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(54) Titre : PROTEINES TOXIQUES POUR DES ESPECES D'INSECTES HEMIPTERES

(54) Title: PROTEINS TOXIC TO HEMIPTERAN INSECT SPECIES

(57) **Abrégé/Abstract:**

An insect inhibitory recombinant polypeptide comprising a sequence having at least 95% amino acid identity to SEQ ID NO: 2 (TIC807), wherein the insect inhibitory recombinant polypeptide exhibits about 15 fold or greater Hemipteran inhibitory activity against a Hemipteran pest species relative to the Hemipteran inhibitory activity of SEQ ID NO: 2, and wherein the insect inhibitory recombinant polypeptide comprises: a) at least one modification at an amino acid residue selected from the group consisting of Val10, Asn12, Ile14, Asn22, Asn23, Gly24, Ile25, Gln26, Gly27, Phe30, Gln38, Ile39, Asp40, Thr41, Ile43, Ile52, Thr93, Ser95, Ser97, Phe138, Phe147, Gln149, Ser151, Ser159, Asn180, Thr182, Ser193, Thr194, Glu195, His196, Tyr197, Ser198, His199, Tyr200, Ser201, Gly202, Tyr203, Pro204, Ile205, Leu206, Thr207, Trp208, Ile209, Ser210, Tyr216, Gly218, Pro219, Met221, Ser222, Trp223, Tyr224, Phe225, Asn239, Val244, Tyr246, Thr247, Val251, Gln253, Ser255, and His287 relative to SEQ ID NO:2; and (b) a contiguous triple deletion in residue range 196-201 relative to SEQ ID NO:2.



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ABSTRACT

An insect inhibitory recombinant polypeptide comprising a sequence having at least 95% amino acid identity to SEQ ID NO: 2 (TIC807), wherein the insect inhibitory recombinant polypeptide exhibits about 15 fold or greater Hemipteran inhibitory activity against a Hemipteran pest species relative to the Hemipteran inhibitory activity of SEQ ID NO: 2, and wherein the insect inhibitory recombinant polypeptide comprises: a) at least one modification at an amino acid residue selected from the group consisting of Val10, Asn12, Ile14, Asn22, Asn23, Gly24, Ile25, Gln26, Gly27, Phe30, Gln38, Ile39, Asp40, Thr41, Ile43, Ile52, Thr93, Ser95, Ser97, Phe138, Phe147, Gln149, Ser151, Ser159, Asn180, Thr182, Ser193, Thr194, Glu195, His196, Tyr197, Ser198, His199, Tyr200, Ser201, Gly202, Tyr203, Pro204, Ile205, Leu206, Thr207, Trp208, Ile209, Ser210, Tyr216, Gly218, Pro219, Met221, Ser222, Trp223, Tyr224, Phe225, Asn239, Val244, Tyr246, Thr247, Val251, Gln253, Ser255, and His287 relative to SEQ ID NO:2; and (b) a contiguous triple deletion in residue range 196-201 relative to SEQ ID NO:2.

PROTEINS TOXIC TO HEMIPTERAN INSECT SPECIES

This application is a division of Canadian Serial No. 2,868,815 filed April 5, 2013.

FIELD OF THE INVENTION

[0003] The present invention generally relates to the field of insect inhibitory proteins. In particular, the present invention relates to proteins exhibiting insect inhibitory activity against agriculturally relevant pests of crop plants and seeds, particularly Hemipteran species of insect pests.

BACKGROUND OF THE INVENTION

[0004] Insect inhibitory proteins derived from *Bacillus thuringiensis* (*Bt*) are non-toxic to humans, vertebrates, and plants. These proteins are also biodegradable, safe, and effective in controlling pest insects. Some of these proteins have been and are being used to control agriculturally relevant pests of crop plants by spraying plants with formulations containing these proteins or with microorganisms that express them, treating seeds with treatments containing these proteins, or expressing these proteins in crop plants and seeds of crop plants as plant-incorporated protectants.

[0005] Certain Hemiptera species, particularly *Amrasca*, *Empoasca* and *Lygus* bugs, are pests of cotton and alfalfa, and typically are only controlled using broad spectrum chemistries, *e.g.*, endosulfan, acephate, and oxamyl, which can persist in and are harmful to the environment. A few *Bt* proteins have been developed in formulations or as transgenic traits in crop plants

for commercial use by farmers to control Coleopteran and Lepidopteran pest species, but no *Bt* proteins have been developed for use in commercial control of Hemipteran pest species.

[0006] Hemipteran specific toxic proteins have been reported in the art. TIC807 is a *Bacillus thuringiensis* protein disclosed in U.S. Patent Application Publication No. US 2008-0295207 A1 as being toxic to Hemipteran pest species. A Cry51Aa1 protein reported as toxic to Lepidopteran species that closely resembles the amino acid sequence of TIC807 has also been disclosed (Huang et al., (2007) J. Invertebr. Pathol. 95(3), 175-180), but no Hemipteran specific activity was reported. Baum et al. disclosed TIC853, a protein reported to be toxic to *Lygus* pest species (U.S. Patent Application Publication No. US 2010-0064394 A1). A protein referred to as AXMI-171 was reported to exhibit some limited inhibition of Hemipteran insects (U.S. Patent Application Publication No. US2010-0298207 A1, example 18), particularly *Lygus hesperus*.

[0007] All of these proteins exhibit a narrow range of toxicity only against *Lygus hesperus* and exhibit toxic effects against other *Lygus* pest species only in high doses which are not considered to be achievable by expression in plants. Compared to the Hemipteran toxic proteins in the prior art, there is a need for toxin proteins that can be used on and in plants that exhibit a broad host range against Hemipteran pest species and at low concentration effective doses.

BRIEF SUMMARY OF THE INVENTION

[0008] Recombinantly engineered Hemipteran toxic proteins described herein (referred to herein as “engineered toxin proteins”, “engineered toxic proteins”, “engineered Hemipteran toxic proteins”, or “engineered Hemipteran toxin proteins”, are also referred to herein in truncated form as “eHTP’s” when referred to in groups of two or more such proteins, and “eHTP” when referred to singularly) are derivatives of naturally occurring *Bacillus thuringiensis* insecticidal toxins, TIC807 (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and AXMI-171 (SEQ ID NO:206) have been described previously to exhibit bio-control activity directed to Hemipteran pest species, particularly *Lygus hesperus* insect species (references cited elsewhere herein). The recombinant Hemipteran insect toxic proteins of the present invention are particularly toxic to insects of the *Amrasca*, *Empoasca* and *Lygus* species of insect pests and to other insect pest species that are phylogenetically related to each of these species of insect pests, and additionally to insect pests that feed on plants using a piercing and sucking mechanism used

by the pest species *Amrasca*, *Empoasca* and *Lygus* species of the order Hemiptera. Unlike the precursor insecticidal toxins TIC807 (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and AXMI-171 (SEQ ID NO:206) from which they are derived, which each require moderately high to high doses of protein to achieve toxic effects upon one *Lygus* species and exhibit very low or virtually undetectable toxic effects upon a second closely related species of *Lygus*, the eHTP proteins of the present invention exhibit surprising and unexpected low dose toxic effects against insect pests of the order Hemiptera, including host range toxic effects that span the spectrum of pests within the order.

[0009] The eHTP's of the present invention each contain at least one amino acid substitution, one amino acid addition, or one amino acid deletion compared to the primary amino acid sequence of one or more of the toxin proteins set forth in any of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184. In certain embodiments, an eHTP is provided that contains at least from about 2 to about 260 fold greater inhibitory activity against a *Lygus* pest species than any one or more of the toxins set forth in any of SEQ ID NO:2 (TIC807), SEQ ID NO:8 (TIC807_M2), SEQ ID NO:182 (Cry51Aa1), SEQ ID NO:184 (TIC853), and/or SEQ ID NO:206 (AXMI-171). Optionally the eHTP exhibits at least about 95% amino acid sequence identity to the toxin protein selected from the group consisting of SEQ ID NO:2 (TIC807) and SEQ ID NO:182 (Cry51Aa1). In certain embodiments, an eHTP is provided that contains at least one amino acid substitution, at least one amino acid addition, or at least one amino acid deletion when compared to the amino acid sequence of any of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184. The eHTP exhibits an increased or greater *Lygus* inhibitory activity and target pest species spectrum compared to the activity and target pest species spectrum of the *Bacillus thuringiensis* proteins of set forth in SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, and SEQ ID NO:184. Each of the aforementioned eHTP's contain at least, collectively or in the alternative: (i) the amino acid substitution, addition, or deletion in a solvent accessible amino acid residue of SEQ ID NO:2; (ii) the amino acid substitution, addition, or deletion within 3 consecutive residues of a solvent accessible amino acid residue of SEQ ID NO:2; or, (iii) an amino acid sequence as set forth in SEQ ID NO:180. The aforementioned eHTP's will each contain at least, with reference to the amino acid sequence positions as numbered according to the amino acid positions of TIC807, one substitution or deletion selected from the group consisting of asparagine at position 12 replaced by aspartic acid, phenylalanine at position 46 replaced by serine, isoleucine at position 52 replaced by methionine, tyrosine at position 54 replaced by

histidine, threonine at position 68 replaced by alanine, glutamine at position 70 replaced by alanine, alanine at position 87 replaced by serine, threonine at position 93 replaced by alanine, serine at position 95 replaced by alanine, glycines at position 105 replaced by alanine, serine at position 117 replaced by alanine, serine at position 119 replaced by alanine, glutamate at position 125 replaced by cysteine, histidine, arginine, phenylalanine, serine, glutamine, lysine, threonine, asparagine, alanine, leucine, valine, methionine, aspartic acid, or tyrosine, glycines at position 128 replaced by alanine, threonine at position 133 replaced by glutamic acid, tyrosine, or tryptophan, isoleucine at position 134 replaced by alanine, valine, leucine, phenylalanine, lysine, cysteine, or methionine, glutamate at position 135 replaced by serine, alanine, valine, tryptophan, or threonine, asparagine at position 137 replaced by histidine, tyrosine, threonine, glutamic acid, serine, alanine, glutamine, glycine, isoleucine, tryptophan, lysine, cysteine, methionine, aspartic acid, phenylalanine, or arginine, phenylalanine at position 138 replaced by valine, Ala139 replaced by serine, Thr145 replaced by alanine, Phe147 replaced by serine, valine, threonine, cysteine, leucine, aspartic acid, alanine, glycine, glutamic acid, isoleucine, tyrosine, methionine, asparagine, glutamine, histidine, alanine, arginine, tryptophan, or proline, glutamine at position 148 replaced by alanine, glutamine at position 149 replaced by aspartic acid, glutamic acid, cysteine, alanine, or phenylalanine, alanine at position 150 replaced by serine, leucine, valine, glycine, aspartic acid, tryptophan, glutamic acid, asparagine, tyrosine, phenylalanine, proline, lysine, threonine, glutamine, or arginine, serine at position 151 replaced by alanine, aspartate at position 153 replaced by alanine, glutamate at position 155 replaced by cysteine, isoleucine, lysine, aspartic acid, histidine, tyrosine, glutamine, lysine, asparagine, threonine, alanine, phenylalanine, arginine, methionine, proline, tryptophan, serine, or valine, asparagine at position 157 replaced by cysteine, aspartic acid, tryptophan, tyrosine, methionine, alanine, phenylalanine, valine, leucine, proline, glutamic acid, threonine, glycine, isoleucine, or arginine, isoleucine at position 158 replaced by alanine, serine at position 159 replaced by alanine or threonine, serine at position 167 replaced by arginine or alanine, valine at position 175 replaced by alanine, methionine at position 177 replaced by alanine, asparagine at position 180 replaced by aspartic acid, threonine at position 182 replaced by alanine, leucine at position 187 replaced by alanine, histidine at position 196 deleted, tyrosine at position 197 deleted, serine at position 198 deleted, histidine at position 199 deleted, tyrosine at position 200 replaced by alanine, tyrosine at position 200 deleted, Ser201 replaced by alanine, serine at position 201 deletion, tryptophan at position 208 replaced by alanine, serine at position 217 replaced by asparagine, proline at position 219 replaced by arginine, tryptophan at position

