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Application for Patent for

**INSECTICIDAL COMPOSITIONS AND METHODS FOR MAKING INSECT-
RESISTANT TRANSGENIC PLANTS**

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BACKGROUND OF THE INVENTION

The present invention relates generally to the field of plant molecular biology, and more particularly to novel polynucleotide sequences and proteins encoded from such sequences derived from *Bacillus thuringiensis* and that encode ET29, TIC809, ET37, TIC810 and TIC812 proteins that exhibit toxicity to coleopteran species and to insects within the super-order referred to as Hemiptera. Coleopteran toxic proteins include ET29, TIC809 (an amino acid sequence variant of ET29), and ET37 (a homologue of ET29). TIC810 and TIC812 and nucleotide sequences encoding these proteins are also provided herein. When TIC810 or TIC812 are combined together with ET29, TIC809 or ET37, insecticidal compositions are provided that exhibit surprisingly greater potency against coleopteran species as compared to the presentation of only ET29, TIC809, or ET37 alone, and the combination of the two (TIC810 with either ET29, TIC809, or ET37, or TIC812 with either ET29, TIC809, or ET37) surprisingly provides a Hemiptera toxic composition, particularly when provided in the diet of species such as *Lygus hesperus* (western tarnished plant bug, WTPB). Methods of making and using polynucleotides encoding these and related proteins in the development of transgenic plants and plant cells that are resistant to Coleoptera and Hemiptera insect infestation are also disclosed.

Environmentally-sensitive methods and compositions for controlling or eradicating insect infestation are desirable in many instances because crops of commercial interest are often the targets of insect attack, particularly attack from coleopteran and lepidopteran insect pests. This is particularly true for farmers, nurserymen, growers, and commercial and residential areas which seek to control insect populations using environmentally friendly methods and compositions. Controlling or eradicating Hemiptera infestations of crops is also of commercial importance, and is increasing in importance as biotechnology approaches for coleopteran and Lepidopteran pest control methods become more widely available, particularly because fewer chemical insecticidal applications are utilized, which result in broad spectrum insecticidal activity.

The insecticidal properties of the bacterium *Bacillus thuringiensis* have been long recognized. *B. thuringiensis* is well known for producing proteinaceous parasporal crystals, or δ -endotoxins, that exhibit specific toxicity to a variety of lepidopteran, coleopteran, and dipteran larvae (English et. al., US Patent No. 6,063,597). Compositions comprising *B. thuringiensis* strains that produce insecticidal proteins have been used commercially as

environmentally acceptable insecticides because they exhibit toxicity to specific target insects, and fail to exhibit toxicity to plants, animals and other non-target organisms.

More than 250 different δ -endotoxins have been isolated and characterized. Sequences encoding some of these δ -endotoxins have been used to construct genetically engineered *B. thuringiensis* products in which one or more insecticidal proteins are expressed that exhibit specific insecticidal activity to target pests, and have been approved for agricultural use as topically applied insecticidal compositions. Transgenic plants expressing one or more Bt insecticidal delta endotoxin proteins for use in controlling one or more insects within a specific class, such as Lepidopteran or coleopteran pests, have been approved for commercialization and have been successful. However, there is a risk that populations of target pest insects that feed on these transgenic plants will develop resistance to one or more of the toxins produced by the plants, and so there remains a need for identifying new insecticidal proteins that can be used alone or together with others that manifest their toxic effects through different modes of action. New insecticidal compositions are desirable for producing transgenic plants that express one or more *B. thuringiensis* insecticidal proteins toxic to the same insect species, providing a means for managing resistance and delaying or eliminating the development of resistance of any particular susceptible insect species to any of the one or more insecticidal agents expressed within a transgenic plant.

Most Bt toxins exhibit toxicity to lepidopteran species. Few have been shown to be effective against coleopteran species, and other than cytolytic toxins which exhibit no host range specificity, no Bt toxins have been shown to exhibit insecticidal activity to lepidopteran or coleopteran species and to Hemipteran species of insect pests. Thus there is a need for identifying new coleopteran and/or Hemipteran specific insecticidal compositions, and methods for controlling infestations by members of the Coleoptera and Hemiptera insect families, particularly for Coleoptera, by members of the family Chrysomelidae, more particularly, by the genus *Diabrotica* in the family Chrysomelidae that may include those that are from the genus *Diabrotica* including *Diabrotica virgifera* (western corn rootworm, WCR), *Diabrotica undecempunctata* (southern corn rootworm, SCR), *Diabrotica barberi* (Northern Corn Rootworm, NCR), *Diabrotica virgifera zeae* (Mexican Corn Rootworm, MCR), *Diabrotica balteata* (Brazilian Corn Rootworm, BZR) and Brazilian Corn Rootworm complex (BCR) consisting of *Diabrotica viridula* and *Diabrotica speciosa*, and particularly by members of the super-order Hemiptera, which includes any insect pest within the sub-order Heteroptera, including insects commonly referred to as stink bugs, Lygus bugs

(including *Lygus hesperus*, *Lygus lineolus*, and *Lygus elisus*), assassin bugs, bed bugs, and flower bugs, and the sub-order Homoptera, including insects commonly known as cicadas, aphids, leafhoppers, scale insects and whiteflies.

SUMMARY OF THE INVENTION

The present invention provides polypeptide compositions isolated from *B. thuringiensis* that exhibit insecticidal activity against Coleoptera and Hemiptera insect pests, and provides nucleotide sequences encoding such polypeptides. The present invention provides control of Coleoptera and Hemiptera insect infestation by co-expressing at least two *B. thuringiensis* proteins in plants either as independent proteins or as a fusion protein of the two, resulting in surprisingly high levels of accumulation of the insecticidal proteins for providing effective control of target Coleoptera and Hemiptera insect pests and provides improvements in insect resistance management of Coleoptera and Hemiptera insect infestations. Additionally, a method of increasing the level of *in planta* accumulation of a *B. thuringiensis* insecticidal protein or variant thereof is provided that also provides the additional benefit of an absence of abnormal plant morphology for transgenic plants expressing these proteins.

In accomplishing the foregoing, a polynucleotide molecule is provided as set forth in SEQ ID NO:1, isolated from *B. thuringiensis* strain EG5078. The polynucleotide molecule encodes an insecticidal protein as set forth in SEQ ID NO:2, designated herein as ET37, exhibiting coleopteran insect pest inhibitory bioactivity.

A polynucleotide molecule as set forth in SEQ ID NO:3 is also provided that is isolated from *B. thuringiensis* strain EG4096 that encodes a TIC810 amino acid sequence as set forth in SEQ ID NO:4.

Yet another polynucleotide sequence is provided as set forth in SEQ ID NO:5 that encodes a TIC812 amino acid sequence as set forth in SEQ ID NO:6, which is isolated from *B. thuringiensis* strain EG5078. TIC812 is substantially identical to TIC810.

Specifically contemplated herein is an isolated polynucleotide molecule encoding an insecticidal protein or an insecticidal fragment thereof that exhibits at least from about 70% to about 99% or greater sequence similarity to a polypeptide sequence as set forth in SEQ ID NO:4 and SEQ ID NO:6, or any percentage there between. The insecticidal protein or insecticidal fragment is encoded by an isolated polynucleotide molecule derived from *B. thuringiensis*, and comprises a polynucleotide sequence that exhibits at least about 70%, about 80%, about 90% or about 99% or more sequence identity or any percentage in between,

or that hybridizes under stringent conditions, to a polynucleotide sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:5 or the complement thereof.

5 In still another embodiment, there is provided a polynucleotide molecule for use in achieving improved expression of an insecticidal protein in a plant. The insecticidal protein preferably exhibits biological activity in controlling a Coleopteran or a Hemipteran insect pest or both, but may be active against other than a Coleopteran or Hemipteran insect pest, and may be encoded from a nucleotide sequence engineered for expression in a plant cell, such as is set forth in SEQ ID NO:15 (TIC810) or SEQ ID NO:19 (ET37).

10 Other polynucleotide sequences are provided for herein, for use in achieving stably transformed plant cells expressing one or more of the proteins of the present invention. Such nucleotide sequences include but are not intended to be limited to SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:28, SEQ ID NO:31, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:43, and SEQ ID NO:46.

15 Oligonucleotide sequences are provided for use in identifying related nucleotide sequences in other bacteria, and in particular in other *Bacillus* bacterial strains including *Bacillus thuringiensis* and *Bacillus laterosporus* and *Bacillus entomocidus*.

Insecticidal proteins are provided that are exemplified by the amino acid sequences as set forth at SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:14, and SEQ ID NO:47 (TIC127). In particular embodiments, SEQ ID NO:4 (TIC810) and SEQ ID
20 NO:6 (TIC812) are provided as accessory proteins, chaperones, or proteins that otherwise stabilize and enhance the expression and accumulation of a second protein expressed contemporaneously along with these accessory proteins. TIC809, ET29 and ET37 are all insecticidal proteins that exhibit greater levels of stability and therefore improved levels of accumulation when each are contemporaneously expressed along with a TIC810 or a TIC812
25 amino acid sequence. These sequences may be expressed together in the same subcellular compartment, i.e., both in the cytoplasm or both targeted for insertion into and accumulation in the chloroplast or plastid of a plant cell, or contemporaneously expressed to accumulate in different subcellular compartments, such as a TIC810 in the cytoplasm and an ET29 or TIC809 targeted for insertion into and accumulation in the plant chloroplast or plastid. In
30 addition, the combination of any one of the TIC809, ET29, or ET37 proteins along with either of the TIC810 or TIC812 proteins results in a composition that is surprisingly stabilized for high level expression and accumulation of both protein components in plant cells and exhibits insecticidal activity directed to controlling plant pests in the orders

Coleoptera and Hemiptera. The combination of proteins may also be expressed as a peptide fusion, for example, such as SEQ ID NO:47.

In a further embodiment, the present invention relates to a biologically pure culture of a *B. thuringiensis* wild type strain selected from the group consisting of EG5078, from which the polynucleotide sequence as set forth in SEQ ID NO:1 encoding the ET37 protein and SEQ ID NO:5 encoding the TIC812 protein are isolated, and EG4096, from which SEQ ID NO:3 encoding the TIC810 protein and SEQ ID NO:7 encoding the ET29 protein are isolated. EG4096 and EG5078 have been deposited with the Northern Regional Research Laboratory of Agricultural Research Service Center Collection (NRRL), USDA, 1815 North University Street, Peoria, IL 61604, pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure and have been assigned the accession Numbers NRRL-B-21582 and NRRL-B-30841 respectively. EG4096 was deposited on May 30, 1996 and EG5087 was deposited on May 3, 2005.

The present invention also relates to a recombinant DNA construct for expression in a plant or plant cell comprising a double gene cassette simultaneously expressing a first polynucleotide sequence and a second polynucleotide sequence wherein the first polynucleotide sequence encodes a polypeptide sequence selected from the group consisting of ET29, TIC809, and ET37 or insecticidally active fragments thereof, and wherein the second polynucleotide sequence encodes a polypeptide sequence selected from the group consisting of TIC810, and TIC812, and insecticidally active fragments thereof. The first polynucleotide sequence is selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:17 and the second polynucleotide sequence is selected from the group consisting of SEQ ID NO:15 and SEQ ID NO:19, wherein the second polynucleotide sequence is co-expressed with the first polynucleotide sequence to enhance or improve expression of the first polynucleotide sequence, and to facilitate insecticidal activity directed to controlling Coleopteran and Hemipteran plant pest infestation.

The present invention also relates to a host cell transformed to contain either the expression vector or the recombinant DNA construct of the present invention, disclosed herein. The host cell may be selected from the group consisting of a bacterial cell, a fungal cell, and a plant cell. In one aspect of the embodiment, the host cell is a bacterial cell such as a *B. thuringiensis* cell that is transformed to contain the expression vector of the present invention. In another aspect of the embodiment, the host cell is a transgenic plant cell transformed to contain the recombinant DNA construct of the present invention. The recombinant DNA construct may comprise polynucleotide sequences that encode a

combination of two of the four proteins simultaneously which polypeptide sequences are set forth in SEQ ID NO:14 (TIC809), SEQ ID NO:16 (TIC810), SEQ ID NO:18 (ET37) and SEQ ID NO:20 (TIC812). Preferable combinations of the proteins may include TIC809 and TIC810, TIC809 and TIC812, ET37 and TIC810, and ET37 and TIC812 wherein co-expression of the polynucleotide sequence encoding TIC810 or TIC812 with the polynucleotide sequence encoding TIC809 (or ET29) or ET37 will result (a) in increased accumulation of TIC809 (or ET29) or ET37 protein in the plant cell, (b) in normal cell growth, (c) in a transgenic plant regenerated from the host plant cell, (d) in a normal phenotype, and (e) in increased levels of Coleopteran and Hemipteran insect resistance.

The transgenic plant cell of the present invention may comprise a maize plant cell, a wheat plant cell, a rye plant cell, a barley plant cell, an oat plant cell, a buckwheat plant cell, a sorghum plant cell, a rice plant cell, a sugarcane plant cell, a pigeon pea plant cell, a peanut plant cell, an onion plant cell, a garlic plant cell, a grass plant cell (including bent grass, fescue, brome, Timothy, orchard, Bermuda, zoysia, and the like), an Arabidopsis plant cell, a broccoli plant cell, a sunflower plant cell, a canola plant cell, a pea plant cell, a cowpea plant cell, a bean plant cell, a coffee plant cell, a soybean plant cell, a cotton plant cell, a linseed plant cell, a cauliflower plant cell, an asparagus plant cell, a lettuce plant cell, a cabbage plant cell, a tobacco plant cell, a spice plant cell (including curry, mustard, sage, parsley, pepper, thyme, cilantro, bay, cumin, turmeric, nutmeg, cinnamon, and the like), a sugar beet plant cell, a potato plant cell, a sweet potato plant cell, a carrot plant cell, a turnip plant cell, a celery plant cell, a tomato plant cell, an egg plant cell, a cucumber plant cell, a squash or melon plant cell and the like, a fruit tree plant cell (including apple, apricot, peach, pear, plum, orange, lemon, lime, and the like), a nut tree plant cell (including acorn, hickory, Brazil, pecan, walnut, hazelnut, and the like), a grape plant cell, a berry plant cell (including blackberry, blueberry, strawberry, cranberry, and the like), and flower plant cells.

