escaped mortality developed into flies. 40% of the paired matings using such flies gave eggs. However, the eggs were sterile and failed to hatch. The toxicity of δ-endotoxin to advance stage insect larvae has not been reported in literature till date. The high level of toxicity may be due to higher stability of chimeric toxin in the mid gut of insect larvae or improved receptor binding and pore-forming ability of δ-endotoxin. The example again established the potential of the chimeric toxin against *Spodoptera sp.* Since *Helicoverpa* does not prefer to eat tobacco leaf, toxicity experiment with transgenic tobacco plants could not be conducted on *Helicoverpa*.

Example 4
High toxicity of the novel chimeric Cry1E prepared from leaves of transgenic tobacco plants expressing δ-endotoxin

Total soluble protein was prepared from leaves of transgenic tobacco. Fresh leaf tissue was powered under liquid N_2 and then suspended in 5 volumes of protein extraction buffer (TrisCl, 20 mM, pH 9.5; EDTA 2 mM, pH 8.0; NaCl, 50 mM; DTT, 1 mM; PVP 2% and PMSF, 100 mM). The suspension was mixed well and centrifuged twice (20,000xg, 20 min and 4°C). The supernatant was loaded on Sepharose Q column (10 cm x 2.5 cm). The protein was eluted with increasing gradient of NaCl in extraction buffer and 100 fractions of 5 ml were collected. The δ-endotoxin was detected with ELISA. The fractions containing the δ-endotoxin were pooled. A known amount of the plant-purified δ-endotoxin was mixed in semi-synthetic diet. The toxicity trials were conducted with 3-day old larvae of *Spodoptera litura* and *Helicoverpa armigera*, as described in previous examples. The result showed that EC_{50} (the concentration required for 50% killing) for *Spodoptera* and *Helicoverpa* were 42.39±1.72 ng/ml and 283.11±8.29 ng/ml of semi-synthetic diet. The result further established the high level of toxicity to the larvae of *Spodoptera* and *Helicoverpa*. The point is noteworthy that insecticidal crystal protein made in plant tissue is much more toxic as compared to the same protein made in *E. coli*. Probably the δ-endotoxin folds much better in plant cytoplasm, the role of some unidentified chaperons in such folding cannot be overruled.

Example 5

Stability of chimeric δ-endotoxin in plant tissue

Total soluble leaf protein of transgenic plant was extracted as discussed earlier and incubated at 4°C and 28°C. They were used for toxicity trials. The samples were taken out after every two days in case of former and every day in case of later. The total crude protein was mixed in semi synthetic diet and toxicity experiment was carried out with neonatal larvae of *Spodoptera litura*. The insect mortality data was recorded after 7 days of feeding and LC_{50} was calculated. The results are shown in Tables 6, and 7 here below.

Table 6

S.No	Incubation period of crude protein at 4°C (in days)	LD_{50} (in ng/ml of semi- synthetic diet)
1.	2	48.71 ± 2.4
2.	4	57.87 ± 2.96
3.	6	66.44 ± 3.65
4.	8	70.19 ± 3.55
5.	12	73.82 ± 3.1
6.	16	74.37 ± 3.67

Table 7

S.No	Incubation period of crude protein at 28°C (in days)	LD_{50} (in ng/ml of semi-synthetic diet)
1.	1	76.44 ± 3.3
2.	2	98.13 ± 4.6
3.	3	143.46 ± 8.5
4.	4	---
5.	5	---

The result established the stability of chimeric δ-endotoxin designed by us against the plant proteases. The chimeric toxin was stable for more than 16 days at 4°C and 3 days at 28°C, and caused more than 80% mortality. Increase in LC_{50} with time was presumably due to some degradation of the toxin.

Main advantages of the present invention are:

1. Cry1Ea δ-endotoxin was engineered to obtain a novel chimeric toxin, herein said chimeric Cry1E. The toxicity of the chimeric protein was several fold higher as compared to the parent toxins or other δ-endotoxin reported to function against *Spodoptera*. Its larvicidal activity was very high when it is made in plants.

2. The gene encoding chimeric toxin was designed to express both in *E. coli* and plants. Hence, it can be used in the engineering of a microbe for the expression of chimeric toxin, which can be used in the preparation of the microbial formulation. The same gene can also be used in the genetic engineering of the plants for the trade of insect resistance. We have shown that the sequence designed by us gives very high level of expression (0.5% of the total soluble protein) of the chimeric toxin in transgenic tobacco and cotton leaves (results not included).