223 replaced by tyrosine, phenylalanine at position 235 replaced by alanine, asparagine at position 239 replaced by alanine, aspartate at position 241 replaced by alanine, threonine at position 243 replaced by alanine, valine at position 244 replaced by isoleucine, threonine at position 245 replaced by alanine, tyrosine at position 246 replaced by phenylalanine, threonine at position 247 replaced by alanine or lysine, serine at position 249 replaced by alanine or arginine, valine at position 250 replaced by alanine, valine at position 251 replaced by alanine, serine at position 252 replaced by alanine, arginine at position 273 replaced by tryptophan, threonine at position 274 replaced by alanine, isoleucine at position 275 replaced by alanine, arginine at position 282 replaced by alanine, histidine at position 287 replaced by alanine or phenylalanine, serine at position 293 replaced by alanine, asparagine at position 295 replaced by alanine, glutamate at position 299 replaced by alanine, methionine at position 300 replaced by alanine, threonine at position 303 replaced by alanine, proline at position 305 replaced by alanine, isoleucine at position 306 replaced by alanine, and threonine at position 308 replaced by alanine, or wherein the protein comprises any combination of the referenced substitutions and/or deletions. eHTP's contain at least one amino acid substitution, one amino acid addition, or one amino acid deletion at an amino acid residue of SEQ ID NO:2, or the corresponding amino acid position of SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184, selected from the group consisting of (i) an amino acid residue having a relative solvent-accessibility of from at least about 15% to at least about 36%; and (ii) an amino acid residue located within a distance of about 3 consecutive residues from an amino acid having from at least about 15% to at least about 36% relative solvent-accessibility. An eHTP of the present invention contains at least one amino acid substitution, addition, or deletion at an amino acid residue selected from the group consisting of Thr93, Ser95, Ser97, Phe147, Gln149, Ser151, Asn180, Thr182, Val251, Gln253, and Ser255 of SEQ ID NO:2. Any of the aforementioned eHTP's can contain at least one additional amino acid substitution, addition, or deletion at an amino acid residue selected from the group consisting of Val10, Ile14, Asn22, Asn23, Gly24, Ile25, Gln26, Gly27, Phe30, Gln38, Ile39, Asp40, Thr41, Ile43, Ser193, Thr194, Glu195, His196, Tyr197, Ser198, His199, Tyr200, Ser201, Gly202, Tyr203, Pro204, Ile205, Leu206, Thr207, Trp208, Ile209, Ser210, Tyr216, Ser217, Gly218, Pro219, Pro220, Met221, Ser222, Trp223, Tyr224, Phe225, Asn239, and Val244 of SEQ ID NO: 2 or the corresponding amino acid residue position of SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184. Any of the aforementioned eHTP's may contain one or more modifications selected from the group consisting of S95A, F147A, Q149E, V251A, P219R, and a deletion of any three consecutive amino acids from amino acid residues 196-

201 as set forth in SEQ ID NO:2. Any of the eHTP's of the present invention can be further modified to exhibit increased solubility compared to the underlying naturally occurring *Bacillus thuringiensis* protein as set forth in any of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184 in which the eHTP contains at least one or more amino acid sequence modifications relative to the amino acid sequence as set forth in SEQ ID NO:2. The modification(s) contain at least a lysine substitution at one or more of the amino acid positions defined as 58, 59, 198, 199, 201, or 202 in SEQ ID NO:2; a glutamic acid residue substitution at one or more of the amino acid positions defined as 198, 248, or 301 in SEQ ID NO:2; or an arginine residue substitution at one or more of the amino acid positions defined as 246, 250, or 253 in SEQ ID NO:2. An eHTP having an amino acid sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:119, SEQ ID NO:120, SEQ ID NO:121, SEQ ID NO:122, SEQ ID NO:123, SEQ ID NO:124, SEQ ID NO:125, SEQ ID NO:126, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:130, SEQ ID NO:131, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:134, SEQ ID NO:135, SEQ ID NO:136, SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, SEQ ID NO:141, SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:146, SEQ ID NO:147, SEQ ID NO:148, SEQ ID NO:149, SEQ ID

NO:150, SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153, SEQ ID NO:154, SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157, SEQ ID NO:158, SEQ ID NO:159, SEQ ID NO:160, SEQ ID NO:161, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, SEQ ID NO:165, SEQ ID NO:166, SEQ ID NO:167, SEQ ID NO:168, SEQ ID NO:169, SEQ ID NO:170, SEQ ID NO:171, SEQ ID NO:172, SEQ ID NO:173, SEQ ID NO:174, SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177, SEQ ID NO:178, SEQ ID NO:179, SEQ ID NO:202, and SEQ ID NO:204, or an insect inhibitory fragment thereof, is a preferred embodiment of the present invention. The target Hemipteran pest species inhibited by the eHTP's of the present invention include at least *Lygus hesperus*, *Lygus lineolaris*, *Empoasca fabae* and *Amrasca devastans*, as well as other pests within the order Hemiptera that are phylogenetically related to each other or which use a piercing and sucking approach for feeding on plants.

[0010] Methods of controlling a Hemipteran pest by contacting the pest with a Hemipteran inhibitory amount of a eHTP of the present invention, as well as an insect inhibitory composition that contains at least a Hemipteran controlling amount (or Hemipteran inhibitory amount) of one or more of the eHTP's of the present invention, are also provided. In certain embodiments, an insect inhibitory composition comprising any of the eHTP's disclosed herein is provided. In certain embodiments of these methods, the Hemipteran pest is in a cotton field, a soybean field or an alfalfa field. Hemipteran toxic or Hemipteran controlling compositions can contain at least one or more eHTP along with a supplemental agent that is selected from the group consisting of an insect inhibitory protein, an insect inhibitory dsRNA molecule, and an insect inhibitory chemistry. Each of these agents can exhibit Hemipteran controlling properties, can exhibit properties for controlling pests unrelated to Hemipteran species such as Lepidopteran species or Coleopteran species, or may exhibit dual mode of action properties in which one or more Hemipteran species and one or more Lepidopteran or Coleopteran species are simultaneously controlled.

[0011] Recombinant polynucleotides that encode eHTP's of the present invention are provided. Microbes are also provided that contain the polynucleotides of the present invention, and such polynucleotides within such microbes are functionally positioned within expression cassettes designed to express the eHTP's of the present invention from operably linked functional genetic regulatory elements. Microbes are intended to include bacterial cells, as well as transgenic plant cells. Such transgenic plant cells can be regenerated into whole plants, or plant parts that also contain the recombinant polynucleotide. Methods of controlling a Hemipteran pest by exposing the pest to the microbe, whether bacterial cell or

transgenic plant cell, plant or plant part, each of which expresses a Hemipteran inhibitory amount of an eHTP are also provided. The recombinant polynucleotide may contain a nucleotide sequence 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, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:186, SEQ ID NO:187, SEQ ID NO:188, SEQ ID NO:189, SEQ ID NO:190, SEQ ID NO:191, SEQ ID NO:192, SEQ ID NO:193, SEQ ID NO:194, SEQ ID NO:195, SEQ ID NO:196, SEQ ID NO:197, SEQ ID NO:198, SEQ ID NO:199, SEQ ID NO:200, SEQ ID NO:201, and SEQ ID NO:203, or other sequences that can be assembled to encode one or more of the proteins of the present invention. In certain embodiments, the recombinant polynucleotide can further comprise a nucleotide sequence encoding one or more insect inhibitory agents that are different from the eHTP encoded by the recombinant polynucleotide. The transgenic plant part is a seed, a boll, a leaf, a flower, pollen, a stem, a root, or any portion thereof. The transgenic plant part may be a non-regenerable portion of the seed, boll, leaf, flower, stem, or root. Also provided are methods of controlling a Hemipteran pest, comprising exposing the transgenic microbe, bacteria, plant cell, plant or plant part to the target pest, wherein the microbe, bacteria, plant cell, plant or plant part expresses a Hemipteran inhibitory amount of a eHTP encoded by the recombinant polynucleotide.

[0012] Processed plant products that contain a detectable amount of a recombinant polynucleotide encoding an eHTP or any distinguishing Hemipteran controlling portion thereof are also provided. Such processed products include, but are not limited to, plant biomass, oil, meal, animal feed, flour, flakes, bran, lint, hulls, and processed seed. The processed product may be non-regenerable.

[0013] Methods of making a transgenic plant by introducing the recombinant polynucleotide into a plant cell and selecting a transgenic plant that expresses an insect inhibitory amount of an eHTP encoded by a recombinant polynucleotide are also provided. The methods include introducing the recombinant polynucleotide encoding any of the eHTP's provided herein into a plant cell and selecting a transgenic plant that expresses an insect inhibitory amount of the eHTP encoded by the recombinant polynucleotide.

[0013a] One embodiment of the present invention provides an insect inhibitory polypeptide comprising the amino acid sequence as set forth in SEQ ID NO: 34. Further provided is a polynucleotide encoding the insect inhibitory polypeptide and a host cell comprising the polynucleotide.

[0013b] Also provided is an insect inhibitory composition comprising the insect inhibitory polypeptide and a polynucleotide encoding the insect inhibitory polypeptide.

[0013c] The present invention also provides a method of controlling a Hemipteran pest comprising presenting the Hemipteran pest with an inhibitory amount of the insect inhibitory polypeptide of the invention.

[0013d] Also provided are seed and plant cells comprising the polynucleotide or the insect inhibitory polypeptide of the invention.

[0014] Other embodiments, features, and advantages of the invention will be apparent from the following detailed description, the examples, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 illustrates the mortality of *Lygus* species plotted against eHTP protein concentration. Figure 1A illustrates the mortality of *Lygus hesperus* populations in response to various concentrations of four different eHTP's compared to a control sample containing the naturally occurring TIC807 protein. Figure 1B illustrates the mortality of *Lygus lineolaris* populations in response to various protein concentrations of three different eHTP's compared to a control sample containing the naturally occurring TIC807 protein.

[0016] Figure 2 illustrates a ribbon diagram of the atomic structure of a Hemipteran toxic protein of the present invention showing the relative positions of the result effective changes increasing toxic effects and/or broadening host range specificity compared to the relative position of the same amino acid position within a TIC807 or related protein. Two surface patches are illustrated by spheres encircling particular residue positions within the atomic structure in the ribbon diagram: [1] one sphere has an atomic radius of from about 9.2 to about 12.2 Angstroms from the beta carbon atom of S95 (relative to the S95 position as set forth in SEQ ID NO:2); [2] another sphere has an atomic radius of from about 9.2 to about 12.2 Angstroms from the beta carbon atom of P219 (relative to the P219 position as set forth in SEQ ID NO:2). Changes to the amino acids within the ribbon structure that fall within these spheres are result effective in causing increased toxic properties and broader host range toxic effects compared to a protein having a naturally occurring amino acid at that particular position.