In another embodiment, the present invention relates to a transgenic plant transformed to contain a recombinant DNA construct, disclosed herein. The transgenic plant may be regenerated from the transgenic plant cell of the present invention, or may be from a transgenic seed that is obtained from the regenerated transgenic plant or its offspring. The transgenic plant is selected from the group consisting of a monocot plant and a dicot plant that may include monocots such as maize, wheat, rye, barley, oats, buckwheat, sorghum, rice, sugarcane, onion, garlic, grass, or dicots such as sunflower, canola, peas, cowpeas, pigeon peas, beans, soybeans, coffee, broccoli, cotton, linseed, cauliflower, Arabidopsis, asparagus, lettuce, tobacco, spice plants (including curry, mustard, sage, parsley, pepper, thyme, cilantro,

bay, cumin, turmeric, nutmeg, cinnamon and the like), sugar beet, potato, sweet potato, carrot, turnip, celery, tomato, egg plant, cucumber, squash or melon, fruit tree plant (including apple, apricot, peach, pear, plum, orange, lemon, lime and the like), berry plants (including blackberry, blueberry, strawberry, cranberry and the like), nut tree plants (including acorn, hickory, Brazil, pecan, walnut, hazelnut, and the like), grape plants, and flower plants.

In another embodiment, the present invention relates to a transgenic seed from the transgenic plant transformed to contain a recombinant DNA construct. The transgenic seed may be from the transgenic plant that is regenerated from the transgenic plant cell of the present invention, or may be from offspring of the regenerated transgenic plant, or from hybrids created as a result of crossing or breeding the transgenic plant with a non-transgenic plant. In one aspect, the transgenic seed may be coated with a seed coating and wherein the seed coating comprises a herbicidal composition, a fungicide seed coating, a bactericide seed coating, an insecticide seed coating, a plant hormone seed coating, a nutrient seed coating, a microbial inoculum seed coating, a color seed coating, an avian repellent seed coating, a rodent repellent seed coating, an insecticidal protein seed coating, a bacterial seed coating containing an insecticidal protein, a single stranded RNA seed coating, a double stranded RNA seed coating, a micro RNA seed coating or a small interfering RNA seed coating. One means for enabling and stabilizing a seed coating comprising such single or dsRNA compositions is to combine such RNA molecules with complementary DNA molecules so that stabilized DNA-RNA molecular hybrids are presented in the seed coating composition, enabling the presentation of the dsRNA or single stranded RNA to a pest feeding on the seed or the microenvironment within the realm of the sprouting seed or micro roots of an emerging sprouting shoot upon germination of the coated seed.

In accordance with one embodiment of the present invention, there is provided a method for generating a plant resistant to Coleopteran and/or Hemipteran insect infestation, comprising the steps of: a) inserting into the genome of a plant cell a first nucleic acid molecule that functions in the plant to encode a first protein selected from the group consisting of SEQ ID NO:14 (TIC809) and SEQ ID NO:18 (ET37), and a second nucleic acid molecule that functions in the plant to encode a second protein selected from the group consisting of SEQ ID NO:16 (TIC810) and SEQ ID NO:20 (TIC812);

b) obtaining the plant cell containing the nucleic acid molecules of step (a); and

c) generating from the plant cell a transgenic plant that expresses both proteins, wherein the transgenic plant exhibits Coleopteran and/or Hemipteran pest resistance compared to a plant lacking said molecules.

5 In another embodiment, the present invention also provides a method for controlling a Coleopteran and/or Hemipteran insect pest infestation of a plant, comprising providing in the diet of the insect pest a plant, plant tissue or plant cells expressing a TIC809, ET2, or ET37 protein along with a TIC810 or TIC812 protein.

The polynucleotide and polypeptide compositions and methods disclosed herein will find particular benefit when used against Coleopteran and Hemipteran insect pests selected
10 from the group of Coleopteran families consisting of Chrysomelidae, Cucujidae, Scarabaeidae, Trogositidae, Tenebrionidae, Curculionidae, Elateridae and Bruchidae, and from members of the order Hemiptera including specifically the members of the sub-orders Heteroptera and Homoptera. In one aspect of the invention, the Coleopteran insects are those from the family Chrysomelidae. The exemplary Coleopteran insects in the family
15 Chrysomelidae may include those that are from the genus *Diabrotica* including *D. virgifera* (WCR), *D. undecempunctata* (SCR), *D. barberi* (NCR), *D. virgifera zeae* (MCR), *D. balteata* (BZR) and Brazilian Corn Rootworm complex (BCR) consisting of *D. viridula* and *D. speciosa*.

A nucleic acid sequence molecule may be constructed to incorporate a third structural
20 gene sequence encoding a third agent (dsRNA or protein) as a means for providing in the same plant an additional agronomic trait exhibiting activity directed to controlling more than one plant pest, such as exhibiting Coleopteran and/or Hemipteran insect control and an additional trait for a Lepidopteran insect resistance, resistance to bacterial, viral, or fungal infestation, nematode resistance, or for providing a supplemental trait such as a herbicide
25 resistance trait, a yield trait, a stress trait, a feed enhancement or trait that results in the enhancement of feed processing, and the like. The method may consist of the steps of inserting into the genome of a plant cell a first nucleic acid molecule encoding a protein that is selected from the group consisting of SEQ ID NO:14 (TIC809) and SEQ ID NO:18 (ET37). The first nucleic acid molecule is linked to a second nucleic acid molecule that
30 encodes a protein selected from the group consisting of SEQ ID NO:16 (TIC810) and SEQ ID NO:20 (TIC812). A third nucleic acid molecule that is introduced into the plant genome encodes an agent that provides for an agronomic trait that is other than that provided for by the first and second nucleic acid molecules, including but not limited to Lepidopteran insect resistance, resistance to bacterial, viral, or fungal infestation, nematode resistance, or for

providing a supplemental trait such as a herbicide resistance trait, a yield trait, a stress trait, a feed enhancement or trait that results in the enhancement of feed processing, and the like.

In addition to providing the proteins of the present invention in a composition for controlling Coleoptera or Hemiptera insect pest infestation, a polynucleotide sequence transcribing a ribonucleotide acid (RNA) molecule that functions, when ingested by an invertebrate pest, to control invertebrate pest infestation by inhibition of a biological function within the pest as a second mode of action or as an insect resistance management feature of the composition is also specifically contemplated. The RNA molecule may comprise a dsRNA molecule, a siRNA molecule, a miRNA molecule, or an ssRNA molecule, and should be specific for inhibiting an essential gene of a target pest such as a pest targeted by the compositions of the present invention.

The compositions and methods disclosed by the present invention provide many advantages over the prior art including those specifically outlined above. These advantages may include: obtaining improved control of susceptible insect pests including not only those that infest plants, obtaining a greater number of commercially viable insect resistant plant lines; achieving season long protection from insect pathogens; and increasing the incidence of morphologically-normal transformed plants.

DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is a *B. thuringiensis* polynucleotide sequence encoding an insecticidal ET37 protein.

SEQ ID NO:2 is an ET37 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:1.

SEQ ID NO:3 is a polynucleotide sequence encoding an insecticidal TIC810 protein.

SEQ ID NO:4 is a TIC810 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:3.

SEQ ID NO:5 is a *B. thuringiensis* polynucleotide sequence encoding a TIC812 protein.

SEQ ID NO:6 is a TIC812 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:5.

SEQ ID NO:7 is a *B. thuringiensis* polynucleotide sequence encoding an ET29 protein.

SEQ ID NO:8 is an ET29 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:7.

SEQ ID NO:9 is a *B. thuringiensis* polynucleotide sequence encoding TIC810 from nucleotide position 1 to nucleotide position 657 and encoding ET29 from nucleotide position 716 to 1411.

5 SEQ ID NO:10 is a *B. thuringiensis* polynucleotide sequence encoding TIC812 from nucleotide position 1 to 657 and encoding ET37 from nucleotide position 716 to 1411.

SEQ ID NO:11 is a polynucleotide sequence encoding TIC810 from nucleotide position 1 to nucleotide position 657 and encoding ET37 from nucleotide position 716 to 1411.

10 SEQ ID NO:12 is a polynucleotide sequence encoding TIC812 from nucleotide position 1 to 657 and encoding ET29 from nucleotide position 716 to 1411.

SEQ ID NO: 13 is a polynucleotide sequence constructed for expression in a plant cell that encodes a TIC809 protein.

SEQ ID NO:14 is a TIC809 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:13.

15 SEQ ID NO:15 is a polynucleotide sequence constructed for expression in a plant cell that encodes a TIC810 protein.

SEQ ID NO:16 is a TIC810 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:15.

20 SEQ ID NO:17 represents a polynucleotide sequence constructed for expression in a plant cell that encodes an ET37 protein.

SEQ ID NO:18 is an ET37 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:19.

SEQ ID NO:19 is a polynucleotide sequence constructed for expression in a plant cell that encodes a TIC812 protein.

25 SEQ ID NO:20 is a TIC812 amino acid sequence encoded by the polynucleotide sequence as set forth in SEQ ID NO:17.

SEQ ID NO:21 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a TIC810 amino acid sequence and is referred to herein as pr370.

30 SEQ ID NO:22 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a TIC810 amino acid sequence and is referred to herein as pr371.

SEQ ID NO:23 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a TIC810 amino acid sequence and is referred to herein as pr375.

SEQ ID NO:24 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a TIC810 amino acid sequence and is referred to herein as pr376.

SEQ ID NO:25 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a ET29 amino acid sequence and is referred to herein as pr365.

SEQ ID NO:26 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a ET29 amino acid sequence and is referred to herein as pr372.

5 SEQ ID NO:27 is a thermal amplification primer for use in amplifying a nucleotide sequence encoding a TIC810_ET29 or a TIC812_ET37 operon sequence and is referred to herein as pr421.

10 SEQ ID NO:28 is a synthetic nucleotide sequence present in transformation vector pMON64138 and consisting of a first plant expression cassette encoding a TIC809 protein and a second plant expression cassette encoding a TIC810 protein.

SEQ ID NO:29 is a TIC809 amino acid sequence encoded from the first plant expression cassette as set forth in SEQ ID NO:28.

SEQ ID NO:30 is a TIC810 amino acid sequence encoded from the second plant expression cassette as set forth in SEQ ID NO:28.

15 SEQ ID NO: 31 is a synthetic nucleotide sequence present in pMON64139 and consisting of a first plant expression cassette encoding a chloroplast targeted TIC809 and a second plant expression cassette encoding a chloroplast targeted TIC810.

SEQ ID NO:32 is a TIC809 amino acid sequence encoded from the first plant expression cassette as set forth in SEQ ID NO:31.

20 SEQ ID NO:33 is a TIC810 amino acid sequence encoded from the second plant expression cassette as set forth in SEQ ID NO:31.

SEQ ID NO:34 is a synthetic nucleotide sequence present in pMON70513 and consists of a plant expression cassette encoding a TIC809 amino acid sequence.

25 SEQ ID NO:35 is a TIC809 amino acid sequence encoded from the plant expression cassette as set forth in SEQ ID NO:34.

SEQ ID NO:36 is a synthetic nucleotide sequence present in pMON70514 and consists of a plant expression cassette encoding a chloroplast targeted TIC809 amino acid sequence.

30 SEQ ID NO:37 is a TIC809 amino acid sequence encoded from the plant expression cassette as set forth in SEQ ID NO:36.

SEQ ID NO:38 is a synthetic nucleotide sequence present in pMON64144 and consists of a plant expression cassette encoding a chloroplast targeted TIC809 amino acid sequence.

SEQ ID NO:39 is a TIC809 amino acid sequence encoded from the plant expression cassette as set forth in SEQ ID NO:38.

5 SEQ ID NO:40 is a synthetic nucleotide sequence present in pMON64150 and consists of a first plant expression cassette encoding a chloroplast targeted TIC809 amino acid sequence and a second plant expression cassette encoding a chloroplast targeted TIC810 amino acid sequence.

SEQ ID NO:41 is a TIC809 amino acid sequence encoded from the first plant expression cassette as set forth in SEQ ID NO:40.

10 SEQ ID NO:42 is a TIC810 amino acid sequence encoded from the second plant expression cassette as set forth in SEQ ID NO:40.

SEQ ID NO:43 is a synthetic nucleotide sequence present in pMON64151 and consists of a first plant expression cassette encoding a TIC809 amino acid sequence and a second plant expression cassette encoding a TIC810 amino acid sequence.

15 SEQ ID NO:44 is a TIC809 amino acid sequence encoded from the first plant expression cassette as set forth in SEQ ID NO:43.

SEQ ID NO:45 is a TIC810 amino acid sequence encoded from the second plant expression cassette as set forth in SEQ ID NO:43.

20 SEQ ID NO:46 is a nucleotide sequence encoding a TIC127 peptide which corresponds to a fusion between TIC809 (encoded by nucleotide position 1-696) and TIC810 (encoded by nucleotide position 754-1407), in which a short linker sequence (encoded by nucleotide position 697-753) has been introduced to allow for the two proteins to be separated by proteolysis after expression in a plant cell (or upon ingestion in the gut of a target insect pest).