3. The transgenic plants expressing the chimeric toxin exhibited very high degree of protection against the larvae of *Spodoptera litura* in all developmental stages. They died within 2-3 days of feeding on the transgenic plants. Such high level of toxicity of transgenic plants against any lepidopteran insect has not been reported till date. This protein may also be effective against many other insect larvae. Our results show that the toxin was effective against *Helicoverpa* also.

4. Potential of the chimeric toxin in transgenic plants was further established by short-term feeding on transgenic plants. Feeding for 4 hours caused 100% mortality of *Spodoptera* larvae at all the developmental stages. The feeding for extremely short (up to one hour) periods, delayed larval development and interfered with metamorphosis. Such protein may be extremely valuable in protecting agronomically important crops and forests. The gene coding chimeric toxin can be used in the development of transgenic plants and/or for production of the toxin in a microbe, which can be used in microbial formulations.

5. Since the transgenic plants expressing the novel chimeric toxin caused 100% mortality of *Spodoptera* larvae within a very short period of feeding, the probability of the development of resistance in insects against this δ-endotoxin will be extremely low.

Information for sequence ID No. 1

1. Sequence characteristics

A Length : 641
B Type : Amino Acid
C Topology : Linear
D Molecular type : Protein

2. Sequence description : Sequence ID No. 1

1 M A I V N N Q N Q C V P Y N C L N N P E N E I L D I E R S N
31 S T V A T N I A L E I S R L L A S A T P I G G I L L G L F D
61 A I W G S I G P S Q W D L F L E Q I E L L I D Q K I E E F A
91 R N Q A I S R L E G I S S L Y G I Y T E A F R E W E A D P T
121 N P A L K E E M R T Q F N D M N S I L V T A I P L F S V Q N
151 Y Q V P F L S V Y V Q A A N L H L S V L R D V S V F G Q A W
181 G F D I A T I N S R Y N D L T R L I P I Y T D Y A V R W Y N
211 T G L D R L P R T G G L R N W A R F N Q F R R E L T I S V L
241 D I I S F F R N Y D S R L Y P I P T S S Q L T R E V Y T D P
271 V I N I T D Y R V G P S F E N I E N S A I R S P H L M D F L
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Information for sequence ID NO. 2

1. Sequence characteristics

A Length : 1990 bp
B Type : Nucleic Acid
C Standards : Double
D Topology : Linear
E Molecule Type : Synthetic
F Original source : *Bacillus thuringiensis*
G Feature : Chimeric gene

2. Sequence description : Sequence ID No. : 2

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<140> 2,480,319

<141> 2000-03-28

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Claims:

1. A chimeric δ endotoxin protein Cry1E of SEQ ID No. 1.

2. The protein as claimed in claim 1, wherein said chimeric protein of SEQ ID No. 1 is designed from δ endotoxins Cry1Ea and Cry1Ca of *Bacillus thuringiensis*.

3. The protein as claimed in claim 1, wherein said chimeric protein of length of 641 residues is consisting of residues 1 to 529 from endotoxin Cry1Ea of same position, residues 530 to 599 from Cry1Ca of position 533 to 602, residues 600 to 616 from Cry1Ea of position 588 to 604 and residues 617 to 641 of a synthetic polypeptide.

4. The protein as claimed in claim 1, wherein the last 25 amino acid residues improve the stability against the proteases of plants.

5. The protein as claimed in claim 1, wherein said chimeric protein is stable at temperature ranging between 4-35°C.

6. A chimera gene of SEQ ID No. 2.

7. The chimera as claimed in claim 6, wherein said chimera encodes chimeric protein of SEQ ID No. 1.

8. The chimera as claimed in claim 6, wherein said chimera is a 1.99-kb double stranded DNA.

9. The chimera as claimed in claim 6, wherein said chimera contains plant preferred codon distributed evenly to facilitate efficient translation.

10. The chimera as claimed in claim 6, wherein said chimera contains plant preferred translation initiation codon of ATGGCT at 5' end.

11. The chimera as claimed in claim 6, wherein said chimera contains plant preferred translation termination codon of TAATGA.

12. The chimera as claimed in claim 6, wherein said chimera contains 33 restriction sites distributed uniformly throughout the length of the gene at a distance of about 40-80 bp.