[0017] Figure 3 is a chart view illustrating the population mortality of *Lygus* species for thirteen different eHTP's compared to each other and to the naturally occurring TIC807 protein.

DETAILED DESCRIPTION

[0018] This application describes eHTP's (engineered Hemipteran species toxic proteins). The eHTP's of the present invention are to be distinguished from proteins such as TIC807, TIC853, Cry51Aa1 and AXMI-171, which are known in the art and are not to be considered to be within the scope or definition of the term eHTP, as the prior art proteins are not engineered to exhibit improved toxic properties directed to one or more Hemipteran pest species and do not exhibit broad host range levels of inhibitory activity. eHTP's surprisingly and unexpectedly exhibit high levels of toxic activity against Hemipteran and related pest species. An additional feature of these eHTP's that is even more unexpected and surprising is the finding that these proteins exhibit broader host range toxic properties compared to

progenitor proteins which provide the foundational basis for the eHTP's of the present invention. The foundational or baseline scaffold toxin proteins, such as TIC807 (SEQ ID NO:2), Cry51Aa1 (SEQ ID NO:8), TIC853 (SEQ ID NO:184), and AXMI-171 (SEQ ID NO:206) do not exhibit the breadth and scope of biological anti-Hemipteran activity or host range of the eHTP proteins of the present invention.

[0019] More than 2000 different amino acid sequence variants of Hemipteran toxic proteins derived from *Bacillus thuringiensis* species were tested to identify the specific amino acid insertions, substitutions, or deletions described herein which confer expanded Hemipteran species host range inhibitory spectrum and also provide dramatically increased Hemipteran species inhibitory activity when compared to the spectrum and activity of the baseline scaffold protein, TIC807, TIC853, and Cry51Aa1. Amino acid residues are identified in the baseline scaffold proteins that (a) can be modified to yield enhanced Hemipteran inhibitory spectrum and/or improved *Lygus* inhibitory activity relative to one or more of the scaffold proteins, (b) accumulate in surface patches of a folded insect inhibitory protein exhibiting the fold structure of one or more of the scaffold proteins, and /or (c) occur in specific positions of one or more of the scaffold protein amino acid sequence that are result effective in decreasing the resulting eHTP proteins' mean effective dose for controlling a Hemipteran species and broadening the range of Hemipteran species that are affected by the eHTP protein.

[0020] The Hemipteran pest species are intended to mean insects that feed upon plants and plant tissues by slashing or piercing the outer surface of the target plant, and then consume macerated plant exudates pooling in the slash or pierce location by sucking or wicking the pooled exudates. Such insects include adults and nymphs, including but not limited to the following listing of plant bugs: the Family Miridae, cicadas from the Family Cicadidae, leafhoppers (*e. g.*, *Empoasca spp.*, *Amrasca spp.*) from the Family Cicadellidae, planthoppers from the families Fulgoroidea and Delphacidae, treehoppers from the Family Membracidae, psyllids from the Family Psyllidae, whiteflies from the Family Aleyrodidae, aphids from the Family Aphididae, phylloxera from the Family Phylloxeridae, mealybugs from the Family Pseudococcidae, scales from the families Coccidae, Diaspididae and Margarodidae, lace bugs from the Family Tingidae, stink bugs from the Family Pentatomidae, cinch bugs (*e. g.*, *Blissus spp.*) and other seed bugs from the Family Lygaeidae, spittlebugs from the Family Cercopidae squash bugs from the Family Coreidae, and red bugs and cotton stainers from the Family Pyrrhocoridae. Other pests from the order *Hemiptera* include *Acrosternum hilare* (green stink bug), *Anasa tristis* (squash bug), *Blissus leucopterus leucopterus* (chinch bug), *Corythuca gossypii* (cotton lace bug), *Cyrtopeltis modesta* (tomato bug), *Dysdercus*

suturellus (cotton stainer), *Euschistus servus* (brown stink bug), *Euschistus variolarius* (one-spotted stink bug), *Graptostethus spp.* (complex of seed bugs), *Leptoglossus corculus* (leaf-footed pine seed bug), *Lygus lineolaris* (tarnished plant bug), *Lygus hesperus* (Western tarnish plant bug), *Nezara viridula* (southern green stink bug), *Oebalus pugnax* (rice stink bug), *Oncopeltus fasciatus* (large milkweed bug), and *Pseudatomoscelis seriatus* (cotton fleahopper). More specifically, the Family Cicadellidae includes, but is not limited to the tribe Empoascini, e.g. *Amrasca biguttula*, *Amrasca devastans*, *Austroasca viridigrisea*, *Asymmetrasca decedens*, *Empoasca decipiens*, *Empoasca distinguenda*, *Empoasca dolichi*, *Empoasca fabae*, *Empoasca kerri*, *Empoasca kraemeri*, *Empoasca onukii*, *Empoasca sakaii*, *Empoasca smithi*, *Empoasca vitis*, *Jacobiasca lybica*, *Sonasca Solana*, tribe Erythroneurini, e.g. *Empoasca nagpurensis*, *Thaiaassamensis*, *Zygnidia quyumi*, tribe Nirvaniae, e.g. *Sophonia rufofascia*, Family Delphacidae, e.g. *Nilapoarvata lugens*, *Sogatella furcifera*, *Unkanodes sapporonus*, and Family Lophopidae, e.g. *Zophiuma lobulata*.

[0021] eHTP's of the present invention contain one or more amino acid sequence modifications compared to one or more of the scaffold proteins, including substitutions and deletions, of amino acid residues at seventy-two (72) different amino acid positions. Such modifications provide eHTP's with increased toxicity and/or an enhanced inhibitory spectrum against Hemipteran insects when compared to one or more of the scaffold proteins which include but are not limited to TIC807 (SEQ ID NO:2), or related protein such as TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), and TIC853 (SEQ ID NO:184). eHTP's include, but are not limited to, modifications of at least one amino acid substitution or one amino acid deletion at any of these seventy-two positions, described as "X" in the amino acid sequence set forth as SEQ ID NO:180 but do not include the amino acid sequences of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184. eHTP's of the present invention also exhibit enhanced Hemipteran inhibitory spectrum and/or improved Hemipteran inhibitory activity when compared to the spectrum and activity of the baseline or scaffold proteins.

[0022] eHTP's include at least one amino acid modification of the relative positions of TIC807 (SEQ ID NO:2) as set forth above in paragraph [0009]. eHTP's can also include at least two, three, four, or more of these aforementioned amino acid substitutions and/or deletions and can also include at least two, three, four, or more of these amino acid substitutions and/or deletions as well as a deletion of any three contiguous amino acids within residues 196-201 of SEQ ID NO:2. Accordingly, eHTP's include proteins set forth as SEQ

ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77, SEQ ID NO:78, SEQ ID NO:79, SEQ ID NO:80, SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, SEQ ID NO:84, SEQ ID NO:85, SEQ ID NO:86, SEQ ID NO:87, SEQ ID NO:88, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91, SEQ ID NO:92, SEQ ID NO:93, SEQ ID NO:94, SEQ ID NO:95, SEQ ID NO:96, SEQ ID NO:97, SEQ ID NO:98, SEQ ID NO:99, SEQ ID NO:100, SEQ ID NO:101, SEQ ID NO:102, SEQ ID NO:103, SEQ ID NO:104, SEQ ID NO:105, SEQ ID NO:106, SEQ ID NO:107, SEQ ID NO:108, SEQ ID NO:109, SEQ ID NO:110, SEQ ID NO:111, SEQ ID NO:112, SEQ ID NO:113, SEQ ID NO:114, SEQ ID NO:115, SEQ ID NO:116, SEQ ID NO:117, SEQ ID NO:118, SEQ ID NO:119, SEQ ID NO:120, SEQ ID NO:121, SEQ ID NO:122, SEQ ID NO:123, SEQ ID NO:124, SEQ ID NO:125, SEQ ID NO:126, SEQ ID NO:127, SEQ ID NO:128, SEQ ID NO:129, SEQ ID NO:130, SEQ ID NO:131, SEQ ID NO:132, SEQ ID NO:133, SEQ ID NO:134, SEQ ID NO:135, SEQ ID NO:136, SEQ ID NO:137, SEQ ID NO:138, SEQ ID NO:139, SEQ ID NO:140, SEQ ID NO:141, SEQ ID NO:142, SEQ ID NO:143, SEQ ID NO:144, SEQ ID NO:145, SEQ ID NO:146, SEQ ID NO:147, SEQ ID NO:148, SEQ ID NO:149, SEQ ID NO:150, SEQ ID NO:151, SEQ ID NO:152, SEQ ID NO:153, SEQ ID NO:154, SEQ ID NO:155, SEQ ID NO:156, SEQ ID NO:157, SEQ ID NO:158, SEQ ID NO:159, SEQ ID NO:160, SEQ ID NO:161, SEQ ID NO:162, SEQ ID NO:163, SEQ ID NO:164, SEQ ID NO:165, SEQ ID NO:166, SEQ ID NO:167, SEQ ID NO:168, SEQ ID NO:169, SEQ ID NO:170, SEQ ID NO:171, SEQ ID NO:172, SEQ ID NO:173, SEQ ID NO:174, SEQ ID NO:175, SEQ ID NO:176, SEQ ID NO:177, SEQ ID NO:178, SEQ ID NO:179, SEQ ID NO:202, and SEQ ID NO:204, and insect inhibitory fragments thereof.

[0023] eHTP's of the present invention exhibit any amino acid sequence different from any one or more of the scaffold proteins, including SEQ ID NO:2 (TIC807), in at least one amino acid position where the different amino acid residue either (i) has a relative amino acid

solvent-accessibility of at least from about 15% to at least about 36% compared to the same residue positions in any one or more of the scaffold proteins; and/or (ii) is located within a distance of about 3 consecutive amino acid residues from an amino acid having at least from about 15% to at least about 36% relative solvent-accessibility compared to the corresponding amino acid residue positions in the primary amino acid sequence of one or more of the scaffold proteins, and exhibits broadened Hemipteran inhibitory spectrum and/or increased Hemipteran inhibitory activity when compared to the activity correlated with one or more of the scaffold proteins. The words “increased spectrum” are intended to mean, with reference to two different proteins exhibiting toxic effects to a particular single pest, the protein exhibiting increased spectrum exhibits toxic effects to that particular single pest as well as to one or more other pests within the same phylogenetic order or to one or more other pests in one or more different phylogenetic orders other than the order to which the particular single pest belongs. The words “increased Hemipteran inhibitory activity” are intended to mean that a particular protein exhibiting such increased activity requires, under standardized conditions, a lower amount of that protein to achieve a particular effect, such as mortality, stunting, morbidity, cessation of feeding, or another measureable phenotypic effect upon a particular single pest, than a control protein.