25 SEQ ID NO:47 is a TIC127 amino acid sequence.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

30 Polynucleotide sequences derived from *B. thuringiensis* are provided herein that encode ET29, ET37, TIC809, TIC810 and TIC812 proteins as well as a fusion between the ET29 derivative TIC809 and TIC810. Synthetic nucleotide sequences constructed for expression in plants are also provided that encode ET29, TIC809, ET37, TIC810 and TIC812 amino acid sequences as well as the TIC127 protein fusion between TIC809 and TIC810. Methods of making and using the polynucleotide sequences in the development of transgenic plants and plant cells that are resistant to Coleopteran and Hemipteran insect infestation are also disclosed. The proteins are also provided as insecticidal compositions either in

formulations for topical application in various agricultural or animal environments, or as insecticidal compositions produced by a preferred host cell such as a bacterial cell, a plant cell, or a yeast or fungal cell. With reference to the term "derived", it is intended that a sequence is directly isolatable from a particular source, or after isolation from a particular source, a sequence such as a nucleotide sequence is modified to encode a protein that is substantially the same as the sequence that was isolated from a particular source. Alternatively, an amino acid sequence can be substantially the same as an amino acid sequence isolated from a particular source, or encoded from a particular nucleotide sequence. An amino acid sequence can be a chimera of a number of different amino acid sequences that have been each individually isolated from a particular source, but various segments of such different amino acid sequences have been cobbled together to produce the chimera. In this sense, the chimera is derived from each of the various different amino acid sequences. A nucleotide sequence can be similarly derived from other nucleotide sequences. A nucleotide sequence can be derived from other nucleotide sequences as a consequence of its production or having been obtained by reference to one or more other nucleotide sequences. Similarly, amino acid sequences can be obtained or produced by reference to one or more other amino acid sequences, and thus be derived in that manner.

Synthetic polynucleotide sequences of the present invention are preferably designed for *in planta* expression of insecticidal proteins in plant tissues and in plant cells. In particular, the insecticidal proteins of the present invention are referred to as ET29, ET37, TIC809, TIC810, TIC812 and TIC127 proteins. An amino acid sequence of any of these proteins is intended to be within the scope of the present invention so long as it exhibits insecticidal activity at least equivalent to that of the full length protein from which it was derived.

In one embodiment, the present invention relates to a biologically pure culture of a *B. thuringiensis* bacterium containing a nucleotide sequence encoding one or more of the proteins disclosed herein. In particular, the nucleotide sequences are those set forth in SEQ ID NO:1 encoding ET37 (SEQ ID NO:2), SEQ ID NO:3 encoding TIC810 (SEQ ID NO:4), SEQ ID NO:5 encoding TIC812 (SEQ ID NO:6), and SEQ ID NO:7 encoding ET29 (SEQ ID NO:8). Also, fusions are contemplated and specifically embodied herein, such as TIC127 (SEQ ID NO:46 encoding SEQ ID NO:47). A biologically pure culture may also include those that are transformed with a polynucleotide sequence of the present invention or with two or more polynucleotide sequences, at least a first polynucleotide sequence being selected from the group consisting of an ET37 coding sequence and an ET29 coding sequence, and at

least a second polynucleotide sequence being selected from the group consisting of a TIC810 coding sequence and a TIC812 coding sequence. Exemplary bacterial strains, i.e., EG4096 and EG5078, have been deposited in the Northern Regional Research Laboratory of Agricultural Research Service Center Collection (NRRL), USDA, 1815 North University Street, Peoria, IL 61604, pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure and have been assigned the accession numbers as indicated in Table 1.

Table 1. Exemplary *B. thuringiensis* strains

Bt Strain	Nature of Strains	Toxins contained	NRRL numbers	Deposit Dates
EG4096	Wild type	ET29, TIC810	NRRL-B-21582	May 30, 1996
EG5078	Wild type	ET37, TIC812	NRRL B-30841	May 3, 2005

The naturally occurring (native) polynucleotide sequence encoding ET37 is set forth at SEQ ID NO:1. This sequence exhibits about 99% sequence identity to the polynucleotide sequence encoding an ET29 insecticidal protein, as disclosed in US Pat. No. 6,093,695, and as disclosed herein at SEQ ID NO:7. The ET37 amino acid sequence encoded from SEQ ID NO:1 is set forth at SEQ ID NO:2. Insecticidal activity of the ET37 protein is demonstrated herein in bioassays using Coleopteran insects of the genus *Diabrotica*, including WCR and SCR, and Hemipteran insects of the genus *Lygus*. In the course of conducting sequence analysis of the extra-chromosomal plasmids on which the ET37 and ET29 coding sequences are located in their respective strains, a single open reading frame was identified upstream of each of the ET37 and ET29 open reading frames, these corresponding respectively to sequences encoding the proteins TIC812 and TIC810. The native polynucleotide molecule (SEQ ID NO:3) encoding a TIC810 protein (SEQ ID NO:4) is positioned immediately upstream of the ET29 coding sequence in *B. thuringiensis* strain EG4096. The native polynucleotide molecule (SEQ ID NO:5) encoding a TIC812 protein (SEQ ID NO:6) is positioned immediately upstream of the ET37 coding sequence in *B. thuringiensis* strain EG5078.

ET29, ET37, TIC810 and TIC812 all may be distantly related to the Cyt insecticidal toxin family, however from a phylogenetic perspective, the ET29 and 37 proteins are much closer to each other than to other Cyt proteins, and the TIC810 and 812 proteins are also much closer to each other than to other Cyt proteins. The ET37 amino acid sequence shares

about 99% sequence similarity with that of ET29. TIC810 and TIC812 exhibit about 97% amino acid sequence similarity to each other. TIC810 exhibits about 33% amino acid sequence similarity with ET29 and ET37. Similarly, the TIC812 protein exhibits about 32% amino acid sequence similarity with ET29 and ET37. The similarity comparison is based upon Pairwise alignments between the proteins using the Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA.

In accordance with the present invention, certain combinations of expression of the TIC810, TIC812, ET37 and ET29 proteins in a host cell function to achieve a desirable elevated level of insecticidal protein accumulation in the host cell, resulting in improved insecticidal activity directed to certain target Coleopteran and Hemipteran insect pests. A polynucleotide sequence encoding ET29 can be co-expressed with a polynucleotide sequence encoding a TIC810 protein to achieve enhanced expression and or accumulation of the ET29 insecticidal protein in a host cell. Similarly, a polynucleotide sequence encoding ET37 can be co-expressed with a polynucleotide sequence encoding TIC812 to achieve an enhanced expression and or accumulation of the ET37 insecticidal protein in a host cell. It is envisioned that TIC812 is interchangeable with TIC810 as an insecticidal agent, and as a chaperone or accessory protein required for stabilization, accumulation, and improved host range bioactivity of either ET37 or ET29. Co-expression of TIC810 or TIC812 along with ET37 or ET29 results in improved expression and/or accumulation of ET37 or ET29. Such combinations are referred to herein as stabilized insecticidal compositions. Furthermore, the stabilized compositions exhibit a greater host range, at least with reference to its insecticidal efficacy directed to Coleopteran and Hemipteran insect pests, than any of the individual components of the compositions. A recombinant cell in which a first protein consisting of either ET37 or ET29 and a second protein consisting of either TIC810 or TIC812 exhibits increased levels of ET29/ET37 accumulation, provides levels of Coleopteran and Hemipteran insect resistance that were previously unattainable with the insecticidal proteins ET37 and ET29, and results in an absence of abnormal morphology and/or phenotype of the cell or organism consisting of such recombinant cells, all in comparison to a cell expressing either ET29 or ET37 in the absence of TIC810 or TIC812.

The above insecticidal combinations may also be expressed in a plant along with at least one additional insecticidal protein different from either protein comprising the combination, exhibiting a mode of action different from either protein comprising the combination, and toxic to the same insect species as the proteins of the combination. This second combination, which includes the additional insecticidal protein, provides a means of

Coleopteran insect resistance management. Such additional proteins include but are not limited to the Coleopteran toxins Cry3Bb and variants, Cry22A, TIC901, TIC1201, TIC407, TIC417, CryET70, the binary toxins PS149B1, ET33/34, and ET80/76, and various other proteins that have been shown to exhibit Coleopteran insecticidal activity such as patatins, Cry3Aa variants, and non-specific insecticidal compositions isolatable from bacterial species such as *Xenhorabdus* and *Photorhabdus*.

ET29 or ET37 protein each may be combined with TIC810 or TIC812 and co-expressed in a plant with an agent that exhibits insecticidal activity directed to other than a Coleopteran insect pest species, achieving desired control of more than one type of common plant pests selected from the group consisting of Lepidopteran insect pests, and Hemipteran insect pests. Furthermore, such combinations could be combined with still other agents that are effective in controlling virus pests, bacterial pests, fungal pests, and the like. The agents contemplated in such combinations can be expressed along with the ET29/ET37 and TIC810/TIC812 combination or provided through application of insecticidal or pesticidal agents in an agriculturally acceptable formulation, perhaps with a carrier such as an emollient, colloid, spray, powder, mixture, or dust. In situations in which the compositions would be useful in controlling animal pests such as fleas, ticks, lice, mites and the like, it is useful to include along with the ET29/ET37 and TIC810/TIC812 combination an agent that is effective for controlling the same or other pests to which the combination is directed, and so such applications should be provided in a pharmaceutically acceptable formulation. Particular formulations of the insecticidal combinations of the present invention are contemplated for use in topical and/or systemic application to field crops, grasses, fruits and vegetables, and ornamental plants. In one embodiment, the insecticidal composition comprises an oil flowable suspension of bacterial cells that is expressing one or more of the novel insecticidal proteins disclosed herein. Exemplary cells may be *B. thuringiensis* strains EG4096, EG5078, sIC8134 or sIC8135, however, any such bacterial host cell such as *B. megaterium*, *B. subtilis*, *E. coli*, or *Pseudomonas* spp. expressing an insecticidal composition would be useful.

The insecticidal compositions of the present invention may be combined with other biotechnology methods, such as double stranded RNA mediated gene suppression technology, to achieve desired control of one or more pests of a particular plant. Specific nucleotide sequences selected from the sequences native to the cells of a particular pest that are involved in an essential biological pathway are expressed in a plant cell in such a way as to result in the formation of a double stranded RNA, or even a stabilized double stranded

RNA. In this manner, upon ingestion by the pest of a pesticidally effective amount of the RNA, i.e., one or more plant cells expressing the double stranded RNA derived from the cells of such pest, suppression of one or more essential biological pathways is suppressed in the pest. The pest contemporaneously ingests along with the dsRNA, a pesticidally effective amount of the insecticidal compositions described herein, resulting in the provision of an effective insect or pest resistance management system which avoids the likelihood of the onset of resistance because the two pesticidal agents are functioning through different modes of action. A particular recombinant plant cell expressing a composition corresponding to the Coleopteran and Hemipteran insecticidal proteins of the present invention can also express as dsRNA molecules one or more sequences derived from the genome of a targeted Coleopteran pest and one or more sequences derived from the genome of a targeted Hemipteran pest, resulting in the provision of multiple insecticidally effective amounts of the proteins of the present invention and of the dsRNA's designed for suppression of one or more essential genes in the targeted Coleopteran and/or Hemipteran pests. Plants consisting of the gene encoding one or more the proteins of the present invention alone or in combination with additional pest controlling agents such as dsRNA agents exhibit improved yield and drought tolerance compared to plants lacking such pesticidal agents. This may be because these traits result in stabilized root masses that are more uniform, strong and healthy and provides greater nutrient and moisture gathering capacity in comparison to root masses lacking such agents.

A chimeric protein may be synthesized in which a sequence encoding ET29 or ET37 and a sequence encoding TIC810 or a TIC812 is fused together, providing for expression of the stabilized insecticidal composition as a single protein. It is contemplated that the chimeric protein may not be stabilized or exhibit insecticidal bioactivity unless the two fused peptides are unlinked. This physical separation can be accomplished by including as a spacer between the proteins a unique peptide sequence that is the target for any number of proteases known in the art. The chimeric protein can also be linked to other sequences that affect the stability of the chimera, resulting in formation of a crystalline form or inclusion body that consists substantially of the chimera-fusion peptide to the exclusion of other contaminating compositions, peptides, or molecules. Such chimeras or fusions are exemplified herein in Example 11 as TIC127 (sequential fusion of TIC809 and TIC810 linked by a short peptide) and TIC128 (sequential fusion of TIC810 and TIC809) and are shown to exhibit Coleopteran and Hemipteran insecticidal bioactivity.

Expression vectors for use in a host cell are also provided. Expression vectors comprising sequences that result in the expression of a combination of at least two of the polynucleotide sequences disclosed herein are provided in exemplary embodiments. In one embodiment, an expression vector is an isolated and purified polynucleotide molecule comprising a combination of two different polynucleotide sequences, each sequence containing a promoter functional in a desired host cell that is operatively linked to a nucleotide segment encoding a TIC809, TIC810, TIC812, ET29, or ET37. In some embodiments, a transcription termination and polyadenylation sequence may be included 3' of the nucleotide segment encoding one of these proteins.

Expression vectors for use in a bacterial host cell are provided, e.g., in an *E. coli* cell or a *Bacillus* cell including one from *B. thuringiensis*, *B. megaterium*, *B. subtilis*, or related *Bacillus* species. Bacterial host cell expression vectors can contain one nucleotide sequence expressing one or more of the proteins of the present invention in series, in much the same way that proteins can be expressed in most bacterial cells, i.e., in a polycistronic expression cassette. Alternatively, a bacterial expression vector may consist of a nucleotide sequence encoding only one of the proteins of the present invention.