13. The chimera as claimed in claim 12, wherein restriction sites are recognized by enzymes selected from a group consisting of *Hind* III, *Eco*RI, and *Bam*HI.

14. The chimera as claimed in claim 6, wherein said chimera is divided into 58 overlapping oligonucleotides of length 40 to 65 base pairs (bp) each located at a distance of 6 to 26 bp.

15. The chimera as claimed in claim 6, wherein said chimera contains overlapping nucleotides with an overlap of 13 to 18 nucleotides with the immediately adjacent oligonucleotides on the complementary strand.

16. The chimera as claimed in claim 6, wherein said chimera has T_m value ranging between 44 to 55°C.

17. A method of overexpressing insecticidal chimeric protein Cry1E in microbes, said method comprising:

(a) cloning gene Cry1E of SEQ ID No.2 encoding said chimeric protein in a vector,

(b) transforming microbe with said cloned vector, and

(c) overexpressing said chimeric protein into said microbe.

18. The method as claimed in claim 17, wherein said chimera is expressed into a microbe selected from a group consisting of bacteria, algae, and fungi.

19. The method as claimed in claim 17, wherein restriction enzymes for said cloning are selected from a group consisting of *Hind* III, *Eco*RI, N*col*, *Mfe*I and *Bam*HI.

20. The method as claimed in claim 17, wherein overexpression of said protein is induced by using isopropylthiogalactoside (IPTG).

21. The method as claimed in claim 17, wherein said protein is overexpressed at 15°C to avoid mis-folding of said proteins.

22. The method as claimed in claim 17, wherein said vector is selected from a group consisting of Plasmids, viral DNA, and cosmids.

23. The method as claimed in claim 17, wherein expression of said chimera in the microbe is confirmed by RT-PCR, western Analysis, and ELISA.

24. The method as claimed in claim 17, wherein the presence of said chimera in the microbe is confirmed by PCR and southern Analysis.

25. A method of treating plants infected with insects using insecticidal chimeric protein of claim 1, said method comprising:

(a) incorporating gene encoding chimera protein Cry1E into plant infected with insects,

(b) exposing transgenic plant to insects, and

(c) determining insecticidal activity of said transgenic plants,

wherein the insects are selected from the group consisting of *Spodoptera sp.*, and *Helicoverpa sp*.

26. The method as claimed in claim 25, wherein plants are selected from a group consisting of tobacco, cotton, chickpea, pegeonpea, groundnut, cauliflower, cabbage, chilli, and capsicum.

27. The method as claimed in claim 25, wherein the chimeric protein is about 0.5% of the total soluble protein of the plants.

28. The method as claimed in claim 25, wherein said chimeric protein is stable in said transgenic plant.

29. The method as claimed in claim 25, wherein insects exposed to said chimeric protein show weight loss before death.

30. The method as claimed in claim 25, wherein said chimeric protein shows insecticidal property against insect at all developmental stages.

31. The method as claimed in claim 25, wherein said chimeric protein is multifold more potent insecticide as compared to parental proteins.

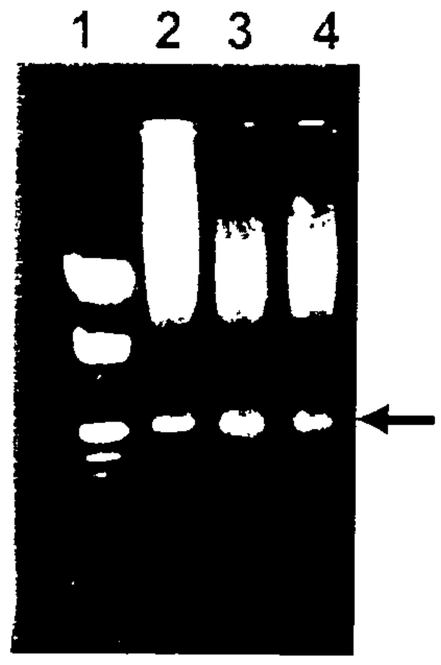
32. The method as claimed in claim 25, wherein insecticidal activity of said chimeric protein shows mortality of insect pests ranging between 80-100% within about 4 hours of exposure.

33. The method as claimed in claim 25, wherein insects exposed to said chimeric protein for about 1 hour show delayed development, infertility and disrupted metamorphosis.