[0024] eHTP's exhibit an amino acid sequence that differs from one or more of the scaffold proteins, including particularly TIC807, in at least one amino acid residue located within at least one of the two different surface patches of a folded insect inhibitory protein (see Figure 2 and Table 3 data). One surface patch is defined as including the amino acid residues encompassed within a sphere having an atomic radius of from about 9.2 to about 12.2 Angstroms (Figure 2, sphere [1]) relative to the beta-carbon (Cb) atom of Ser95 as set forth in SEQ ID NO:2 when that protein is folded into a three dimensional structure under physiological conditions; which includes residues Thr93, Ser95, Ser97, Phe147, Gln149, Ser151, Asn180, Thr182, Val251, Gln253, and Ser255. As used herein, the phrase “Cb atom” refers to the beta-carbon atom in the amino acid residue side chain. The Cb atom is thus the first carbon in the protein side chain that is present in all amino acid residues with the exception of Glycyl residues. With reference to Figure 1, eHTP's can include, but are not limited to, one or more conservative or non-conservative substitutions of surface patch [1] amino acid residues T93, S95, S97, F147, Q149, S151, N180, T182, V251, Q253, and S255 or the equivalent amino acids within one or more of the scaffold proteins, particularly SEQ ID NO:2 (TIC807). eHTP's can include, but are not limited to, one or more substitutions of surface patch [1] amino acid residues such as: T93A; S95A, S95V, S95L, or S95I; F147T,

F147C, F147D, F147G, F147E, F147Y, F147M, F147N, F147Q, F147H, F147R, F147W, F147P, F147A, F147V, F147L, or F147I; Q149A, Q149C, Q149F, Q149E or Q149D; S151A; N180D; T182A; V251E or V251A, and/or Q253R. The other or second surface patch that has been identified as amino acid residues that are receptive to modifications which are result effective in conferring improved Hemipteran inhibitory bioactivity in the form of eHTP's of the present invention is defined as including the amino acid residues encompassed within a sphere having an atomic radius of from about 9.2 to about 12.2 Angstroms (Figure 2, sphere [2]) relative to the beta-carbon atom of Pro219 or the equivalent amino acid position in one or more of the scaffold proteins, particularly as set forth in SEQ ID NO:2, when any one of the applicable scaffold proteins is folded into a three dimensional structure under physiological conditions, which includes residues Val10, Ile14, Asn22, Asn23, Gly24, Ile25, Gln26, Gly27, Phe30, Gln38, Ile39, Asp40, Thr41, Ile43, Ser193, Thr194, Glu195, His196, Tyr197, Ser198, His199, Tyr200, Ser201, Gly202, Tyr203, Pro204, Ile205, Leu206, Thr207, Trp208, Ile209, Ser210, Tyr216, Ser217, Gly218, Pro219, Pro220, Met221, Ser222, Trp223, Tyr224, Phe225, Asn239, and Val244. Such eHTP's can include, but are not limited to, one or more conservative or non-conservative amino acid residues substitutions and/or one or more amino acid deletions within surface patch [2] including Val10, Ile14, Asn22, Asn23, Gly24, Ile25, Gln26, Gly27, Phe30, Gln38, Ile39, Asp40, Thr41, Ile43, Ser193, Thr194, Glu195, His196, Tyr197, Ser198, His199, Tyr200, Ser201, Gly202, Tyr203, Pro204, Ile205, Leu206, Thr207, Trp208, Ile209, Ser210, Tyr216, Ser217, Gly218, Pro219, Pro220, Met221, Ser222, Trp223, Tyr224, Phe225, Asn239, and Val244 of SEQ ID NO:2 (TIC807). eHTP's can include, but are not limited to, one or more substitutions and/or deletions within the amino acid residues located within surface patch [2] such as: a deletion of any three contiguous amino acid residues in the sequence His196, Tyr197, Ser198, His199, Tyr200, Ser201; Ser217Asn, Ser217Gln, Ser217Arg; and/or Pro219Arg, Pro219Asn, Pro219Gln. eHTP's can include, but are not limited to, one or more amino acid residue substitutions and/or deletions within surface patch [2] such as: a deletion of any three contiguous HisTyrSer residues in the sequence His196, Tyr197, Ser198, His199, Tyr200, Ser201; Ser217Asn, Ser217Gln, Ser217Arg; and/or Pro219Arg, Pro219Asn, Pro219Gln. An eHTP can have at least one amino acid modification in each of the two aforementioned surface patches of the folded insect inhibitory protein. eHTP can have one, or a combination of more than one modification at residues T93, S95, F147, Q149, S151, N180, T182, H196, Y197, S198, H199, Y200, S201, W208, S217, P219, W223, N239, V244, or V251 relative to SEQ ID NO:2 (TIC807). Conservative amino acid changes can be made by substituting an acidic,

basic, neutral polar, or neutral non-polar –type amino acid with another amino acid of the same type. Non-conservative amino acid changes can be made by substituting an acidic, basic, neutral polar, or neutral non-polar amino acid-type with an amino acid of a different type. Furthermore, of the eHTP proteins listed in Table 4B, all 267 are amino acid sequence variants that exhibit increased toxicity to *Lygus* spp. when compared to one or more of the scaffold proteins, including scaffold protein TIC807. Only ten of these amino acid sequence variants exhibit modified amino acid residues compared to one or more of the scaffold proteins that are positioned outside of the two referenced surface patches.

[0025] The prior art teaches solubility problems associated with the scaffold proteins. eHTP's exhibit improved solubility compared to the scaffold proteins, and generally exhibit increased solubility at a pH of less than 9.0, in contrast to the observed solubility profile of one or more of the scaffold proteins. This increased solubility at more physiological pH is evident when the eHTP is expressed in *E. coli*, in a plant cell, in a plant cell cytoplasm, a plant cell apoplast, or in or targeted for import into a plastid of a plant cell. Amino acid modifications that improve solubility relative to one or more of the scaffold proteins, including SEQ ID NO:2 (TIC807) include but are not limited to, substitution of a lysine amino acid residue at one or more of the following amino acid positions in TIC807 or the applicable residue in any of the other scaffold proteins: 58, 59, 198, 199, 201, or 202; or, substitution of a glutamic acid amino acid residue at one or more of amino acid positions 198, 248 or 301; or, substitution of a arginine amino acid residue at one or more of amino acid positions 246, 250 or 253.

[0026] Insect inhibitory compositions comprising the above described eHTP's are also provided. Such compositions may further comprise at least one additional insect inhibitory agent different from the eHTP included in the composition. The insect inhibitory agent is selected from any number of insect inhibitory agents including an insect inhibitory protein, an insect inhibitory dsRNA molecule, and one or more chemical agents useful in controlling insect pests. Examples of additional inhibitory agents includes, but are not limited to, a TIC1415 protein, a dsRNA directed towards Hemipteran orthologs of *Nilaparvata lugens* V-ATPase-E, 21E01, a dsRNA directed towards Hemipteran orthologs of actin ortholog, ADP/ATP translocase, α -tubulin, ribosomal protein L9 (RPL9) or V-ATPase A subunit, AXMI-171 (US20100298207A1), Cry3A, Cry4Aa, Cry11Aa, and Cyt1Aa, DIG11, DIG5, Cry7, eCry3.1Ab, mCry3A, Cry8, Cry34/Cry35, Cry3, DIG2, Cry1, Cry1A.105, Cry2, Cry1F, VIP3, 5307, and Cry9. Chemical agents useful in controlling Hemipteran species include but are not limited to pyrethrins and synthetic pyrethroids; oxadiazine derivatives;

chloronicotinyis; nitroguanidine derivatives; triazoles; organophosphates; pyrrols; pyrazoles; phenyl pyrazoles; diacylhydrazines; biological/fermentation products; and carbamates. Known pesticides within these categories are listed in The Pesticide Manual, 11th Ed., C. D. S. Tomlin, Ed., British Crop Protection Council, Farnham, Surrey, UK (1997).

[0027] Pyrethroids that are useful in the present composition include pyrethrins and synthetic pyrethroids. The pyrethrins that are preferred for use in the present method include, without limitation, 2-allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one ester of 2,2-dimethyl-3-(2methyl propenyl)-cyclopropane carboxylic acid, and/or (2-methyl-1-propenyl)-2-methoxy-4-oxo-3-(2 propenyl)-2-cyclopenten-1-yl ester and mixtures of cis and trans isomers thereof (Chemical Abstracts Service Registry Number ("CAS RN") 8003-34-7).

[0028] Synthetic pyrethroids that are preferred for use in the present invention include (s)-cyano(3-phenoxyphenyl)methyl 4-chloro alpha (1-methylethyl)benzeneacetate (fenvalerate, CAS RN 51630-58-1), (S)-cyano (3-phenoxyphenyl) methyl (S)-4-chloro-alpha-(1-methylethyl) benzeneacetate (esfenvalerate, CAS RN 66230-04-4), (3-phenoxyphenyl)-methyl(+)-cis-trans-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate (permethrin, CAS RN 52645-53-1), (±) alpha-cyano-(3-phenoxyphenyl) methyl(+)-cis,trans-3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropane carboxylate (cypermethrin, CAS RN 52315-07-8), (beta-cypermethrin, CAS RN 65731-84-2), (theta cypermethrin, CAS RN 71697-59-1), S-cyano (3-phenoxyphenyl) methyl (±) cis/trans 3-(2,2-dichloroethenyl) 2,2 dimethylcyclopropane carboxylate (zeta-cypermethrin, CAS RN 52315-07-8), (s)-alpha-cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethyl cyclopropanecarboxylate (deltamethrin, CAS RN 52918-63-5), alpha-cyano-3-phenoxybenzyl 2,2,3,3-tetramethyl cyclopropoanecarboxylate (fenpropathrin, CAS RN 64257-84-7), (RS)-alpha-cyano-3-phenoxybenzyl(R)-2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate (tau-fluvalinate, CAS RN 102851-06-9), (2,3,5,6-tetrafluoro-4-methylphenyl)-methyl-(1 alpha, 3 alpha)-(Z)-(±)-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate (tefluthrin, CAS RN 79538-32-2), (±)-cyano (3-phenoxyphenyl) methyl (±)-4-(difluoromethoxy)-alpha-(1-methyl ethyl) benzeneacetate (flucythrinate, CAS RN 70124-77-5), cyano(4-fluoro-3-phenoxyphenyl)methyl 3-[2-chloro-2-(4-chlorophenyl)ethenyl]-2,2-dimethylcyclopropanecarboxylate (flumethrin, CAS RN 69770-45-2), cyano(4-fluoro-3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate (cyfluthrin, CAS RN 68359-37-5), (beta cyfluthrin, CAS RN 68359-37-5), (transfluthrin, CAS RN 118712-89-3), (S)-alpha-cyano-3-phenoxybenzyl(Z)-(1R-cis)-2,2-dimethyl-3-[2-(2,2,2-trifluoro-trifluoromethyl)-