Promoters that function in bacteria are well-known in the art. An exemplary promoter for the *Bacillus* crystal proteins may include any of the known crystal protein gene promoters, including the ET29 gene promoter (US Pat. No. 6,093,695), and promoters specific for *B. thuringiensis* sigma factors (Baum and Malvar, *Molec. Microbiol.*, 18(1): 1-12, 1995). Alternatively, mutagenized or recombinant crystal protein-encoding gene promoters may be engineered by those skilled in the art and used to promote expression of the novel polynucleotide sequences disclosed herein. For purpose of the present invention, the promoter used herein for expression of the polynucleotide sequences is cry1A promoter.

Recombinant DNA constructs for expression in a plant cell are provided. Such constructs typically contain two or more plant functional expression cassettes that are linked together in such a way as to enable the simultaneous introduction of the two or more expression cassettes into the same locus in a plant genome, or alternatively may contain two or more plant functional expression cassettes that are linked within the construct or vector, but are capable of being introduced independently into different loci within a plant genome. These expression cassettes may be referred to as a first and a second expression cassette expressing, respectively, a first polynucleotide sequence encoding a first protein and a second polynucleotide sequence encoding a second protein. It is intended that the first protein can be any of the proteins disclosed herein, and the second protein can be any of the other

proteins disclosed herein other than the first protein. Exemplary polynucleotide sequences are provided as set forth in SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17 and SEQ ID NO:19.

Promoters used in the recombinant DNA construct of the present invention should exhibit the ability to drive expression of the polynucleotide sequences encoding the insecticidal agents when introduced into plant cells. Promoters that are useful in expressing the polypeptide sequences in plants can be inducible, constitutive, tissue-specific or developmentally specific promoters for use in a monocot or a dicot plant. In one embodiment, the promoter selected for use may be a constitutive promoter and, for the purpose of the present invention, the promoter may specifically comprise an enhanced cauliflower mosaic virus (CaMV 35S) promoter. In another embodiment, the promoter selected may be a tissue-specific promoter and, for the purpose of the present invention, the promoter may specifically comprise a root-specific promoter Rcc3 isolated from rice (US Patent Application Serial No. 11\075,113).

A vector or construct may also include elements that function to regulate the level and timing of expression of the gene of interest to which they are linked, in addition to one or more promoters. For example, the construct may include an intron sequence. The intron sequence employed in the present invention may include a rice actin intron (U.S. Patent No. 5,641,876). The recombinant DNA construct may also have a translation leader sequence between the promoter and the coding sequence. The vector or construct may also include, within the coding region of interest, a nucleic acid sequence that acts, in whole or in part, to terminate transcription of that region. In one embodiment, the polyadenylation sequence of the present invention may be from the 3' untranslated region of a wheat heat shock protein gene (tahsp17).

A recombinant DNA construct may include other elements as well. For example, the construct may contain DNA segments that provide replication function and one or more selectable markers for use in bacterial cells. The construct may also comprise a screenable marker, a selectable marker and other elements as appropriate for selection of plant or bacterial cells having the recombinant DNA constructs of the invention. The recombinant DNA constructs are designed with suitable selectable markers that can confer antibiotic or herbicide tolerance to the cell. The antibiotic tolerance polynucleotide sequences include, but are not limited to, polynucleotide sequences encoding for proteins involved in tolerance to kanamycin, neomycin, hygromycin, and other antibiotics known in the art. An antibiotic tolerance gene in such a vector may be replaced by a herbicide tolerance gene encoding for 5-

enolpyruvylshikimate-3-phosphate synthase (EPSPS, described in U.S. Patent Nos. 5,627,061, and 5,633,435; Padgett et al. *Herbicide Resistant Crops*, Lewis Publishers, 53-85, 1996) or other selectable marker genes and their equivalents such as basta tolerance, bar tolerance, methotrexate resistance, glyphosate oxidoreductase, glyphosate acetyl transferase, 5 phosphonate acetylase (*phnO* and alleles derived from *Enterobacteriaceae*), and dicamba tolerance genes, and the like. Plants expressing the insect tolerance properties of the present invention that are coupled with one or more such selectable markers are particularly useful for commercial purposes.

10 The polynucleotide sequences of the present invention may be used to transform a plant cell that can be regenerated to produce a transgenic plant that exhibits improved insect resistance when compared to the plant or plant cell from which the transgenic plant is derived. The polynucleotide sequences of the present invention may be modified to improve their expression in the plant host cell. Expression of the polynucleotide sequences of the present invention in the plant cell may achieve accumulations of the insecticidal proteins in 15 the cytoplasm, or can result in the insecticidal proteins being accumulated into a subcellular organelle such as a chloroplast, a plastid or a mitochondrion.

In accomplishing the foregoing, the polynucleotide sequences encoding the proteins of the present invention as set forth in SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18 and SEQ ID NO:20 and SEQ ID NO:47 have been improved for expression in plants. Such 20 polynucleotide sequences exemplified herein are set forth in SEQ ID NO:13 (TIC809), SEQ ID NO:15 (TIC810), SEQ ID NO:17 (ET37), SEQ ID NO:19 (TIC812), and SEQ ID NO:46 (TIC127), and furthermore in expression cassette sequences as set forth at SEQ ID NO:28, SEQ ID NO:31, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, and SEQ ID NO:43. Co-expression of at least one of a TIC810 or TIC812 protein along with one or 25 more of a ET29, TIC809, or ET37 insecticidal protein improves (or assists, acts as a chaperone to, stabilizes, or may otherwise interact with the insecticidal protein as an accessory protein) with the expression and/or accumulation of the insecticidal protein. Co-expression in a transgenic plant results in the absence of low levels of expression and/or accumulation and absence of phytotoxic effects observed when only an insecticidal protein 30 such as ET29, ET37, or TIC809 are expressed alone.

There are many methods for introducing the recombinant DNA construct comprising the combination of the polynucleotide sequences into cells and suitable methods are believed to include virtually any method by which DNA can be introduced into a plant cell, such as by *Agrobacterium* infection, direct delivery of DNA such as, for example, by PEG-mediated

transformation of protoplasts (Omirulleh et al., Plant Mol. Biol., 21:415-428, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation or by microprojectile bombardment and the like.

5 In consideration of insect resistance management (IRM), the compositions and methods of the present invention are useful in producing transgenic plants which express two or more *B. thuringiensis* proteins toxic to the same insect species and which confers a level of resistance management for delaying the onset of resistance of any particular susceptible insect species to one or more of the insecticidal agents expressed within the transgenic plant. Alternatively, expression of a *B. thuringiensis* insecticidal protein toxic to a particular target
10 insect pest along with a different proteinaceous agent toxic to the same insect pest but which confers toxicity by a means different from that exhibited by the *B. thuringiensis* toxin is desirable. Such other different proteinaceous agents may comprise any of Cry insecticidal proteins, Cyt insecticidal proteins, insecticidal proteins from *Xenorhabdus* sp. or *Photorhabdus* sp., *B. thuringiensis* vegetative insecticidal proteins, and the like. One means
15 for achieving this result would be to produce two different transgenic events, one event expressing a combination of the two insecticidal proteins of the present invention, active against Coleopteran and Hemipteran insects, and the other expressing a third insecticidal protein, and breeding the two traits together into a hybrid plant. The third insecticidal protein could be one that exhibits Coleopteran insecticidal activity, one that exhibits
20 Hemipteran insecticidal activity, one that exhibits insecticidal activity directed to both Coleopteran and Hemipteran (and perhaps toxicity to other insect orders as well), or one that exhibits insecticidal activity directed to one or more other orders of insects other than Coleopteran and Hemipteran insect pests including but not limited to Lepidopteran, Orthoptera, Diptera and the like.

25 A pesticidal amount of the insecticidal proteins of the present invention may be provided in the diet of an insect pest. Typically, the diet consists of a plant part upon which the insect normally feeds, such as a plant tissue or plant cell, but may also include other compositions such as an artificial diet formulated for enhancing the development and survival of a particular insect pest. The insecticidal protein can be provided in a composition that is
30 applied to the surface of the diet, or more preferably can be produced by the protein synthesis machinery of a cell and, as described above, accumulated within the plant cell or secreted outside of the plant cell, so long as the amount of the protein toxin provided is an insecticidal amount sufficient to inhibit the insect pest from further feeding, or to inhibit the further growth and development of the insect pest, or to cause mortality to the insect pest.

Transgenic plants can be selfed (self-pollinated) to generate seeds that exhibit a genotype that is homozygous with respect to the transgene encoding the insecticidal protein. Such seeds produce plants and seeds that are only homozygous with respect to the transgene encoding the insecticidal protein. Often, the transgenic plants that are produced are generated in varieties that fail to exhibit the most desirable agronomic qualities. Therefore, homozygous recombinant plants can be crossed with inbred lines that exhibit particular agronomically important qualities.

The present invention will find particular utility in the creation of transgenic plants of commercial interest including various turf grasses, wheat, corn, rice, barley, oats, a variety of ornamental plants and vegetables, as well as a number of nut- and fruit-bearing trees and plants. Specifically, these plants may include maize, wheat, rye, barley, oats, buckwheat, sorghum, rice, onion, grass, sunflower, canola, peas, beans, soybeans, cotton, linseed, cauliflower, asparagus, lettuce, tobacco mustard, sugar beet, potato, sweet potato, carrot, turnip, celery, tomato, egg plant, cucumber, squash, apple, apricot, peach, pear, plum, orange, blackberry, blueberry, strawberry, cranberry and lemon. In general, the invention is useful in monocotyledonous and dicotyledonous plant varieties. The transgenic plant is selected from the group consisting of a monocot plant and a dicot plant that may include monocots such as maize, wheat, rye, barley, oats, buckwheat, sorghum, rice, sugarcane, onion, garlic, grass, or dicots such as sunflower, canola, peas, cowpeas, pigeon peas, beans, soybeans, coffee, broccoli, cotton, linseed, cauliflower, Arabidopsis, asparagus, lettuce, tobacco, spice plants (including curry, mustard, sage, parsley, pepper, thyme, cilantro, bay, cumin, turmeric, nutmeg, cinnamon and the like), sugar beet, potato, sweet potato, carrot, turnip, celery, tomato, egg plant, cucumber, squash or melon, fruit tree plant (including apple, apricot, peach, pear, plum, orange, lemon, lime and the like), berry plants (including blackberry, blueberry, strawberry, cranberry and the like), nut tree plants (including acorn, hickory, Brazil, pecan, walnut, hazelnut, and the like), grape plants, and flowering plants.

DNA sequence information provided herein allows for the preparation of nucleotide sequences or probes and/or primers exhibiting the ability to specifically hybridize to nucleotide sequences disclosed herein, or to homologous sequences encoding proteins related to TIC810, TIC812, ET37 and ET29. The ability of such nucleic acid probes to specifically hybridize to a sequence encoding a related toxin or accessory/chaperonin-like protein provides particular utility in a variety of embodiments. Most importantly, the probes may be used in a variety of assays for detecting the presence of complementary sequences in a given sample. The nature of the regulatory environment under which transgenic plants containing

the genes of the present invention are commercialized provides a particular utility to being able to detect the presence of the sequences encoding the proteins as well as the proteins of the present invention in a biological sample and provides a means for detecting infringement of certain claimed embodiments during the term of any patent that is issued thereon.

5 In certain embodiments, it is advantageous to use oligonucleotide primers, either alone or in pairs or other primer sets. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or mutating a defined segment of a toxin or accessory/chaperonin-like protein coding sequence from *B. thuringiensis* or other sources using thermal amplification methods. Segments of related
10 toxin or accessory/chaperonin-like coding sequences from other species may also be amplified.

 Kits for detecting, in a biological sample, polynucleotide or amino acid sequences of the present invention are also envisioned. Such kits contain one or more polynucleotide sequences each for use as a probe for detecting the presence of a polynucleotide sequence
15 encoding an insecticidal protein of the present invention or fragment thereof. Such kits could also or alternatively contain antibody specific for binding to one or more polypeptides of the proteins of the present invention, as well as reagents for use with the probe or antibody, and the kits would also contain control samples for use in ensuring that the nucleotides or peptides identified with the probe and or antibody and reagents were functioning according to
20 the manufacturers' instructions. All of the reagents necessary for carrying out the methods of identification of either nucleotide sequences or peptides would be packaged together in a kit along with instructions for use. An exemplary kit could contain a TIC810 or related polynucleotide sequence encoding an insecticidal protein along with a sample of the exemplary nucleotide sequence amplification primers pr375 and pr376 as set forth in SEQ ID
25 NO:23 and SEQ ID NO:24, together with the necessary reagents necessary for carrying out an amplification reaction, all packaged together in the kit.

 The antibodies that bind specifically to epitopes presented only by any one of ET37, TIC810 and TIC812 proteins or their homologs may also be used for identifying the presence of any one of ET37, TIC810 and TIC812 proteins or its homologs, for purifying the proteins
30 or homologs, for identifying a nucleotide sequence from which an ET37, TIC810 or TIC812 protein or a homolog is being expressed, and for use in the kits designed to allow the detection an ET37, TIC810 or TIC812 protein or a homolog or the detection of a nucleotide sequence expressing the protein or its homolog. The skilled artisan will readily appreciate

that such antibodies also provide for the identification of fusions of such proteins, such as TIC127 and the like.