34. The method as claimed in claim 25, wherein EC_{50} for *Helocoverpa sp.* is ranging between 250-350 ng/ml of artificial diet of insects.

35. The method as claimed in claim 25, wherein EC_{50} of *Spodoptera sp.* is ranging between 25-50 ng/ml of artificial diet.

36. The method as claimed in claim 25, wherein said method shows no adverse effect on the normal growth of the transformed plants.



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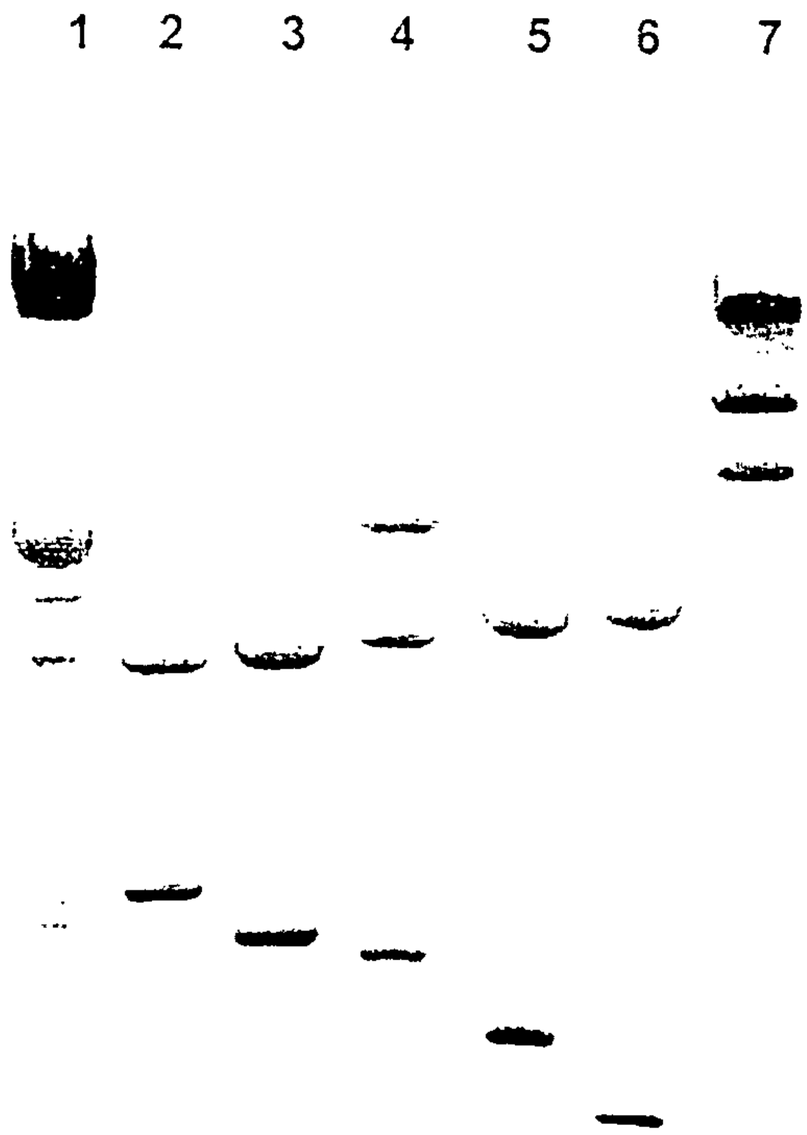