ethoxycarbonyl)vinyl]cyclopropane carboxylate (acrinathrin, CAS RN 101007-06-1), (IR cis) S and (IS cis) R enantiomer isomer pair of alpha-cyano-3-phenoxybenzyl-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate (alpha-cypermethrin, CAS RN 67375-30-8), [IR,3S)3(1'RS)(1',2',2',2'-tetrabromoethyl)]-2,2-dimethyl cyclopropanecarboxylic acid (s)-alpha-cyano-3-phenoxybenzyl ester (tralomethrin, CAS RN 66841-25-6), cyano-(3-phenoxyphenyl) methyl 2,2-dichloro-1-(4-ethoxyphenyl)cyclopropane carboxylate (cycloprothrin, CAS RN 63935-38-6), [1 α , 3 α (Z)]-($\pm$)-cyano-(3-phenoxyphenyl)methyl 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate (cyhalothrin, CAS RN 68085-85-8), [1 α (s), 3 α (z)]-cyano(3-phenoxyphenyl) methyl-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropane carboxylate (lambda cyhalothrin, CAS RN 91465-08-6), (2-methyl [1,1'-biphenyl]-3-yl) methyl 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyclopropanecarboxylate (bifenthrin, CAS RN 82657-04-3), 5-1-benzyl-3-furylmethyl-d-cis(1R,3S,E)2,2-dimethyl-3-(2-oxo,-2,2,4,5 tetrahydro thiophenylidenemethyl)cyclopropane carboxylate (kadethrin, RU15525, CAS RN 58769-20-3), [5-(phenyl methyl)-3-furanyl]-3-furanyl 2,2-dimethyl-3-(2-methyl-1-propenyl) cyclopropane carboxylate (resmethrin, CAS RN 10453-86-8), (1R-trans)-[5-(phenylmethyl)-3-furanyl]methyl 2,2-dimethyl-3-(2-methyl-1-propenyl)cyclopropanecarboxylate (bioresmethrin, CAS RN 28434-01-7), 3,4,5,6-tetra hydro-phthalimidomethyl-(IRS)-cis-trans-chrysanthemate (tetramethrin, CAS RN 7696-12-0), 3-phenoxybenzyl-d,l-cis,trans 2,2-dimethyl-3-(2-methylpropenyl) cyclopropane carboxylate (phenothrin, CAS RN 26002-80-2); (empenthrin, CAS RN 54406-48-3); (cyphenothrin; CAS RN 39515-40-7), (prallethrin, CAS RN 23031-36-9), (imiprothrin, CAS RN 72963-72-5), (RS)-3-allyl-2-methyl-4-oxocyclopent-2-enyl-(1A,3R; 1R,3S)-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropane carboxylate (allethrin, CAS RN 584-79-2), (bioallethrin, CAS RN 584-79-2), and (ZXI8901, CAS RN 160791-64-0). It is believed that mixtures of one or more of the aforementioned synthetic pyrethroids can also be used in the present invention. Particularly preferred synthetic pyrethroids are tefluthrin, lambda cyhalothrin, bifenthrin, permethrin and cyfluthrin. Even more preferred synthetic pyrethroids are tefluthrin and lambda cyhalothrin, and yet more preferred is tefluthrin.

[0029] Insecticides that are oxadiazine derivatives are useful in the subject invention. The oxadiazine derivatives that are preferred for use in the present invention are those that are identified in U.S. Patent No. 5,852,012. More preferred oxadiazine derivatives are 5-(2-chloropyrid-5-ylmethyl)-3-methyl-4-nitroiminoperhydro-1,3,5-oxadiazine, 5-(2-

chlorothiazol-5-ylmethyl)-3-methyl-4-nitroiminoperhydro-1,3,5-oxadiazine, 3-methyl-4-nitroimino-5-(1-oxido-3-pyridinomethyl) perhydro-1,3,5-oxadiazine, 5-(2-chloro-1-oxido-5-pyridiniomethyl)-3-methyl-4-nitroiminoperhydro-1,3,5-oxidiazine; and 3-methyl-5-(2-methylpyrid-5-ylmethyl)-4-nitroiminoperhydro-1,3,5-oxadiazine. Even more preferred is thiamethoxam (CAS RN 153719-23-4).

[0030] Chloronicotinyl insecticides are also useful in the subject invention. Chloronicotinyls that are preferred for use in the subject composition are described in U.S. Patent No. 5,952,358, and include acetamiprid ((E)-N-[(6-chloro-3-pyridinyl)methyl]-N'-cyano-N-methyleneimidamide, CAS RN 135410-20-7), imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimide, CAS RN 138261-41-3), and nitenpyram (N-[(6-chloro-3-pyridinyl)methyl]-N-ethyl-N'-methyl-2-nitro-1,1-ethenediamine, CAS RN 120738-89-8).

[0031] Nitroguanidine insecticides are useful in the present invention. Such nitroguanidines can include those described in U.S. Patent Nos. 5,633,375, 5,034,404 and 5,245,040.

[0032] Pyrrols, pyrazoles and phenyl pyrazoles that are useful in the present invention include those that are described in U.S. Patent 5,952,358. Preferred pyrazoles include chlorfenapyr (4-bromo-2-(4-chlorophenyl)-1-ethoxymethyl-5-trifluoromethylpyrrole-3-carbonitrile, CAS RN 122453-73-0), fenpyroximate ((E)-1,1-dimethylethyl-4[[[(1,3-dimethyl-5-phenoxy-1H-pyrazole-4-yl)methylene]amino]oxy]methyl]benzoate, CAS RN 111812-58-9), and tebufenpyrad (4-chloro-N[[4-1,1-dimethylethyl]phenyl]methyl]-3-ethyl-1-methyl-1H-pyrazole-5-carboxamide, CAS RN 119168-77-3). A preferred phenyl pyrazole is fipronil (5-amino-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(1R,S)-(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile, CAS RN 120068-37-3).

[0033] Diacylhydrazines that are useful in the present invention include halofenozide (4-chlorobenzoate-2-benzoyl-2-(1,1-dimethylethyl)-hydrazide, CAS RN 112226-61-6), methoxyfenozide (RH-2485; N-tert-butyl-N'-(3-methoxy-o-toluoyl)-3,5-xylohydrazide, CAS RN 161050-58-4), and tebufenozide (3,5-dimethylbenzoic acid 1-(1,1-dimethylethyl)-2,(4-ethylbenzoyl)hydrazide, CAS RN 112410-23-8).

[0034] Triazoles, such as amitrole (CAS RN 61-82-5) and triazamate are useful in the method of the present invention. A preferred triazole is triazamate (ethyl [[1-[(dimethylamino)carbonyl]-3-(1,1-dimethylethyl)-1H-1,2,4-triazol-5-yl]thio]acetate, CAS RN 112143-82-5).

[0035] Biological/fermentation products, such as avermectin (abamectin, CAS RN 71751-41-2) and spinosad (XDE-105, CAS RN 131929-60-7) are useful in the present invention.

[0036] Organophosphate insecticides are also useful as one of the components of the present invention. Preferred organophosphate insecticides include acephate (CAS RN 30560-19-1), chlorpyrifos (CAS RN 2921-88-2), chlorpyrifos-methyl (CAS RN 5598-13-0), diazinon (CAS RN 333-41-5), fenamiphos (CAS RN 22224-92-6), and malathion (CAS RN 121-75-5). [0037] In addition, carbamate insecticides are useful in the subject invention. Preferred carbamate insecticides are aldicarb (CAS RN 116-06-3), carbaryl (CAS RN 63-25-2), carbofuran (CAS RN 1563-66-2), oxamyl (CAS RN 23135-22-0) and thiodicarb (CAS RN 59669-26-0).

[0038] When a chemical insecticide is described herein, it is to be understood that the description is intended to include salt forms of the insecticide as well as any isomeric and/or tautomeric form of the insecticide that exhibits the same insecticidal activity as the form of the insecticide that is described.

[0039] The chemical insecticides that are useful in the present invention can be of any grade or purity that pass in the trade as such insecticide. Other materials that accompany the insecticides in commercial preparations as impurities can be tolerated in the subject invention and compositions, as long as such other materials do not destabilize the composition or significantly reduce or destroy the activity of any of the insecticide components or the transgenic event against the target pest(s). One of ordinary skill in the art of the production of insecticides can readily identify those impurities that can be tolerated and those that cannot.

[0040] eHTP's are related by amino acid modifications such that the modified proteins exhibit enhanced Hemipteran inhibitory spectrum and/or improved Hemipteran inhibitory activity against *Lygus* spp., *Empoasca* spp. and/or *Amrasca* spp. compared to the parent protein, TIC807. The phrases "more active", "improved activity", "enhanced specificity", "increased toxic potency", "increased toxicity", "improved Hemipteran inhibitory activity", "enhanced Hemipteran inhibitory activity", "improved *Lygus*, *Empoasca* and/or *Amrasca* inhibitory activity", "greater *Lygus*, *Empoasca* and/or *Amrasca* inhibitory activity", "greater Hemipteran inhibitory activity" and "enhanced *Lygus*, *Empoasca* and/or *Amrasca* inhibitory spectrum" and "enhanced Hemipteran inhibitory spectrum" refer to a comparison of the activity of an eHTP and of the activity of a TIC807 (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and/or a AXMI-171 (SEQ ID NO:206) protein against a Hemipteran insect, wherein activity attributed by the eHTP of the present invention is greater than the activity attributed to the TIC807 protein (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID

NO:184, and/or a AXMI-171 (SEQ ID NO:206) protein. eHTP's provided herein exhibit enhanced Hemipteran inhibitory spectrum and/or improved or greater Hemipteran inhibitory activity when compared to the *Bacillus thuringiensis* proteins of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, and SEQ ID NO:184, where the Hemipteran pest species include *Lygus hesperus*, *Lygus lineolaris*, *Empoasca fabae*, and *Amrasca devastans*. *Amrasca devastans* is also called *Amrasca biguttula biguttula*. eHTP's exhibiting enhanced insect inhibitory spectrum and/or improved insect inhibitory activity compared to TIC807 can be identified by many different methods. In general, exemplary and non-limiting methods for identifying eHTP proteins can comprise :

- (1) administering identical amounts of a test eHTP and of control TIC807 (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and/or an AXMI-171 (SEQ ID NO:206) protein to a test insect under controlled assay conditions; and, measuring and comparing the potency of the test and control proteins; and/or,
- (2) determining the protein doses (e.g., protein concentration in diet) of a test eHTP and of control TIC807 (SEQ-ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and/or an AXMI-171 (SEQ ID NO:206) protein which elicit equivalent insect population responses under controlled assay conditions (i.e. obtaining a dose response curve).

In the second approach, a statistically robust dose response value used for comparison would be the median lethal concentration (LC50) required to kill 50% of a test population. However, in certain embodiments, other values including but not limited to, a median inhibitory concentration ("IC50") required to result in 50% growth inhibition of a test population can be used. In this context, "growth inhibition" can comprise stunting and/or inhibition of Hemipteran development.

[0028] As used herein, the phrase "an insect inhibitory amount", refers to an amount of a composition containing an agent that is effective in achieving any measurable inhibition of insect viability, growth, insect development, insect reproduction, insect feeding behavior, insect mating behavior and/or any measurable decrease in the adverse effects caused by insect feeding on a composition containing the agent. Similarly, a "Hemipteran inhibitory amount" refers to an amount of a protein of the present invention alone or with other agents targeting the applicable Hemipteran species for control, that results in any measurable inhibition of target insects belonging to the order Hemiptera related to viability, growth,

development, reproduction, feeding behavior, mating behavior, and or any measurable decrease in the adverse effects caused by Hemipteran insects feeding on a plant. Likewise, “*Lygus*, *Empoasca* and/or *Amrasca* inhibitory amount” refers to an amount of a composition containing one or more proteins of the present invention, i.e., eHTP’s, or other agent that results in any measurable inhibition, viability, growth, development, reproduction, feeding behavior, mating behavior and/or any measurable decrease in the adverse effects caused by *Lygus*, *Empoasca* and/or *Amrasca* feeding on a composition containing that eHTP. As used herein in the context of an eHTP, an “enhanced Hemipteran inhibitory activity or “greater enhanced Hemipteran inhibitory activity” refers to any measurable increase in the inhibition of Hemipteran viability, growth, development, reproduction, feeding behavior, mating behavior and/or any measurable decrease in the adverse effects caused by Hemipteran feeding on a composition containing that eHTP relative to the corresponding inhibitory activity observed with any one or more of the scaffold proteins, including TIC807, Cry51Aa1(SEQ ID NO:182), TIC853 (SEQ ID NO:184), and/or AXMI-171 (SEQ ID NO:206) proteins. Likewise, “enhanced *Lygus*, *Empoasca* and/or *Amrasca* inhibitory activity” or “greater enhanced *Lygus*, *Empoasca* and/or *Amrasca* inhibitory activity” refers to any measurable increase in the inhibition, viability, growth, development, reproduction, feeding behavior, mating behavior and/or any measurable decrease in the adverse effects caused by the presence of one or more eHTP of the present invention in a composition or plant provided in the diet of *Lygus*, *Empoasca* and/or *Amrasca* relative to the corresponding inhibitory activity observed with an equivalent composition or plant containing only an applicable amount of one or more of the scaffold proteins, including but not limited to TIC807 (SEQ ID NO:2), Cry51Aa1(SEQ ID NO:182), TIC853 (SEQ ID NO:184), and/or AXMI-171 (SEQ ID NO:206) proteins.