Agronomically and commercially important products and/or compositions of matter including but not limited to animal feed, commodities, and cotton or soybean or corn products and by-products that are intended for use as animal feeds or for use as food for human consumption or for use in compositions that are intended for human consumption including but not limited to cotton seed, cotton seed oil, cotton seed solids, and the like, soy meal, soy oil, soy flour, and the like, corn flour, corn meal, corn syrup, corn oil, corn starch, popcorn, corn cakes, cereals containing corn or soy, and corn by-products, and the like are intended to be within the scope of the present invention if these products and compositions of matter contain detectable amounts of the nucleotide sequences set forth herein as being diagnostic for the presence of a sequence encoding an ET29, an ET37, a TIC809, a TIC810, a TIC812, a TIC127, or combinations thereof and the like. Distillers dry goods solids are also contemplated as an agronomically and commercially important product, especially if it contains detectable amounts of a nucleotide sequence encoding one or more of the proteins of the present invention, or detectable amounts of one or more of the proteins of the present invention.

Seed comprising detectable amounts of nucleotide sequences encoding these proteins, or seed or plant parts that can be processed into products that contain detectable amounts of such nucleotide sequences or proteins are within the scope of the present invention.

Those of skill in the art, in light of these examples, should appreciate that many changes can be made to the foregoing disclosure without departing from the spirit and scope of the inventions disclosed.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

EXAMPLES

Example 1

This example illustrates the construction of a nucleotide sequence that functions to achieve expression of an ET29 protein in maize plant cells.

5 ET29 is a protein derived from *B. thuringiensis* and has been previously shown to exhibit corn rootworm insecticidal biological activity when provided in the diet to corn rootworm larvae (U. S. Patent No.'s 6,093,695; 6,537,756; 6,686,452). Native Bt coding sequences have been shown to exhibit unacceptable levels of protein synthesis when expressed in plant cells (US Patent No. 5,500,365). Expression of ET29 protein in corn
10 plant cells, and specifically in cells of corn roots, could provide corn plants with protection from corn rootworm feeding damage. Accordingly, a nucleotide sequence encoding a *B. thuringiensis* ET29 insecticidal protein was constructed that was anticipated to be more highly expressed in plants, avoiding certain inimical nucleotide sequences that have been previously shown to be problematic, while maintaining a nucleotide sequence that encodes
15 the native insecticidal protein with one exception; a supplemental Alanine codon at position two (2) of the coding sequence (SEQ ID NO:13) was included in the synthetic sequence to facilitate ease of cloning. The ALA2 variant ET29 amino acid sequence as set forth at SEQ ID NO:14 has been designated as TIC809 and in bioassays exhibits no less biological activity than the native ET29.

20 The TIC809 coding region as set forth in SEQ ID NO:13 was subcloned into a binary plant transformation vector. Elements upstream of the TIC809 coding region included a enhanced CaMV 35S promoter, a wheat major chlorophyll a/b-binding protein 5' untranslated leader sequence, a rice actin 1 gene first intron and flanking untranslated leader region (UTL) exon sequence, and, optionally, a maize ribulose 1,5-bisphosphate carboxylase
25 small subunit chloroplast transit peptide coding sequence. *In planta* expression of a fusion protein consisting of the chloroplast transit peptide (ctp) linked to the N-terminus of the TIC809 protein enables targeting of the TIC809 protein into plastids. A wheat hsp17 3' untranslated region (UTR) was incorporated downstream of the TIC809 to achieve transcription termination and polyadenylation of mRNA transcripts.

30 Plant transformation vectors contain a glyphosate tolerance selectable marker. The plant transformation vector pMON70513 provides for expression of a cytoplasm soluble TIC809 protein while pMON70514 provides for expression of a plastid targeted TIC809 protein.

Example 2

This example illustrates the comparison of plastid targeted versus non-targeted expression of TIC809 in transient corn protoplast assays, and the subsequent analysis of transgenic plants transformed to express the TIC809 protein targeted to maize plastids.

5 Transient expression assays using corn protoplasts transformed with either pMON70513 or pMON70514 were compared to each other and to an empty vector control. The results indicated low levels of expression of the untargeted TIC809 protein compared to the targeted TIC809 protein. Accordingly, only pMON70514 was analyzed further. Transgenic corn events were produced after *Agrobacterium*-mediated transformation of corn
10 protoplasts. Regenerated corn plants ("R0 plants") were screened for glyphosate tolerance and the copy number of the wheat hsp17 (tahsp17) 3' UTR. Six-leaf stage (V6) root and leaf samples from each transgenic corn event were screened for the presence and amount of ET29 protein using an ELISA method.

Nineteen (19) out of eighty-seven (87) transgenic events analyzed by ELISA
15 displayed a distinctive abnormal phenotype characterized by a chlorotic stalk, and eight (8) of these exhibited tassel or ear abnormalities. The average TIC809 expression level in leaf and root tissue from plants exhibiting an abnormal phenotype was 2.2 and 2.0 parts per million, respectively. The average TIC809 levels in leaf and root from phenotypically normal plants was 1.4 and 1.1 ppm, respectively. These results suggested that higher levels of TIC809
20 protein may be correlated with the observed abnormal phenotypes.

Example 3

This example illustrates cloning of a gene encoding TIC810, a protein expressed from within an operon in *Bacillus thuringiensis* in which the ET29 (*tic809*) gene also resides, and
25 the identification that expression of TIC810 exhibits no corn rootworm insecticidal bioactivity.

et29 was originally cloned on a 7.1 kb *EcoRI* fragment from DNA obtained from *B. thuringiensis* strain EG4096 (U. S. Patent No. 6,686,452), and was retained in plasmid pEG1303, a shuttle vector capable of replication in *B. thuringiensis* and in *E. coli*.
30 Recombinant *B. thuringiensis* strain EG11502 containing pEG1303 produces low levels of ET29 crystal protein when grown in C2 medium (Donovan et al, *Mol. Gen. Genet.* 214: 365-372, 1988).

The ET29 coding sequence was subcloned from the large 7.1 *EcoRI* fragment in pEG1303 as a ~1.5 kb *KpnI*-*ClaI* fragment into the high copy-number shuttle vector

pEG854.9 (Baum et al, (1996) Appl. Env. Microbiol. 62:4367-4373) with the expectation that an increase in the level of ET29 expression would be observed from the smaller fragment. The resulting plasmid, pMON78402, was believed to contain sufficient native DNA 5' and 3' of the ET29 coding region to incorporate any necessary expression elements such as a sporulation dependent promoter. Surprisingly, no protein crystal formation was detected when pMON78402 was introduced into the acrystalliferous *B. thuringiensis* host strain EG10650, suggesting that the 5' region present on pMON78402 may not contain the native ET29 promoter and that ET29 transcription from the clone in pEG1303 was driven from a fortuitous vector-borne promoter. Furthermore, sequencing of the entire 7.1 kb *EcoRI* fragment in pEG1303 revealed the presence of an interrupted open reading frame immediately upstream of the ET29 coding region. The interrupted coding region contained one of the terminal *EcoRI* sites used to clone the 7.1 kb *EcoRI* fragment in pEG1303. A FASTX search of the existing non-redundant protein databases as well as a database of *B. thuringiensis* crystal protein sequences suggested that this partial coding region encodes an amino acid sequence exhibiting approximately 36% sequence identity to that of the ET29 protein. This related protein was designated TIC810. This suggested that the ET29 gene resided within an uncharacterized operon that included at least the upstream TIC810 gene, and that since TIC810 was probably co-expressed with ET29, it too might also exhibit corn rootworm insecticidal activity.

The native ET29 coding sequence is set forth at SEQ ID NO:9 from position 716 through 1408. A single *NheI* site was present within this coding sequence (nucleotides 820-825 as set forth at SEQ ID NO:9). The partial TIC810 coding sequence in pEG1303 is shown as set forth at SEQ ID NO:9 from nucleotide position 369 to 654. The *EcoRI* site that bisected the TIC810 coding sequence is set forth at SEQ ID NO:9 at nucleotides 369 – 374. *NheI* digestion of EG4096 DNA or digestion with *NheI* and compatible restriction enzymes coupled with ligation and inverse PCR allowed for the identification of the nucleotide sequence of the 5' end of the TIC810 coding region.

EG4096 genomic DNA (5 µg) was digested in 50 µL volumes with compatible restriction enzymes in various combinations that include *NheI*, *NheI* + *BlnI*, *NheI* + *SpeI*, and *NheI* + *XbaI*. Ten microliters of the complete digests were mixed with 80 µL sterile water, 10 µL 10X ligase buffer (New England BioLabs, Beverly, MA) and 2 µL T4 ligase and incubated overnight at 4C. The ligation products were used as thermal amplification templates using an Elongase® kit from Invitrogen (Carlsbad, CA) and the divergent primers pr370 and pr371 (as set forth at SEQ ID NO:21 and SEQ ID NO. 22 respectively). SEQ

ID NO:21 corresponds to the reverse complement of nucleotides 650-671 as set forth in SEQ ID NO:9, and SEQ ID NO:22 corresponds to nucleotides 744-764 as set forth in SEQ ID NO:9.

5 Restriction enzymes used for each reaction produced compatible ends. *B. thuringiensis* DNA is AT-rich, and so it was anticipated that the restriction enzymes *SpeI* and *XbaI* would yield smaller PCR products than the *BlnI* and *NheI* restriction enzymes. Only the *SpeI-NheI* and *XbaI-NheI* combinations yielded amplified DNA fragments. The amplified DNA fragments were cloned and sequenced and assembled with the *EcoRI-NheI* segment consisting of nucleotides 369 – 825 as set forth at SEQ ID NO:9. An assembled
10 sequence predicted to encode TIC810 was identified as set forth at SEQ ID NO:3. The TIC810 gene was subsequently amplified directly from EG4096 genomic DNA using a high-fidelity thermostable polymerase. Multiple clones were used to confirm the sequence. The TIC810 gene is predicted to encode a ~25,000 Dalton protein as set forth in SEQ ID NO:4. The deduced TIC810 amino acid sequence exhibits about 33% amino acid sequence identity
15 to the ET29 amino acid sequence, suggesting that TIC810 may also exhibit insecticidal bioactivity.

The expression of TIC810 in an acrystalliferous strain of *B. thuringiensis* was examined. The TIC810 gene was amplified using primers pr375 and pr376 (as set forth in SEQ ID NO:23 and SEQ ID NO:24, respectively), producing an amplicon encoding a
20 TIC810 amino acid sequence variant that contains an ATG translation initiation codon substituted for the native GTG codon. Primer pr375 also incorporates a *SpeI* site 5' to the TIC810 coding region while primer pr376 incorporates an *XhoI* site 3' to the TIC810 coding region amplicon, permitting subcloning of the amplicon into the *B. thuringiensis-E. coli* shuttle vector pMON47407. The amplicon encoding TIC810 was inserted into this vector
25 just down stream of a vector-endogenous *cryIA* promoter. Sequences positioned downstream of the *cryIA* promoter exhibit sporulation-dependent expression in *B. thuringiensis*. The resulting recombinant plasmid pMON78409 containing the sequence encoding the TIC810 protein was introduced into the acrystalliferous *B. thuringiensis* host strain EG10650 by electroporation to generate the recombinant *B. thuringiensis* strain
30 SIC8116. Strain SIC8116 produced parasporal inclusion bodies containing TIC810 protein when grown in C2 sporulation medium. No insecticidal bioactivity was observed when corn rootworm larvae were exposed to artificial media overlaid with sporulated cultures of SIC8116, suggesting that the TIC810 protein did not exhibit insecticidal bioactivity directed to CRW.

Example 4

This example illustrates that ET29 expression under the control of a *cry1A* sporulation dependent promoter results in poor expression levels and aberrant physiological host cell behavior.

The recombinant *B. thuringiensis* strain EG11502, containing the plasmid pEG1303, produced small amounts of the ET29 crystal protein. In an attempt to increase the expression of ET29 in *B. thuringiensis*, the ET29 coding sequence was inserted into the *B. thuringiensis-E. coli* shuttle vector pMON47407. The ET29 coding sequence was amplified from pEG1303 DNA using primers pr365 and pr372 (SEQ ID NO:25 and SEQ ID NO:26, respectively). Following sequence confirmation, the amplified ET29 gene fragment was inserted into the pMON47407 vector backbone in an orientation effective for expression of ET29 from the vector-endogenous *cry1A* promoter. To assess ET29 protein production, the resulting recombinant plasmid, pIC17507, was introduced into *B. thuringiensis* strain EG10650, to produce the recombinant strain SIC8114. Strain SIC8114 produced a lower amount of ET29 protein than did the original ET29 recombinant strain EG11502 and exhibited poor sporulation in C2 sporulation medium. This result suggested that overexpression of ET29 may be detrimental to the host cell, or that some other factor present in the native strain EG4096 and not in the recombinant strain was required for efficient expression and/or accumulation of ET29.

Example 5

This example illustrates the identification of an operon exhibiting homology to the TIC810/ET29 operon.

Other strains of *Bt* were examined by Southern blot analysis for the presence of sequences related to the TIC810/ET29 operon in EG4096. A 5.4 kb *ClaI* restriction fragment from *Bt* strain EG5078 total DNA was found to hybridize to an ET29-specific hybridization probe. This 5.4 kb *ClaI* fragment was cloned into the *B. thuringiensis-E. coli* shuttle vector pEG854 (Baum et al., (1990) Appl. Env. Microbiol. 56:3420-3428) and also into the *E. coli* vector pBluescript IISK to generate the recombinant plasmids pEG1325 and pEG1323, respectively. Sequence analysis of the 5.4 kb insert revealed two tightly linked genes similar to the ET29 and TIC810 genes in *B. thuringiensis* strain EG4096. The 5' proximal coding sequence was designated as *tic812* (as set forth in SEQ ID NO:5) and was predicted to encode a TIC812 polypeptide (as set forth in SEQ ID NO:6). TIC812 exhibits

about 97% amino acid sequence identity to the TIC810 protein. The 3' proximal coding sequence was designated as *et37* (SEQ ID NO:1) and was predicted to encode the ET37 amino acid sequence as set forth in SEQ ID NO:2. ET37 exhibits greater than about 99% amino acid sequence identity to the ET29 protein, differing only at one amino acid position.