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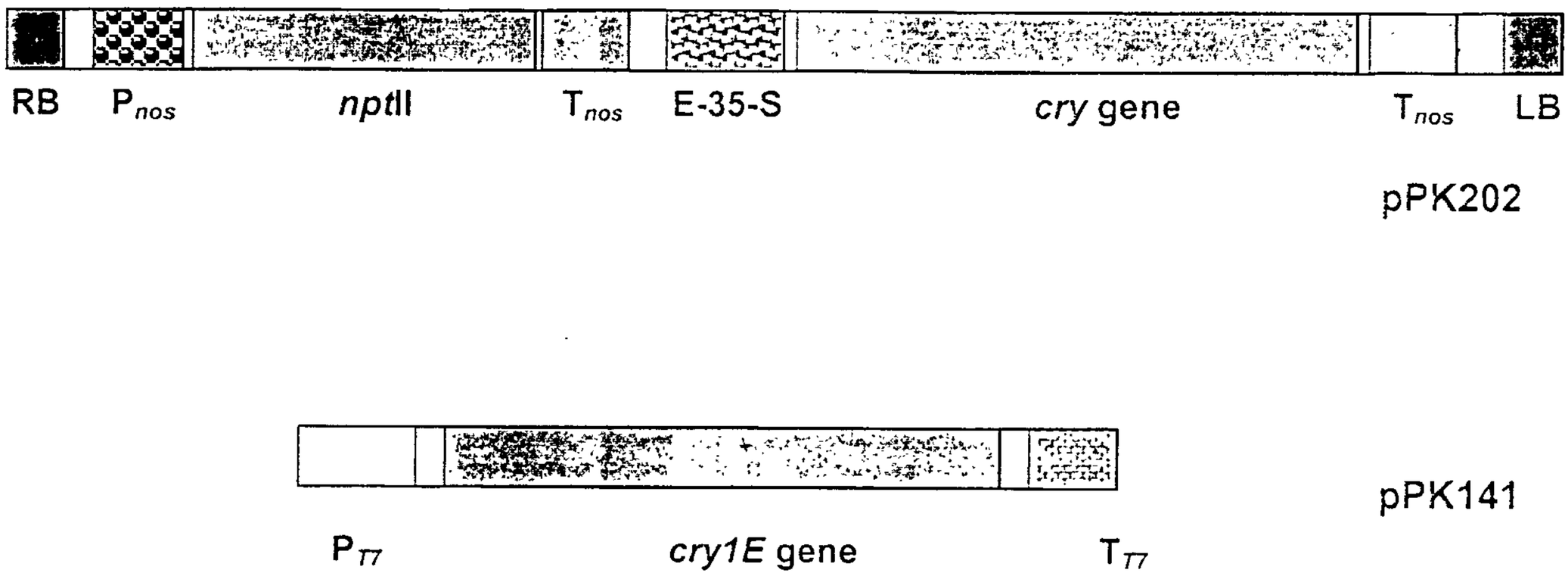
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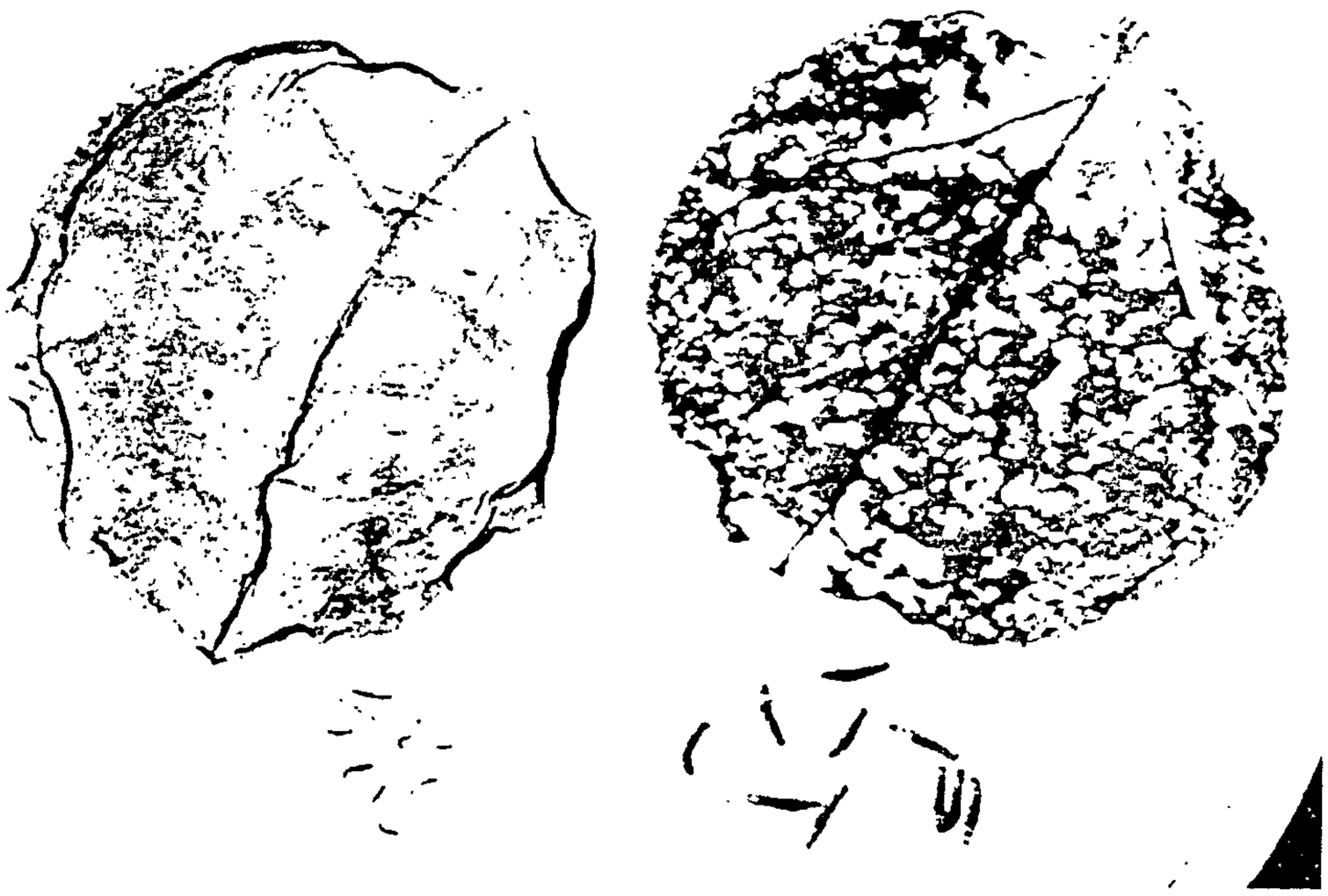
Fig.3