[0029] As used herein in the context of an eHTP, an “enhanced *Lygus*, *Empoasca* and/or *Amrasca* inhibitory spectrum” refers to any measurable increase in the inhibition of a specific *Lygus* spp., *Empoasca* spp. and/or *Amrasca* spp. viability, growth, development, reproduction, feeding behavior, mating behavior and/or any measurable decrease in the adverse effects caused by that *Lygus* spp., *Empoasca* spp. and/or *Amrasca* spp. feeding on a plant relative to the corresponding inhibition of that specific *Lygus* spp., *Empoasca* spp. and/or *Amrasca* spp. observed with the TIC807 protein. In certain embodiments, eHTP provided herein exhibit an enhanced *Lygus* inhibitory spectrum relative to TIC807 in that those eHTP’s can provide increased inhibition of *Lygus lineolaris*.

[0030] An eHTP provided herein can exhibit from about 2 to about 260 fold greater *Lygus*, *Empoasca* and/or *Amrasca* inhibitory activity against a *Lygus*, *Empoasca* and/or *Amrasca* pest species than a protein of SEQ ID NO:2 (TIC807), SEQ ID NO:8 (TIC807_M2), SEQ ID NO:182 (Cry51Aa1), SEQ ID NO:184 (TIC853), and SEQ ID NO:206 (AXMI-171). An eHTP provided herein can exhibit from about 3, 4, 5, 7, 8, 10, 12, 15, 20, 25, 27, 30, 38, 46, 50, 52, 54, 66, 91, 122, 186, 243, or 262 fold greater *Lygus*, *Empoasca* and/or *Amrasca* inhibitory activity against a *Lygus*, *Empoasca* and/or *Amrasca* pest species than a protein of SEQ ID NO:2 (TIC807), SEQ ID NO:8 (TIC807_M2), SEQ ID NO:182 (Cry51Aa1), SEQ ID NO:184 (TIC853), and SEQ ID NO:206 (AXMI-171).

[0031] eHTP's can exhibit an enhanced target pest inhibitory spectrum and/or improved target pest inhibitory activity over SEQ ID NO:2 (TIC807), SEQ ID NO:8 (TIC807_M2), SEQ ID NO:182 (Cry51Aa1), SEQ ID NO:184 (TIC853), and/or a SEQ ID NO:206 (AXMI-171) by causing mortality:

- (i) at a dose of about 0.3 µg/mL to about 70 µg/mL against a *Lygus hesperus* insect species,
- (ii) at a dose of about 0.85 µg/mL to about 100 µg/mL against a *Lygus lineolaris* insect species,
- (iii) measuring at an LC50 value of about 0.3 to about 70 µg/mL against *Lygus hesperus*,
- (iv) measuring at an LC50 value of about 0.85 to about 100 µg/mL against *Lygus lineolaris*, or
- (v) measuring at an LC50 value of more than two-fold lower the LC50 value of TIC807, SEQ ID NO:8, SEQ ID NO:182 (Cry51Aa1), SEQ ID NO:184 (TIC853), and/or a SEQ ID NO:206 (AXMI-171) against *Lygus* spp, *Emrasca* spp. and/or *Amrasca* spp., or
- (vi) at a dose of about 0.69 µg/mL to about 500 µg/mL against a *Amrasca devastans* or *Empoasca fabae* insect species, or
- (vii) measuring at an LC50 value of about 3.5 to about 15 µg/mL against *Amrasca devastans* and/or *Empoasca fabae*.

[0032] Table 4A and 4B tabulate the exemplary eHTP's of the present invention with *Amrasca* and *Lygus* spp. mortality data. Mortality data available for *Lygus* spp. and *Amrasca* spp. are reported either as (a) a µg/mL LC50 value, or as (b) a % mortality at doses of about 1 to about 3 µg/mL for *L. hesperus* or about 100 µg/mL protein for *L. lineolaris*, and about 0.69

to 500 µg/mL for *Amrasca devastans*. The fold increased toxicity compared to TIC807 (SEQ ID NO:2) and TIC807_M2 (SEQ ID NO:8) is provided for exemplary eHTP's where LC50 values were determined.

[0033] The eHTP's of the present invention are particularly useful in controlling insects of the order Hemiptera compared to the scaffold proteins. *Lygus lineolaris* required high doses of TIC807 protein (e.g., in excess of 100 µg/mL) to elicit mortality. The dose response curve for one eHTP of the present invention TIC807_M8 (SEQ ID NO:16), an eHTP that exhibits remarkably improved toxic effects against both *L. lineolaris* and *L. hesperus*, but against *L. lineolaris* the eHTP exhibits a calculated LC50 value of 223 µg/mL. It has not been possible previously to achieve a protein concentration toxic dose that can elicit greater than 50% mortality against *L. lineolaris* species because providing significantly large doses of TIC807 and TIC807_M2 protein in excess of 1000 µg/mL in the diet has not been possible. Therefore, LC50 values against *L. lineolaris* for TIC807 and TIC807_M2 (SEQ ID NO:8) proteins were not determined, but rather estimated as greater than (>) 223 µg/mL (See Tables 1 and 3, Example 4, and Figure 1B).

[0034] Iterative design refers to a semi-random approach for developing and selecting eHTP's including a combination of engineering, testing, and selecting (not necessarily in that order) (see Examples 1 through 4). The word "engineering" is intended to include identifying relevant residues to modify, cloning, and expressing eHTP's described herein. The word "testing" is intended to refer to comparing the *Hemipteran* activity of an eHTP to the activity of a scaffold protein such as TIC807 (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), Cry51Aa1 (SEQ ID NO:182), and/or TIC853 (SEQ ID NO:184); or, comparing an eHTP of the present invention against another protein such as AXMI-171 (SEQ ID NO:206). The word "selecting" is intended to refer to the act of identifying improved variant proteins of the present invention, i.e., eHTP's, and the applicable amino acid residues for "engineering".

[0035] Iterative design includes the elucidation of the atomic structure of proteins of the present invention (for example, as set forth in Figure 2) and the use of the atomic structure to guide and complement semi-random approaches of "selecting" amino acid residues to modify for "engineering", and in this case, has included the identification of amino acid residues at loops and at surface exposed regions of a folded insect inhibitory scaffold protein such as TIC807, TIC853, and Cry51Aa1 that can be modified to confer improvements to insect inhibitory spectrum and activity. Such amino acid residues at loops and at surface exposed regions are selected for "engineering". In this case, iterative design has included the

identification of two different regions within the three dimensional structure of the scaffold protein that harbor an accumulation of relevant amino acid residues that, when modified to contain amino acid residues other than those appearing at those positions in the naturally occurring scaffold protein, result in one or more of the eHTP proteins of the present invention.

[0036] Initially the scaffold protein TIC807 (SEQ ID NO:2) used in this process of iterative design, and 267 different eHTP's were discovered that exhibited increased *Lygus* spp. inhibitory activity compared to the scaffold protein TIC807. TIC807_M8 (SEQ ID NO:16) was discovered in early rounds of the design process. Subsequent rounds of iterative engineering-testing-selecting led to the discovery of other eHTP proteins that exhibited yet greater levels of toxicity against *Lygus* species and also exhibited a broader host range of toxic effects when compared to the scaffold protein. Seven variants (eHTP's) exhibited significantly higher levels of increased toxicity against both *Lygus* species (*L. hesperus* and *L. lineolaris*) when compared to TIC807. LC50 values for these seven, and other, eHTP's constructed herein were determined against *Lygus hesperus* and *Lygus lineolaris* species and compared to LC50 values for scaffold proteins, particularly TIC807. The results are shown in Table 1, and Figure 3 is a bar chart showing graphically the results observed as tabulated in Table 1.

Table 1. LC50 values of select eHTP's compared to TIC807

SEQ ID NO:	Toxin	<i>Lygus hesperus</i>		<i>Lygus lineolaris</i>	
		LC50 value ($\mu\text{g/mL}$)	Toxicity (fold increase)	LC50 value ($\mu\text{g/mL}$)	Toxicity (fold increase)
2	TIC807	73	1	>223*	1
6	TIC807_M1	23	3	100	≥ 2
8	TIC807_M2	5.9	12	>223*	~ 1
10	TIC807_M3	2.9	25	ND	-
12	TIC807_M4	2.4	30	ND	-
14	TIC807_M5	1.1	66	ND	-
18	TIC807_M6	1.45	50	ND	-
20	TIC807_M7	1.4	52	ND	-
16	TIC807_M8	0.8	91	223	≥ 1
28	TIC807_M9	9.9	7	8.3	≥ 27
30	TIC807_M10	0.6	122	4.8	≥ 46
32	TIC807_M11	1.35	54	5.9	≥ 38
36	TIC807_M12	0.4	182	1.2	≥ 186
34	TIC807_M13	0.3	243	0.85	≥ 262

ND = Not Determined. LC50 values are determined by presenting 8-10 different protein concentrations to a population of newly hatched *Lygus* nymphs, allowing nymphs to feed for 5 days, and then scoring for mortality over the dose range provided.

* Toxicity, displayed in terms of a multiple of increased activity compared to the level observed against *Lygus hesperus* using the observed LD50 for TIC807 as the baseline value of 1. Significantly large amounts of protein in excess of 1000 $\mu\text{g/mL}$ have not been possible to provide in *Lygus* diet in order to complete the high range of toxicity dose response to *Lygus lineolaris*. Therefore, an LC50 value was not determined for TIC807 or TIC807_M2. Instead, a 4-dose LC50 estimation in the low range was performed verifying that expected LC50 values for TIC807 and TIC807_M2 are greater than 223 $\mu\text{g/mL}$.

[0037] With reference to Table 1, the iterative design process has provided a means for identifying proteins exhibiting improved toxic properties, not only to *Lygus hesperus*, but also to *Lygus lineolaris*.

[0038] Recombinant polynucleotide compositions that encode eHTP's are also provided. In certain embodiments, eHTP's can be expressed with recombinant DNA constructs in which a polynucleotide molecule with the open reading frame encoding the protein is operably linked to elements such as a promoter and any other regulatory element functional for expression in the system for which the construct is intended. For example, plant-functional promoters can be operably linked to an applicable eHTP coding sequence to enable expression of the protein

in plants. Promoters functional in bacteria are also contemplated for use in expression cassettes. Promoters functional in an applicable bacterium, for example, in an *E. coli* or in a *Bacillus thuringiensis* species can be operably linked to the eHTP coding sequences for expression of the applicable protein in the applicable bacterial strain. Other useful elements that can be operably linked to the eHTP coding sequences include, but are not limited to, enhancers, introns, leaders, encoded protein immobilization tags (HIS-tag), encoded sub-cellular translocation peptides (i.e. plastid transit peptides, signal peptides), encoded polypeptide sites for post-translational modifying enzymes, ribosomal binding sites, and segments designed for use as RNAi triggers for suppression of one or more genes either in plants or in a particular target pest species.