5 The shuttle vector pEG1325, containing both the TIC812 and ET37 genes, was introduced into the acrySTALLIFEROUS *B. thuringiensis* host strain EG10650 using an electroporation procedure. The resulting recombinant strain, EG11541, produced a high level of the ET37 protein when grown in C2 sporulation medium. However, the amount of TIC812 protein present in the spores was approximately only 25% of the amount of ET37
10 protein. The ET37/TIC812 protein mixture produced by strain EG5078 was found to be toxic to corn rootworm larvae when tested in bioassay.

Because of the similarity between the TIC810/ET29 and the TIC812/ET37 operons, and because ET37 and ET29 differ at only one amino acid position, it is unlikely that the two proteins exhibit significantly different insecticidal or cytotoxic properties. The low level of
15 ET29 when expressed alone compared to the greater level of ET37 protein when expressed from its native operon, coupled with the substantial identity between the TIC810 and TIC812 proteins, suggests that the TIC810 and TIC812 proteins may be playing a role in increasing the levels of, or providing some stabilization to, their respective ET29 or ET37 proteins. Therefore, co-expression of TIC812 along with the ET37 protein may be the cause of the
20 observed overproduction of ET37 in strain EG11541. Also, co-expression of TIC810 and ET29 may likewise result in overproduction of ET29.

To test this hypothesis, the individual ET29, ET37, TIC810 and TIC812 coding sequences were amplified and cloned into the *B. thuringiensis* expression vector pMON47407 described above, so that expression of each was under the control of the vector-
25 endogenous *cry1A* promoter. The naturally occurring GTG codon at position 1 of the TIC812 coding sequence was modified to an ATG codon. The TIC810-ET29 tandem coding sequences (SEQ ID NO:9) and the TIC812-ET37 tandem coding sequences (SEQ ID NO:10) were each amplified from genomic DNA and cloned into the TOPO cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and their sequences confirmed. The amplified DNA
30 fragments were then cloned into the vector pMON47407 so that the tandem coding sequences were under the control of the vector-endogenous *cry1A* promoter. Thus, each insert was cloned into pMON47407 in the same orientation and employed the same promoter. The acrySTALLIFEROUS *B. thuringiensis* strain EG10650 was used as the host strain for all expression

studies. The plasmid constructs and recombinant *B. thuringiensis* strains containing these plasmids are listed in Table 2.

Table 2. Plasmids Constructed for Expression Analysis of TIC810/ET29 and TIC812/ET37

Strain	Coding Sequence(s)	Plasmid	Primer pair ¹
EG10650	-	-	-
SIC8114	ET29	pIC17057	pr372-pr365
SIC8116	TIC810	pMON78409	pr375-pr376
SIC8130	ET37	pMON78404	pr372-pr365
SIC8131	TIC812	pMON78405	pr375-pr376
SIC8134	TIC810_ET29	pMON78406	pr421-pr365
SIC8135	TIC812_ET37	pMON78407	pr421-pr365

1 – primer pair used for amplification of the coding sequence inserted into pMON47407

The recombinant strains and EG10650 were each grown in 30 milliliters of C2 medium in a 250 milliliter baffled flask for 3 days at 28C with vigorous agitation. Spores and crystals were collected by low-speed centrifugation, washed once with 30 milliliters of wash buffer (10 mM Tris-HCl, 0.1 mM EDTA, 0.005% Triton X-100, pH 6.8), and resuspended in wash buffer at a final volume of 3 milliliters. These 10X C2 concentrates were then analyzed by SDS-PAGE. Protein concentrations were determined by densitometry using BSA as a standard.

SDS-PAGE analysis indicated that 1) both ET29 and ET37 exhibited poor production when expressed alone; 2) both TIC810 and TIC812 accumulated to high levels when expressed alone; and 3) ET29 and ET37 exhibited dramatically elevated levels of expression when co-expressed along with TIC810 or TIC812, respectively. ET29 protein production was about 4.6 fold higher in the presence of TIC810 than in its absence, and ET37 production was about 6.6 fold higher in the presence of TIC812 than in its absence. Furthermore, strains SIC8134 containing the TIC810_ET29 tandem coding sequence and SIC8135 containing TIC812_ET37 tandem coding sequence exhibited normal sporulation and lysis. These results indicated that TIC810 and TIC812 were required for the high-level production

of ET29 and ET37 in *B. thuringiensis*, respectively, and may be acting as accessory or chaperone proteins.

The 10X TIC812/ET37 spore-crystal suspension was used directly in a bioassay against WCR. Crystal proteins in the suspensions were quantified by SDS-polyacrylamide gel electrophoresis and densitometry using bovine serum albumin as a standard. 200 mL of WCR diet was prepared in a manner similar to that described by Pleau *et al.* (*Entomol. Exp. Appl.* 105:1-11, 2002). Twenty μ L of test sample were applied per well and allowed to dry before applying a single neonate insect larvae per well with a fine bristle paintbrush. Plates were sealed with mylar and ventilated using an insect pin. Twenty-four larvae were tested per sample concentration. The bioassay plates were incubated at 27C, 60% RH, in complete darkness for 5 – 7 days. The number of surviving larvae per treatment was recorded at the end of 5-7 days, depending on the experiment. Surviving larvae were weighed on a microbalance (Cahn C-33). Data were analyzed using JMP[®] 4 statistical software (SAS Institute, Cary, N. C. , USA). The bioassay data are listed in Table 3.

The results suggest that sporulated cultures of wild type *B. thuringiensis* strain EG5078 containing a mixture of ET37 and TIC812 were found to be toxic to Western corn rootworm larvae, causing significant larval mass reduction when compared to the control. A comparable 10X spore suspension from the *cry-* *B. thuringiensis* host strain EG10650 exhibited no activity against WCR larvae in bioassay. A sample containing 500 mg Cry3Bb rootworm insecticidal protein was included as a positive control.

Table 3. Western Corn Rootworm Bioassay vs ET37/TIC812

Sample	Concentration ¹	N	Mean	SD ²	SEM	LO 95%	UP 95%	P> t
UTC ³	0.250	6	0.36	0.05	0.02	0.31	0.41	
Cry3Bb	0.500	3	0.20	0.03	0.02	0.13	0.26	0.0003
TIC812-ET37	1.000	3	0.16	0.04	0.03	0.05	0.27	<0.0001
TIC812-ET37	0.500	3	0.19	0.08	0.05	-0.01	0.39	0.0002
TIC812-ET37	0.250	3	0.25	0.01	0.01	0.22	0.29	0.0084
TIC812-ET37	0.125	3	0.25	0.08	0.04	0.06	0.44	0.0073

1 – mg/ml; 2 – Standard Deviation; 3 – untreated check;

Variances not equal, Levene's method, P>F 0.0053;

There is an effect due to treatment, SLS, P>F 0.0002; and

Means with a P value <0.05 are significantly different from UTC, Planned Contrasts.

Example 6

This example illustrates the co-expression of TIC810 and TIC809 proteins in corn

protoplasts.

Synthetic nucleotide sequences encoding the *B. thuringiensis* TIC810 and ET29 (TIC809) proteins were constructed for expression in plants. The synthetic sequence encoding TIC809 is set forth at SEQ ID NO:13 and the amino acid sequence translation is set forth at SEQ ID NO:14. The synthetic sequence encoding TIC810 is set forth at SEQ ID NO:15, and the amino acid sequence translation is set forth at SEQ ID NO:16. The synthetic coding sequences for TIC809 and TIC810 were cloned into expression vectors for transient expression studies with corn protoplasts. Genetic elements around the coding region(s) of the TIC809 and TIC810 coding sequences were identical, except for the addition of the chloroplast transit peptide (ctp), as described in Example 1. The plasmid constructs are described in part in Table 4 below.

Table 4. Plasmid Constructs for Transient Assay of TIC810/TIC809 Expression

Plasmid	Encoded Cry Protein (s)	Protein Localization
pMON84202	TIC809	Untargeted
pMON84203	ctp-TIC809	Targeted to chloroplasts
pMON64134	TIC809, TIC810	Untargeted
pMON64135	ctp-TIC809, ctp-TIC810	Targeted to chloroplasts
pMON64136	TIC809, ctp-TIC810	TIC809 untargeted; TIC810 targeted to chloroplasts
pMON64137	ctp-TIC809, TIC810	TIC809 targeted to chloroplasts; TIC810 untargeted

Protoplasts were prepared by digesting 12-day corn leaf tissue in 0.6 M mannitol, 10 mM MES pH 5.7, 2% cellulase RS, and 0.3% macerozyme R10 for 2 hours. All transformations were performed using 50 µg DNA and 1.3×10^6 cells.

TIC809 expression in protoplasts was measured using an ELISA with polyclonal antibodies raised against the ET29 protein. Results are shown in Table 5 and represent the average of three duplicate samples.

Table 5. Transient expression of TIC809 in corn protoplasts

Plasmid	Cry Proteins	Mean ²	Standard Error	Comparisons ¹
pMON84202	TIC809	5.1	0.6	c
pMON64134	TIC809, TIC810	120.3	24.9	a
pMON64136	TIC809, ctpTIC810	29.2	3.0	bc
pMON84203	ctpTIC809	111.4	3.2	a
pMON64137	ctpTIC809, TIC810	90.2	18.0	ab
pMON64135	ctpTIC809, ctpTIC810	148.9	15.2	a

1 - Comparisons for all pairs using Tukey-Kramer HSD, alpha = 0.05. Treatments with the same letter are not significantly different from each other.

2 - (ng ET29/mg total protein)

The results indicate that the chloroplast targeted TIC809 protein resulted in an increase in TIC809 expression in the transient system compared to the non-targeted TIC809 expression. By comparing expression from pMON84203 with expression from pMON84202, targeted TIC809 protein expression is observed to be approximately 20-fold higher than that of the untargeted TIC809 protein. However, co-expression of the untargeted TIC810 protein with the untargeted TIC809 also results in a significant increase in the expression of TIC809 protein (compare pMON64134 with pMON84202). In five out of six experiments, targeting both TIC810 and TIC809 to the plastid resulted in equivalent or greater levels of expression and accumulation of TIC809 compared to that amount accumulated when TIC809 is targeted to the plastid alone. In any event, co-expression of TIC809 and TIC810 together in any common cellular location results in increased levels of accumulation of TIC809 compared to levels of TIC809 accumulation when expressed in that compartment in the absence of TIC810.

pMON64136 and pMON64137 were constructed to test the effect of expression of TIC809 when localized to a different subcellular compartment from TIC810. Targeting TIC810 to the plastid had no significant impact on the expression of the non-targeted TIC809 (compare pMON64136 with pMON84202). Similarly, the non-targeted TIC810 had no significant impact on the accumulation of the targeted TIC809 (compare pMON64137 with pMON84203). However, the non-targeted TIC810 increased or stabilized the accumulation

of the non-targeted TIC809. These results suggest that localization of the two proteins to the same space within the cell results in a greater accumulation of the rootworm insecticidal protein TIC809, perhaps as a result of some interaction between the proteins, stabilizing the accumulation of the TIC809 protein. These results are consistent with the observation that co-expression of TIC810 and ET29, or co-expression of TIC812 and ET37, results in high-level expression of ET29 or ET37 respectively in *B. thuringiensis*.

A plasmid containing a luciferase (LUX) gene was included as a control in the transient protoplast expression assays. Although luciferase is generally included in transient assays as an indicator of transformation efficiency, it was observed that luciferase expression levels varied widely depending on the plasmid construct tested, presumably due to phytotoxic effects of the accumulated Bt proteins. Low accumulation of TIC809 was correlated with low luciferase levels. In each case, other than when TIC809 is localized to the chloroplast, co-expression of the untargeted TIC810 in the same compartment with TIC809 resulted in a dramatic increase in both luciferase and TIC809 expression levels (compare pMON64134 with pMON84202). The luciferase data is shown in Table 6.

Table 6. Luciferase levels in transient assay experiment

Plasmid	Encoded Cry protein(s)	Counts per second	Standard error of the mean	Comparisons ¹
pMON84202	TIC809	67.4	1.7	b
pMON64134	TIC809, TIC810	518.6	135.7	a
pMON64136	TIC809, ctpTIC810	70.8	2.3	b
pMON84203	ctpTIC809	408.0	84.7	a
pMON64137	ctpTIC809, TIC810	277.2	2.6	ab
pMON64135	ctpTIC809, ctpTIC810	284.9	32.8	ab

¹ Comparisons for all pairs using Tukey-Kramer HSD, alpha = 0.05. Treatments with the same letter are not significantly different from each other.

Example 7

This example illustrates the results of co-expression of TIC810 and TIC809 in transgenic corn.

TIC809, ctp-TIC809, TIC809-TIC810, and ctpTIC809-ctpTIC810 expression cassettes were introduced into binary plant transformation vectors suitable for use in corn transformation. Other than the sequences encoding the Bt proteins, constructs differ from

each other only with respect to the presence or absence of a chloroplast-targeting peptide coding sequence. A gene conferring glyphosate tolerance was used as a selectable marker for *Agrobacterium*-mediated transformation.

pMON64138 contains a cassette for *in planta* expression of TIC809 and TIC810.