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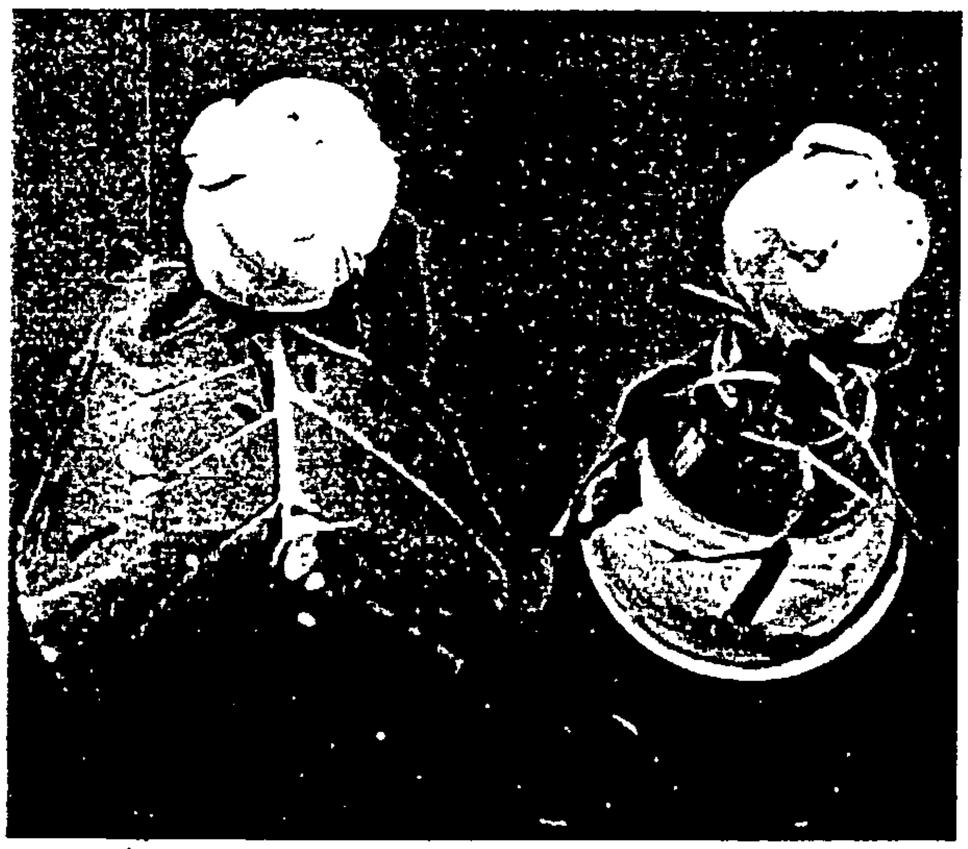

Fig.5

Fig.6