[0039] Exemplary recombinant polynucleotide molecules provided herein include, but are not limited to, SEQ ID NO:186, SEQ ID NO:187, SEQ ID NO:188, SEQ ID NO:189, SEQ ID NO:190, SEQ ID NO:191, SEQ ID NO:192, SEQ ID NO:193, SEQ ID NO:194, SEQ ID NO:195, SEQ ID NO:196, SEQ ID NO:197, SEQ ID NO:198, SEQ ID NO:199, SEQ ID NO:200, SEQ ID NO:35, and SEQ ID NO:201. These sequences encode the respective proteins each having the amino acid sequence as set forth in SEQ ID NO:4 (TIC807_4), SEQ ID NO:6 (TIC807_M1), SEQ ID NO:8 (TIC807_M2), SEQ ID NO:10 (TIC807_M3), SEQ ID NO:12 (TIC807_M4), SEQ ID NO:14 (TIC807_M5), SEQ ID NO:16 (TIC807_M8), SEQ ID NO:18 (TIC807_M6), SEQ ID NO:20 (TIC807_M7), SEQ ID NO:22 (TIC807_22), SEQ ID NO:24 (TIC807_24), SEQ ID NO:26 (TIC807_26), SEQ ID NO:28 (TIC807_M9), SEQ ID NO:30 (TIC807_M10), SEQ ID NO:32 (TIC807_M11), SEQ ID NO:36 (TIC807_M12), and SEQ ID NO:34 (TIC807_M13). Because of the redundancy of the genetic code, the codons of a recombinant polynucleotide molecule encoding for proteins of the present invention may be substituted for synonymous codons (also called a silent substitution); and are within the scope of the present invention. Recombinant polynucleotides encoding any of the eHTP's disclosed herein are thus provided.

[0040] A recombinant DNA construct comprising eHTP coding sequences can also further comprise a region of DNA that codes for one or more insect inhibitory agents which can be configured to be co-expressed along with a DNA sequence encoding an applicable eHTP, a protein different from an eHTP, or an insect or plant gene inhibitory dsRNA molecule. A recombinant DNA construct can be assembled so that all agents designed to be expressed from a particular construct are expressed from one promoter or so that separate agents are each under separate promoter control, or some combination thereof. The proteins of this invention can be expressed from a multi-gene expression system in which one or more

proteins are expressed from a common nucleotide segment on which is also contained other open reading frames and/or promoters depending on the type of expression system selected.

[0041] Recombinant polynucleotide or recombinant DNA construct comprising an eHTP encoding sequence can be delivered to host cells by vectors, e.g., a plasmid, baculovirus, artificial chromosome, virion, cosmid, phagemid, phage, or viral vector. Such vectors can be used to achieve stable or transient expression of an eHTP encoding sequence in a host cell; and, if the case may be, subsequent expression to polypeptide. An exogenous recombinant polynucleotide or recombinant DNA construct that comprises an eHTP encoding sequence and that is introduced into a host cell is also referred to herein as a “transgene”.

[0042] Also provided herewith are transgenic bacteria, transgenic plant cells, transgenic plants, and transgenic plant parts that contain any a recombinant polynucleotide (i.e. transgene) that expresses any one or more eHTP encoding sequence. It is intended that “bacterial cell” or “bacterium” can include, but are not limited to, an *Agrobacterium*, a *Bacillus*, an *Escherichia*, a *Salmonella*, a *Pseudomonas*, or a *Rhizobium* cell. It is intended that “plant cell” or “plant” include an alfalfa, almont, banana, barley, bean, beet, broccoli, cabbage, brassica, brinjal, carrot, cassava, castor, cauliflower, celery, chickpea, Chinese cabbage, celery, citrus, coconut, coffee, corn, clover, cotton, a cucurbit, cucumber, Douglas fir, eggplant, eucalyptus, flax, garlic, grape, guar, hops, leek, legumes, lettuce, Loblolly pine, millets, melons, nectarine, nut, oat, okra, olive, onion, ornamental, palm, pasture grass, papaya, pea, peach, peanut, pepper, pigeonpea, pine, potato, poplar, pumpkin, Radiata pine, radish, rapeseed, rice, rootstocks, rye, safflower, shrub, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugar beet, sugarcane, sunflower, sweet corn, sweet gum, sweet potato, switchgrass, tea, tobacco, tomato, tritcale, turf grass, watermelon, and wheat plant cell or plant. In certain embodiments: transgenic plants and transgenic plant parts regenerated from a transgenic plant cell are provided; transgenic plants can be obtained from a transgenic seed; transgenic plant parts can be obtained by cutting, snapping, grinding or otherwise disassociating the part from the plant; the plant part can be a seed, a boll, a leaf, a flower, a stem, a root, or any portion thereof; and a transgenic plant part provided herein is a non-regenerable portion of a transgenic plant part. As used in this context, a “non-regenerable” portion of a transgenic plant part is a portion that can not be induced to form a whole plant or that can not be induced to form a whole plant that is capable of sexual and/or asexual reproduction. A non-regenerable portion of a plant part is a portion of a transgenic pollen, ovule, seed, boll, leaf, flower, stem, or root.

[0043] Also provided herein are methods of making transgenic plants that contain insect or *Lygus* and/or *Amrasca* inhibitory amounts of an eHTP. Such plants can be made by introducing a recombinant polynucleotide that encodes any of the eHTP proteins provided herein into a plant cell, and selecting a plant derived from said plant cell that expresses an insect or *Hemipteran* inhibitory amount of the eHTP's. Plants can be derived from the plant cells by regeneration, seed, pollen, or meristem transformation techniques.

[0044] Transgenic plants and host cells are provided that express an insect or *Hemipteran* inhibitory amount of the eHTP to control an insect or *Hemipteran* infestation. Any of the aforementioned plant species can be used for protecting a plant from insect or *Hemipteran* infestation provided herein as long as the plant is transformed with a polynucleotide construct designed to express the applicable eHTP.

[0045] Additional aspects of the invention include antibodies, kits, methods for detecting polynucleotides that encode eHTP's or distinguishing fragments thereof, or eHTP's or distinguishing fragments thereof, methods for identifying additional insect inhibitory members of the protein genus of the present invention, formulations and methods for controlling insect growth and/or infestation, and methods for providing such control to plants and other recipient hosts. Each composition, construct, cell, plant, formulation, method or kit provides for the industrial application of the proteins of the present invention, for example, by increasing plant productivity through the commercial use of any of these proteins to inhibit insects.

[0046] A plant product, other than a seed or a fruit or vegetable, is intended as a commodity or other products which move through commerce and are derived from a transgenic plant or transgenic plant part, in which the commodity or other products can be tracked through commerce by detecting nucleotide segments, RNA or proteins that corresponding to an eHTP of the present invention and are produced in or maintained in the plant or plant tissue or part from which the commodity or other product has been obtained. Such commodity or other products of commerce include, but are not limited to, plant parts, biomass, oil, meal, sugar, animal feed, flour, flakes, bran, lint, processed seed, and seed. Plant parts include but are not limited to a plant seed, boll, leaf, flower, stem, pollen, or root. In certain embodiments, the plant part is a non-regenerable portion of said seed, boll, leaf, flower, stem, pollen, or root. Cotton and flax plant bolls and non-regenerable portions thereof that contain the eHTP's are also provided.

[0047] Also provided herewith are processed plant products that contain a detectable amount of an eHTP, an insect inhibitory fragment thereof, or any distinguishing portion thereof.

Without seeking to be limited by theory, it is believed that such processed plant products containing a detectable amount of one or more of the eHTP's provided herein can in certain embodiments exhibit reductions in undesirable microorganisms that can be transmitted by Hemiptera and/or reductions in the undesirable side products of such microorganisms. In certain embodiments, a distinguishing portion thereof can comprise any polypeptide of at least from about 20 to about 100 or more contiguous amino acids as set forth in SEQ ID NO:180, in particular in which the polypeptide does not contain a corresponding polypeptide of contiguous amino acids present in SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:182, or SEQ ID NO:184, and wherein the polypeptide comprises at least one amino acid substitution, addition, or deletion in the corresponding amino acid sequence as set forth in SEQ ID NO:2. Such substitutions, deletions or additions are those as set forth above in paragraph [0009].

[0048] Processed plant products are provided that contain a detectable amount of a recombinant polynucleotide encoding an eHTP, an eHTP or an insect inhibitory fragment thereof, or any distinguishing portion thereof. The processed product is selected from the group consisting of plant biomass, oil, meal, animal feed, flour, flakes, bran, lint, hulls, and processed seed.

[0049] *Hemiptera* infestations of crop plants are controlled by providing in the crop plants a recombinant polynucleotide sequence encoding one or more of the eHTP's of the present invention. Such transgenic crops produce or are treated to contain an insect or *Hemiptera* inhibitory amount of an applicable eHTP, and such crops are imbued with sufficient eHTP by (i) applying any composition comprising or encoding an eHTP to the plant or a seed that gives rise to the plant; and/or (ii) transforming the plant or a plant cell that gives rise to the seed and ultimately, the plant, with a polynucleotide encoding an eHTP. The plant may be a transiently or stably transformed transgenic plant comprising a transgene that expresses an insect or *Hemiptera* inhibitory amount of an eHTP. The plant may be a non-transgenic plant to which a composition comprising an eHTP has been applied. In such methods, the plant is a dicot plant, and more specifically may be a cotton, soybean or alfalfa plant. The Hemipteran insects include adults and nymphs, such as but not limited to the listing of bugs that is set forth above in paragraph [0020].

[0050] Preferably, the *Lygus* spp. is *Lygus hesperus* or *Lygus lineolaris*, the *Empoasca* spp. is *Empoasca fabae*, and the *Amrasca* spp. is *Amrasca devastans*.

[0051] Other features and advantages of the invention will be apparent from the following detailed description, examples, and claims.

EXAMPLES

[0052] In view of the foregoing, those of skill in the art should appreciate that changes can be made in the specific aspects which are disclosed and still obtain a like or similar result.

Example 1: Iterative Engineering-Testing-Selecting Approach

[0053] This example illustrates the random, combinatorial, and inventive aspects of the iterative (also can be referred to as “recursive”) engineering-testing-selecting approach used to identify and describe insect inhibitory proteins exhibiting Coleopteran and/or nematocidal activity or increased toxicity to *Hemipteran insect* species compared to TIC807 (SEQ ID NO:2). Several design approaches were employed to engineer for eHTP’s with greater inhibitory activity against *Lygus* species; approaches that included but were not limited to semi-random modifications, directed modifications of variances in an alignment of TIC807 with other native Bt proteins, and structure/function assisted design. Numerous rounds of engineering and testing were conducted (both consecutively and concurrently) to select for TIC807 protein variants exhibiting increased toxicity. Design approaches were adjusted as data was collected. This iterative engineering-testing-selection approach also included, but was not limited to steps including cloning, expressing, purifying, and bioassay testing of TIC807 control protein compared to the eHTP’s.

[0054] About 267 exemplary eHTP’s having exhibited increased *Lygus* toxicity compared to TIC807 were obtained from more than 2000 groups of candidate eHTP’s (i.e. “test” proteins) that were assayed for improved insect inhibitory activity. The actual total number of candidate eHTP’s tested was much greater than 2000 because testing included recombinant nucleotide segments encoding a number of candidate eHTP’s derived from library mutagenesis that were not sequenced in the selection process.

[0055] Protein stocks of various amounts and purity were prepared depending on the purpose of the test and the testing throughput desired. For example, lower quantity and lower purity protein preparations were prepared for screening higher numbers of variants in bioassay.