- 5 pMON64139 contains a cassette for *in planta* expression of chloroplast targeted TIC809 and chloroplast targeted TIC810. pMON70513 contains a cassette for *in planta* expression of TIC809. pMON70514 contains a cassette for *in planta* expression of a chloroplast targeted TIC809. Regenerated transgenic corn plants obtained after transformation using these four plasmids and glyphosate selection were screened using a TaqMan® assay for the presence of
10 the glyphosate selectable marker gene and the presence of the tahsp17 3' sequence(s). An endpoint PCR assay was used to confirm the presence of the TIC809 and/or TIC810 coding sequences, where relevant, in each event. Events transformed with constructs containing both TIC809 and TIC810 were expected to exhibit two copies of the tahsp17 3' sequence because each coding sequence is bordered on its 3' end by the tahsp17 3' element.
15 Transgenic and control plants containing six leaves (V6 stage) and roots were assayed using an ET29 ELISA to determine the level of accumulation of TIC809.

- Plants regenerated after transformation with pMON70513 containing only the cytoplasm targeted TIC809 coding sequence behaved as indicated above in examples 1 and 2. Plants exhibited substantially abnormal phenotypes and characteristics, typically
20 characterized visually by a chlorotic stalk, among other abnormalities. The TIC809 expression and/or accumulation levels averaged no more than about 2.0 ppm in root tissue.

- Fifteen R0 plants transformed with pMON64138 were assayed for the presence of the glyphosate marker and the presence and copy number of the tahsp17 3' elements, as well as for the presence of the OriV for detection of any vector backbone, and for the presence and
25 intactness of the genes encoding the cytoplasm targeted TIC809 and TIC810 proteins. One event did not contain the full length TIC809 coding sequence and was also determined to exhibit undetectable levels of TIC809 protein. The remaining 14 events displayed an average of about 12 ppm TIC809 (fresh weight) in leaf tissue or root tissue. The levels of TIC809 in root tissue ranged from about 0.2 ppm in one plant to about 45 ppm in the plant
30 exhibiting the greatest level of expression and/or accumulation. This result suggested that the co-expression of TIC810 along with TIC809 in the plant tissues provides for improved levels of expression and/or accumulation of the TIC809 protein. More significantly, abnormal phenotypes were not observed in the plants expressing both TIC809 and TIC810 in the cytoplasm. The expression level of TIC809 was more uniform in root tissue, than in leaf

tissue, and more plants exhibited higher levels of TIC809 expression in the roots than in the leaves.

Fifteen R0 plants were regenerated from transformed plant cells using pMON64139 containing the chloroplast targeted TIC809 and TIC810 coding sequences. One event was identified in the screening analysis that did not contain the full length TIC809 expression cassette, and also did not exhibit detectable levels of TIC809 protein. The remaining 14 events averaged about 4.4 ppm and about 8.6 ppm TIC809 protein in root and leaf tissue respectively. The levels of TIC809 in roots ranged from about 0.2 ppm to about 45 ppm. Corn plants transformed with pMON70514, containing the ctpTIC809 gene, averaged only about 1.7 ppm TIC809 protein in leaf tissue and about 6.3 ppm TIC809 protein in root tissue. Thus, co-expression of the non-targeted TIC810 along with the -targeted TIC809 resulted in higher levels of TIC809 expression and/or accumulation than was achieved with the chloroplast-targeted TIC809 protein. Furthermore, the R0 plants producing elevated levels of TIC809 protein did not exhibit stalk chlorosis or other manifestations of phytotoxicity associated with *in planta* expression of the TIC809 protein alone. Co-expression of chloroplast targeted TIC810 along with chloroplast targeted TIC809 also resulted in increased levels of TIC809 accumulation when compared to levels of chloroplast targeted TIC809 protein expressed in the absence of TIC810.

Eighteen R0 plants transformed to co-express the targeted TIC809 and targeted TIC810 proteins were assayed for the glyphosate tolerance selectable marker gene, the *tahsp17* 3' copy number, presence of OriV (backbone), and for intact TIC809 and TIC810 coding sequences. One event did not contain an intact TIC809 coding sequence and failed to exhibit detectable levels of TIC809 protein. The remaining 17 plants averaged 8.6 and 4.4 ppm TIC809 protein in leaf and in root, respectively. Root expression of TIC809 protein ranged from about 1 ppm to about 9 ppm. Events exhibiting chloroplast targeted TIC809 and TIC810 protein expression failed to exhibit stalk chlorosis or other manifestations of phytotoxicity associated with *in planta* expression of the TIC809 protein alone.

Example 8

This example illustrates maize root enhanced expression of plastid targeted TIC809.

pMON64144 was constructed to contain a chloroplast targeted TIC809 under the operable control of a RCc3 root promoter (US Pat Application Serial No. 11\075,113) and flanked 5' of a CTP coding sequence by a maize heat shock protein HSP70 intron and 3' of

the TIC809 coding sequence by a wheat hsp17 3' transcription termination and polyadenylation sequence. The sequence of the expression cassette is set forth at SEQ ID NO:38.

Corn plants were regenerated after *Agrobacterium* mediated transformation of corn tissue with the vector pMON64144. Regenerated corn plants were screened using a TaqMan® assay for the presence of the glyphosate selectable marker and the wheat 3' flanking sequence. The presence of intact TIC809 coding sequence was confirmed using endpoint PCR assay. Root and leaf samples from 23 R0 corn plants at the six-leaf stage were screened using an ET29 ELISA.

The average level of TIC809 accumulated in root tissue was 0.4 ppm. No TIC809 protein was detected in leaves, suggesting that RCc3 promoter activity is enhanced in root cells. 8 of the 23 R0 plants exhibited a TIC809 protein concentration below the level of detection in root. None of the plants tested exhibited levels greater than about 1ppm TIC809. In contrast, a similar construct under the control of a e35S promoter exhibited on average about 1.4 ppm TIC809 protein in root tissue and about 1.7 ppm in leaves (n=87).

Example 9

This example illustrates *in planta* co-expression of TIC809 and TIC810, each under the control of different promoters.

In Example 7 the TIC809 and TIC810 genes were each expressed *in planta* from separate expression cassettes, expression of each coding sequence being driven from separate but identical e35S promoters. In this example expression cassettes were designed so that expression of TIC809 was substantially localized to the root tissue using a RCc3 promoter, while expression of TIC810 was under the control of an e35S promoter.

pMON64150 contains two expression cassettes. One cassette (SEQ ID NO:40) contained a chloroplast targeted TIC809 coding sequence operably linked at its 5' end to a rice RCc3 promoter and a maize heat shock protein HSP70 intron, and at its 3' end to a wheat hsp17 3' transcription termination and polyadenylation sequence. The other cassette (SEQ ID NO:40) contained a chloroplast targeted TIC810 coding sequence operably linked at its 5' end to an e35S promoter and a rice actin intron sequence, and at its 3' end to a wheat hsp17 3' transcription termination and polyadenylation sequence.

pMON64151 is identical to pMON64150 except that the coding sequences in the two expression cassettes lack the chloroplast targeting peptide coding sequence (SEQ ID NO:43 and SEQ ID NO:40, respectively).

Plants regenerated from corn tissue transformed with either pMON64150 or pMON64151 were tested to confirm the presence and intactness of the TIC809 and TIC810 coding sequences. Leaves and roots from these events were tested at the 6 leaf stage using an ET29 ELISA to determine the levels of TIC809 protein accumulation. Plants transformed with pMON64150 exhibited on average about 1.5 ppm TIC809 per plant, while plants transformed with pMON64151 exhibited on average about 0.4 pm TIC809 per plant. pMON64150 plants exhibited TIC809 root accumulation levels from about 0.4 to about 6 ppm, with more than two thirds of the events exhibiting TIC809 levels at least about 1 ppm. Leaf tissue consistently exhibited levels of TIC809 accumulation below the limits of detection for the assay.

Average root accumulation of TIC809 was greater in pMON64151 events than in events generated using the chloroplast targeted pMON64150 expression cassettes (pMON64151, 6.6ppm vs pMON64150, 1.4ppm). These results are consistent with the results obtained using constructs in which TIC809 was expressed from the e35S promoter (pMON64138 and pMON64139). The greatest difference between plants expressing TIC809 from the RCc3 versus the e35S promoter was the lack of accumulation of TIC809 in leaves when expression was controlled by the RCc3 promoter. Both pMON64150 and pMON64151 events exhibited normal phenotypes.

Example 10

This example illustrates Hemipteran species insecticidal bioactivity of compositions comprising TIC 809 and TIC 810, and homologs thereof as disclosed herein.

Compositions comprising ET29 and/or TIC809 or ET37 have been disclosed herein to be insecticidal to Coleopteran species of insect pests. TIC810 and TIC812 have not demonstrated insecticidal bioactivity to Coleopteran insect species, but as disclosed herein, are useful for facilitating the high level expression and stability of ET29 and or TIC809 and ET37. ET29 had also previously demonstrated insecticidal bioactivity directed to *Ctenocephalides* species, and it is anticipated that ET37 would also demonstrate activity to this species. It was speculated that TIC810 and/or TIC812 also exhibit insecticidal bioactivity and so these proteins were tested in bioassay against other plant pests, for example against Hemipteran insect pest species such as *Lygus hesperus* (Western Tarnished Plant Bug; WTPB).

The WTPB, is a phytophagous, piercing-sucking insect that attacks numerous weeds and crops. The WTPB damages agricultural crops, including cotton, by direct feeding

damage. An assay for testing insecticidal compositions using this class of insects must allow for the insect's natural feeding behavior. The feeding assay employed is based on a 96 well micro-titer plate format using a sachet system as described by Habibi et al., (*Archives of Insect Biochem. and Phys.* 50: 62-74 (2002)). The WTPB artificial diet is supplied by Bio-Serv[®] (Bio-Serv[®] Diet F9644B, Frenchtown, NJ).

Five hundred and eighteen milliliters of autoclaved, boiling water are combined with 156.3 grams of Bio-Serv[®] Diet F9644B in a surface sterilized blender. The contents of four surface sterilized chicken eggs are added and the mixture is blended until smooth, then adjusted to one liter total volume and allowed to cool. Toxin samples are prepared by mixing twenty microliters of sample in the desired concentration with two hundred microliters of blended diet (1:10). Depending upon the number of individual samples desired for testing this amount can be scaled up or down.

A sheet of Parafilm[®] (Pechiney Plastic Packing, Chicago, IL) is placed over a vacuum manifold designed for 96-well format (Analytical Research Systems, Gainesville, FL) and a vacuum of approximately -20 millimeters mercury is applied, which is sufficient to cause extrusion of the Parafilm[®] into the wells. Forty microliters of test sample are added to the Parafilm[®] wells. A sheet of Mylar film (Clear Lam Packaging, Inc., Elk Grove Village, IL) is then placed over the Parafilm[®] and sealed gently with a tacking iron (Bienfang Sealector II, Hunt Corporation, Philadelphia, PA). The Parafilm[®] sachets are then placed over a flat-bottom 96-well plate containing WTPB eggs suspended in agarose. Upon hatching, WTPB nymphs will feed by piercing the sachet that is presented above them. Extraoral digestion as a result of extrusion of WTPB oral secretions into the sachet may lead to proteolysis and degradation of diet contents prior to ingestion by the insect. To assure intact protein was being presented to the insect in its diet, the diet sachets are replaced every two days. This enhancement allows for longer presentation of the intact diet contents over the course of the feeding assay. Insect diet sachets are replaced on days two, four and six. Stunting and mortality scores are determined on day 8 and compared to the untreated check (UTC).

The proteins ET29 (or TIC809), TIC810, ET37 and TIC812 (US Patent Application number 60/713,111), were tested individually and in combinations such as, for example, TIC809 plus TIC810, for their toxicity to the WTPB. Crystal proteins were expressed in the acrySTALLIFEROUS *Bacillus thuringiensis* strain EG10650 and purified over sucrose step gradients to eliminate spores and cell debris. Sucrose step gradients (10 mL each of 55-, 70- and 79-% sucrose in 10 mM Tris-HCl, 0.1 mM EDTA, 0.005% Triton X-100, pH 7.5) were

prepared in 25- by 89- mm Ultra-Clear centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA). Spore-crystal suspensions were layered on top of the gradients and centrifuged at 18,000 rpm (4°C) for 4-18 hr in an ultracentrifuge equipped with a SW28 rotor. Protein crystals were recovered from either the 55-70% or the 70-79% sucrose interface and
5 suspended in 25 mM Tris-HCl pH 7.5. Initial bioassays contained the purified Bt insecticidal proteins at a final concentration of 200 parts per million (ppm). The Coleopteran specific toxin, Cry3Bb1 (Donovan et al., Appl. Environ. Microbiol. 58: 3921-3927 (1992)), the Lepidopteran specific toxin Cry1Ac (Baum et al., Appl. Environ. Microbiol. 56:3420-3428 (1990), and the Lepidopteran specific toxin Cry1Bb1 (U.S Patent No. 5,322,687) were
10 each included as negative controls in the feeding assay. Surprisingly, WTPB nymphs exhibited stunting and mortality when exposed to the combination of the TIC810 plus ET29 proteins and as expected, no stunting and mortality were detected when exposed only to ET29, or to any of the other BT proteins Cry3Bb1, Cry1Ac, or Cry1Bb1, all of which exhibited no significant difference when compared to the untreated control.

15 The *Lygus* bioassays were expanded to include individual crystal preparations of TIC810, TIC812, and a mixture of TIC812 and ET37. Similar to the results described above, only the combination of the two proteins exhibited significant insecticidal activity. TIC810 plus ET29 and TIC812 plus ET37 exhibited significant mortality and mass reduction when compared to untreated controls or to the bioassays using only the individual proteins.
20 However, combinations of TIC810 plus ET29 (or TIC809) exhibited greater mortality and mass reduction than the TIC812 plus ET37 combination.

The results of these bioassays indicated that neither TIC810 nor TIC812 alone are toxic to WTPB, and that a mixture of either TIC810 and ET29 or of TIC812 and ET37 is toxic to WTPB, similar to the results observed when tested against corn rootworm larvae.

25 Example 11

This example illustrates construction of cassettes for expressing TIC809 and/or TIC810, and homologs thereof.

Plant transformation vectors are constructed to achieve high-level expression of
30 rootworm-toxic TIC809 and/or ET37 proteins in plants. Vectors containing TIC812 and ET37 coding sequences may be used to co-express TIC812 and ET37 protein, thereby achieving insect protected plants exhibiting high levels of *in planta* ET37 protein production. Co-expression of TIC810 along with ET37 is sufficient for achieving stable high levels of accumulation of ET37 in a host cell. Similarly, co-expression of TIC812 along with ET29 or

even TIC809 is sufficient to achieve stable high levels of accumulation of ET29 or TIC809 in a host cell. As indicated hereinabove,

It is anticipated that proteins of the Cyt1 and Cyt2 class that exhibits from about
5 15 to about 100 per cent amino acid sequence similarity to ET29 and/or ET37 will exhibit improved expression and/or accumulation when expressed in a host cell along with TIC810, TIC812, or an orthologous or homologous protein exhibiting an amino acid sequence that exhibits from about 50 to about 100% amino acid sequence similarity to TIC810 or TIC812, and that any negative phenotypic effects caused by such Cyt protein expression will be
10 ameliorated by co-expression of such Cyt protein with TIC810, TIC812, or variants thereof.

The above specification describes preferred embodiments of the present invention. It will be understood by those skilled in the art that, without departing from the scope and spirit of the present invention and without undue experimentation, the present invention can be
15 performed within a wide range of equivalent parameters. While the present invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. The present invention is intended to cover any uses, variations, or adaptations of the invention following the principles of the invention in general. Various permutations and combination of the elements provided in all the claims that follow
20 are possible and fall within the scope of this invention.

All publications, patents and published patent applications referred to in this specification are herein incorporated by reference as if each individual publication or patent was specially and individually stated to be incorporated by reference.

Claims

What is claimed is:

1. An isolated and purified nucleotide sequence encoding an insecticidal protein, said protein being selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:47.
2. The isolated and purified nucleotide sequence of claim 1 selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, and SEQ ID NO:46.
3. An isolated and purified insecticidal protein selected from the group consisting of an ET29, an ET37, a TIC810, a TIC812, a TIC809, and a TIC127.
4. A plant or plant cell transformed with a nucleotide sequence encoding an insecticidal protein, said insecticidal protein being selected from the group consisting of an ET29, an ET37, a TIC810, a TIC812, a TIC809, and a TIC127.
5. Progeny or seed from the plant of claim 4, wherein said progeny or seed comprises said nucleotide sequence.
6. A vector comprising a nucleotide sequence encoding a protein selected from the group consisting of an ET29, ET37, TIC810, TIC812, TIC809, and TIC127.
7. A method for enhancing the accumulation of a first insecticidal protein in a host cell, said method comprising contemporaneously expressing said first protein selected from the group consisting of ET29, TIC809, and ET37 with a second insecticidal protein selected from the group consisting of TIC810 and TIC812, wherein said contemporaneous expression enhances the accumulation of the first protein compared to the accumulation of said first protein expressed in the absence of said second protein.
8. A composition exhibiting insecticidal activity comprising a first protein selected from the group consisting of ET29, ET37, and TIC809, and a second protein selected from the group consisting of TIC810 and TIC812.

9. The composition of Claim 8 wherein said first protein is TIC809 and said second protein is TIC810.
10. The composition of claim 8 wherein the ratio of said first protein to said second protein is from about 1:1 to about 2:1.
11. The composition of claim 8 provided in the diet of a Coleopteran or a Hemipteran plant pest in an agriculturally acceptable formulation.
12. The composition of Claim 11 wherein said formulation consists of both proteins expressed in a plant cell.
13. The composition of claim 11 wherein said Coleopteran pest is a corn rootworm and said Hemipteran pest is a Lygus bug.
14. A commodity product comprising a detectable amount of a first protein selected from the group consisting of ET29, ET37 and TIC809 and a detectable amount of a second protein selected from the group consisting of TIC810 and TIC812.
15. A commodity product comprising a detectable amount of (1) a first nucleotide sequence encoding a first protein selected from the group consisting of ET29, ET37 and TIC809 and second nucleotide sequence encoding a second protein selected from the group consisting of TIC810 and TIC812; (2) a first protein selected from the group consisting of ET29, ET37, and TIC809 and a second protein selected from the group consisting of TIC810 and TIC812; (3) both (1) and (2); or (4) any combination of (1) and (2).
16. The commodity product of any of claims 14-15 selected from the group consisting of cotton seed, cotton seed oil, cotton seed meal, soybean seed, soybean oil, soybean meal, soybean flour, distillers' dry goods solids, corn seed, corn flour, corn meal, corn syrup, corn oil, corn starch, animal feed, and cereals manufactured in whole or in part to contain soybean and/or corn by-products.

17. A method for making a plant cell resistant to an insect pest comprising transforming said plant cell to express an insecticidally effective amount of a toxin composition, said toxin composition comprising a first protein selected from the group consisting of ET29, ET37 and TIC809 and a second protein selected from the group consisting of TIC810 and TIC812.

18. The method of claim 17 wherein said plant cell is regenerated into a fertile transgenic plant.

19. The method of claim 17 wherein said plant cell is selected from the group consisting of a monocot plant cell and a dicot plant cell.

20. The method of claim 19, wherein (1) said monocot plant cell is selected from the group consisting of corn, wheat, oat, rice, sorghum, milo, buckwheat, rye, grass (fescue, timothy, brome, orchard, St. Augustine, Bermuda, bentgrass), and barley plant cell, and (2) said dicot plant cell is selected from the group consisting of alfalfa, apple, apricot, asparagus, bean, berry, blackberry, blueberry, canola, carrot, cauliflower, celery, cherry, chickpea, citrus tree, cotton, cowpea, cranberry, cucumber, cucurbit, egg plant, fruit tree, grape, lemon, lettuce, linseed, melon, mustard, nut bearing tree, okra, orange, pea, peach, peanut, pear, plum, potato, soybeans, squash, strawberry, sugar beet, sunflower, sweet potato, tobacco, tomato, turnip, and vegetable plant cells.

21. The method of claim 18, wherein progeny plants and seeds are produced from said transgenic plant, and wherein said progeny plants and seed comprise said first and second protein.

22. A host cell comprising a nucleotide sequence encoding a cyt protein, said protein being selected from the group consisting of a TIC809, a ET37, a TIC810, a TIC812, and a TIC127.

23. The host cell of claim 22 selected from the group consisting of a plant cell, a fungal cell and a bacterial cell.

24. The host cell of claim 23 wherein said plant cell is selected from the group consisting of a monocot plant cell and a dicot plant cell.

25. The host cell of claim 24 wherein (1) said monocot plant cell is selected from the group consisting of corn, wheat, oat, rice, sorghum, milo, buckwheat, rye, grass (fescue, timothy, brome, orchard, St. Augustine, Bermuda, bentgrass), and barley plant cell, and (2) said dicot plant cell is selected from the group consisting of alfalfa, apple, apricot, asparagus, bean, berry, blackberry, blueberry, canola, carrot, cauliflower, celery, cherry, chickpea, citrus tree, cotton, cowpea, cranberry, cucumber, cucurbit, egg plant, fruit tree, grape, lemon, lettuce, linseed, melon, mustard, nut bearing tree, okra, orange, pea, peach, peanut, pear, plum, potato, soybeans, squash, strawberry, sugar beet, sunflower, sweet potato, tobacco, tomato, turnip, and vegetable plant cells.

26. An expression cassette for use in expressing an insecticidal protein in a host cell, wherein said expression cassette comprises in operable linkage a promoter sequence functional in said host cell and a nucleotide sequence encoding said protein, said protein being selected from the group consisting of TIC809, TIC810, TIC812, ET37, and TIC127.

27. The expression cassette of Claim 26 wherein said host cell is selected from the group consisting of a bacterial cell, a fungal cell, a mammalian cell, and a plant cell.

28. The expression cassette of Claim 27 wherein

(a) said bacterial cell is selected from the group consisting of a *Bacillus* species cell, a *Enterobacteriaceae* species cell, a *Pseudomonas* species cell, a *Clostridium* species cell, and a *Rhizobium* species cell, and a *Agrobacterium* species cell; and

(b) said plant cell is selected from the group of plants consisting of a dicotyledonous plant and a monocotyledonous plant, said dicotyledonous plant being further selected from the group consisting of alfalfa, apple, apricot, asparagus, bean, berry, blackberry, blueberry, canola, carrot, cauliflower, celery, cherry, chickpea, citrus tree, cotton,

cowpea, cranberry, cucumber, cucurbit, egg plant, fruit tree, grape, lemon, lettuce, linseed, melon, mustard, nut bearing tree, okra, orange, pea, peach, peanut, pear, plum, potato, soybeans, squash, strawberry, sugar beet, sunflower, sweet potato, tobacco, tomato, turnip, and vegetable, and said monocotyledonous plant being further selected from the group consisting of corn, wheat, oat, rice, sorghum, milo, buckwheat, rye, grass (fescue, timothy, brome, orchard, St. Augustine, Bermuda, bentgrass), and barley.

29. The expression cassette of Claim 26 wherein said host cell is a plant cell and said expression cassette further comprises in operable linkage a sequence selected from the group consisting of an expression enhancer sequence, an untranslated leader sequence, an intron sequence, a chloroplast targeting peptide encoding sequence, and a transcription termination and polyadenylation sequence.
30. A vector comprising the expression cassette of any of Claims 26 through Claim 29.
31. A transgenic plant or plant cell resistant to insect infestation comprising a nucleotide sequence encoding a first cyt toxin selected from the group consisting of ET29, TIC809, and ET37 and a second cyt toxin selected from the group consisting of TIC810 and TIC812.
32. The transgenic plant or plant cell of claim 31 wherein said transgenic plant is selected from the group consisting of a dicot plant or dicot plant cell and a monocot plant or monocot plant cell, said dicot plant or dicot plant cell being further selected from the group consisting of alfalfa, apple, apricot, asparagus, bean, berry, blackberry, blueberry, canola, carrot, cauliflower, celery, cherry, chickpea, citrus tree, cotton, cowpea, cranberry, cucumber, cucurbit, egg plant, fruit tree, grape, lemon, lettuce, linseed, melon, mustard, nut bearing tree, okra, orange, pea, peach, peanut, pear, plum, potato, soybeans, squash, strawberry, sugar beet, sunflower, sweet potato, tobacco, tomato, turnip, and vegetable, and said monocot plant being further selected from the group

consisting of corn, wheat, oat, rice, sorghum, milo, buckwheat, rye, grass (fescue, timothy, brome, orchard, St. Augustine, Bermuda, bentgrass), and barley.

33. The transgenic plant or plant cell of claim 32 resistant to (1) coleopteran insect infestation, wherein said coleopteran insect is selected from the group consisting of a Chrysomelidae, Cucujidae, Scarabaeidae, Trogositidae, Tenebrionidae, Curculionidae, Elateridae and Bruchidae, and (2) hemiptera insect infestation, wherein said hemiptera are selected from the sub-orders Heteroptera and Homoptera.
34. The transgenic plant or plant cell of claim 33, wherein said Chrysomelidea is a Diabrotica species selected from the group consisting of *D. virgifera* (western corn rootworm), *D. undecimpunctata* (southern corn rootworm), *D. barberi* (northern corn rootworm), *D. virgifera zea* (Mexican corn rootworm), *D. balteata* (brazilian corn rootworm) and Brazilian Corn Rootworm complex (BCR) further comprising *D. viridula* and *D. speciosa*.
35. Progeny or seed of the transgenic plant or plant cell of claim 33, wherein said progeny or seed comprise said nucleotide sequence.
36. A method for controlling coleopteran or hemipteran insect infestation of a plant by providing in the diet of the insect one or more plant cells transformed with a nucleic acid sequence comprising in operable linkage a plant functional promoter and a nucleotide sequence encoding a fusion protein, wherein said fusion protein comprises a first amino acid sequence selected from the group consisting of a SEQ ID NO:2, SEQ ID NO:8, and SEQ ID NO:14, and a second amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.
37. A method for protecting a crop in a field from insect infestation comprising growing a transgenic crop plant comprising an insecticidally effective amount of a first protein and a second protein, said first protein being selected from the group consisting of an ET37, an ET29, and a TIC809 and said second protein being selected from the group consisting of TIC810 and TIC812, and providing the proteins together in the diet of the insect to inhibit the insect from surviving on said transgenic crop plant.

38. The method of claim 37 wherein said insect is selected from the group consisting of a coleopteran insect and a Hemipteran insect.
39. The method of claim 37, wherein said transgenic crop plant further comprises an additional insecticidal agent toxic to the same insect as the first and second proteins, wherein said additional insecticidal agent is selected from the group consisting of a *Bacillus* toxin, a *Xenorhabdus* toxin, a *Photorhabdus* toxin, and a dsRNA specific for suppression of one or more essential genes in said insect.
40. The method of claim 37-39 wherein the yield of said crop is improved compared to the yield of an isogenic crop lacking said insecticidal proteins.