Larger quantity and higher purity protein stocks were prepared for high-powered bioassays. Testing trended towards the high-powered bioassays as principally relevant residue positions of the improved variants were elucidated. Initially, about 2000 variants were tested on *Lygus hesperus*. Based on data from *L. hesperus* approximately 600 variants were designed and then further tested on *Lygus lineolaris*. Of these, about 267 variants (Table 4B) demonstrated increased toxicity against *Lygus hesperus* and/or *Lygus lineolaris* when compared to TIC807. These 267 variants included twenty-two (22) variants that were confirmed to demonstrate increased toxicity against both *Lygus* species. Further confirmation and dose response testing narrowed the selection to seven (7) variants that were subsequently characterized using an 8-dose replicated bioassay to determine LC50 values against both *Lygus* species.

[0056] The selection process included dynamic updates of testing data, constantly adjusting engineering approaches, and performing iterative rounds. Concurrently, labor intensive cloning, protein expression, protein purification, and bioassay experiments were employed test the candidate eHTP's.

Example 2: Engineering Approaches

Alignment based approaches

[0057] A multiple sequence alignment of protein members of Cry51: Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and TIC807 (SEQ ID NO:2) were used to identify regions of variability, e.g., positions 195 to 201 and positions 211 to 219, relative to SEQ ID NO:2 (TIC807). These regions were targeted for saturation mutagenesis through use of degenerate oligonucleotide primers encoding random amino acid residues in these regions. Construct libraries were prepared for subsequent protein expression in host cells.

[0058] A multiple sequence alignment of Cry51Aa1 (SEQ ID NO:182), TIC853 (SEQ ID NO:184), and TIC807 (SEQ ID NO:2) was used in combination with a BLOSUM 80 substitution matrix to calculate average pair-wise distances for each position variant to TIC807. Residue positions with lower average pair-wise distances were substituted with alternative amino acid residues using degenerate oligonucleotide primers encoding for alternative amino acid residues, e.g., G28X, G31X, F46X, E125X, F138X, F147X, S167X, Y216X, P218X, G234X, T247X, D268X, and T308X. Construct libraries were prepared for subsequent protein expression in host cells.

Scanning approaches

[0059] Polynucleotide constructs were engineered to express a single Alanine substitution or a double Alanine substitution (Alanine-<parent residue>-Alanine) at every possible position over the full-length of SEQ ID NO:2 (TIC807). See Table 2 for a hypothetical example.

Table 2. A hypothetical example of single and double Alanine scans on a scaffold protein containing the amino acid sequence XXXXAXX.

	Single Alanine Scan	Double Alanine Scan
1	AXXXaXX	AXAXaXX
2	XAXXaXX	XAXAaXX
3	XXAXaXX	XXAXSXX
4	XXXAaXX	XXXAaAX
5	XXXXSXX	XXXXSXA
6	XXXXaAX	-
7	XXXXaXA	-

X = parent residue

a = parent residue is an Alanine residue

A = Modified to an Alanine residue

S = Modified to a Serine residue

[0060] Where an Alanine residue was already present in TIC807, a Serine was substituted instead. Protein variants that exhibited increased toxicity compared to TIC807 were further tested by combination and saturation mutagenesis at those Alanine-substituted residues that conferred increased toxicity. Scanning approaches were also performed on improved combination variants having accumulated modifications from previous iterative rounds of engineering-testing-selecting, *e. g.*, TIC807_M2 (SEQ ID NO:8) having mutations F46S, Y54H, S167R, S217N, and a contiguous triple deletion in residue range 196-201 was further engineered by an additional round of single Alanine substitutions to further improve upon the improved TIC807_M2. Principally relevant residues were identified and further tested by combination and saturation mutagenesis (*e. g.*, A150X, E125X, E155X, F147X, I134X, N157X, Q149X, T133X, E135X, and N137X). Variants engineered by these combined approaches exhibited further improvements to increased toxicity compared to TIC807 and were further combined with other design approaches that took advantage of the atomic structure of TIC807 (SEQ ID NO:2).

Surface exposed residues

[0061] The atomic structure of proteins of the present invention was determined in the midst of the Iterative Engineering-Testing-Selecting approach; and, the relative solvent-accessibility (%SA) of each residue was determined using Molsoft's ICM-Browser (Molsoft L.L.C., 11199 Sorrento Valley Road, S209, San Diego, CA 92121). Shown in Table 3 in columns (A) and (B), actual %SA was calculated for proteins having the respective amino acid sequences set forth as SEQ ID NO:185 (TIC807_L11M) and SEQ ID NO:8 (TIC807_M2). The predicted %SA for residues of TIC807 and TIC853 are listed in Table 3 in columns (A) and (C), respectively. Altogether, the %SA values reported in Table 3 are calculated as a percentage of the solvent-accessible surface area probed by a water molecule over the maximal solvent accessible area in standard extended conformation (Gly-XXX-Gly) for each residue in each position of the atomic structure. Table 3 aligns the residues of each protein by aligned residues in a Clustal W alignment. %SA greater than 100 can occur when maximal solvent accessible area in standard extended conformation (Gly-XXX-Gly) for each residue is less than the actual solvent accessible area probed by a water molecule. %SA greater than 100 are reported in the table as 100%.

[0062] Combined engineering-testing-selecting approaches described herein resulted in a number of principally relevant residues that accumulate in a surface patch ([2] of Figure 2) of residues having a radius of about 9.2 – 12.2 Angstroms around the Cb atom of P219 of SEQ ID NO:2 (TIC807): V10, I14, N22, N23, G24, I25, Q26, G27, F30, Q38, I39, D40, T41, I43, S193, T194, E195, H196, Y197, S198, H199, Y200, S201, G202, Y203, P204, I205, L206, T207, W208, I209, S210, Y216, S217, G218, P219, F220, M221, S222, W223, Y224, F225, N239, and V244 of SEQ ID NO:2 (TIC807). At least half of these residues exhibit %SA values of greater or equal to fifteen (15).

Table 3. Relative % Solvent Accessibility (SA) of Amino Acids of eHTP's & Scaffold Proteins.

(A)			(B)			(C)		
TIC807_L11M (SEQ ID NO:185)			TIC807_M2 (SEQ ID NO:8)			TIC853 (SEQ ID NO:184)		
Position amino residue	and acid	Calculated %SA per residue	Position amino residue	and acid	Calculated %SA per residue	Position amino residue	and acid	Estimated %SA per residue
2ALA		82.1#	2ALA		64.8#	2ALA		60.9#

(A)			(B)			(C)		
TIC807_L11M (SEQ ID NO:185)			TIC807_M2 (SEQ ID NO:8)			TIC853 (SEQ ID NO:184)		
Position and amino acid residue	Calculated %SA per residue		Position and amino acid residue	Calculated %SA per residue		Position and amino acid residue	Estimated %SA per residue	
3ILE	23.3		3ILE	28.9		3ILE	24.3	
4LEU	26.4		4LEU	31.9		4LEU	27.9	
5ASP	26.0		5ASP	22.7		5ASP	29.9	
6LEU	1.0		6LEU	3.4		6LEU	3.7	
7LYS	25.1		7LYS	16.2		7LYS	17.1	
8SER	46.4#		8SER	37.6#		8SER	44.9#	
9LEU	8.9		9LEU	5.2		9LEU	6.3	
10VAL ^{pI}	0.3		10VAL	0.6		10VAL	0.0	
11MET	25.0		11LEU	17.0		11LEU	16.6	
12ASN [*]	49.8#		12ASN	43.2#		12ASP	39.7#	
13ALA	0.0		13ALA	0.0		13ALA	0.0	
14ILE ^{pI}	0.0		14ILE	0.0		14ILE	0.0	
15ASN	23.7		15ASN	24.9		15ASN	19.2	
16TYR	29.5		16TYR	47.1#		16TYR	52.5#	
17TRP	14.1		17TRP	18.2		17TRP	20.1	
18GLY	4.3		18GLY	1.4		18GLY	1.0	
19PRO	63.6#		19PRO	57.3#		19PRO	59.0#	
20LYS	57.3#		20LYS	77.2#		20LYS	100#	
21ASN	36.3#		21ASN	28.4		21ASN	61.5#	
22ASN ^{pI}	16.9		22ASN	10.1		22ASN	15.2	
23ASN ^{pI}	0.3		23ASN	0.8		23ASN	0.0	
24GLY ^{pI}	42.0#		24GLY	43.2#		24GLY	43.3#	
25ILE ^{pI}	10.1		25ILE	13.8		25ILE	7.6	
26GLN ^{pI}	92.4#		26GLN	86.2#		26GLN	94.7#	
27GLY ^{pI}	62.0#		27GLY	73.9#		27GLY	62.8#	
28GLY	49.0#		28GLY	50.6#		28TYR	47.7#	
29ASP	66.0#		29ASP	68.1#		29ASN	80.7#	
30PHE ^{pI}	4.5		30PHE	4.1		30PHE	1.5	
31GLY	37.2#		31GLY	41.4#		31ASN	61.1#	
32TYR	25.2		32TYR	25.3		32TYR	21.5	
33PRO	70.7#		33PRO	76.0#		33PRO	78.5#	
34ILE	4.8		34ILE	5.5		34ILE	2.6	
35SER	42.2#		35SER	29.1		35SER	27.0	
36GLU	54.2#		36GLU	47.2#		36GLU	50.2#	
37LYS	81.0#		37LYS	79.5#		37ARG	87.5#	
38GLN ^{pI}	12.8		38GLN	14.5#		38GLN	9.0	
39ILE ^{pI}	7.8		39ILE	7.9		39ILE	5.1	
40ASP ^{pI}	52.4#		40ASP	55.3#		40ASP	49.8#	
41THR ^{pI}	0.3		41THR	0.0		41THR	0.2	
42SER	53.1#		42SER	56.0#		42SER	53.0#	
43ILE ^{pI}	13.1		43ILE	23.5		43ILE	25.1	
44ILE	8.3		44ILE	12.0		44ILE	8.1	
45THR	30.7		45THR	37.8#		45THR	45.7#	
46PHE [*]	20.0		46SER	43.7#		46SER	40.5#	
47THR	48.1#		47THR	45.2#		47THR	43.7#	
48HIS	73.5#		48HIS	65.3#		48HIS	78.3#	
49PRO	9.4		49PRO	12.6		49SER	9.0	

(A)			(B)			(C)		
TIC807_L11M (SEQ ID NO:185)			TIC807_M2 (SEQ ID NO:8)			TIC853 (SEQ ID NO:184)		
Position amino residue	and acid	Calculated %SA residue per	Position amino residue	and acid	Calculated %SA residue per	Position amino residue	and acid	Estimated %SA residue per
50ARG		58.7#	50ARG		53.7#	50ARG		61.5#
51LEU		13.7	51LEU		8.1	51LEU		3.0
52ILE*		32.4	52ILE		31.5	52MET		43.7#
53PRO		22.2	53PRO		26.5	53PRO		22.8
54TYR*		52.7#	54HIS		42.2#	54HIS		45.5#
55ASP		57.5#	55ASP		59.2#	55ASP		55.5#
56LEU		15.0	56LEU		18.6	56LEU		15.1
57THR		62.0#	57THR		73.2#	57THR		80.0#
58ILE		67.6#	58ILE		60.9#	58ILE		68.0#
59PRO		26.6	59PRO		21.9	59PRO		20.7
60GLN		28.8	60GLN		20.7	60GLN		21.3
61ASN		71.9#	61ASN		73.6#	61ASN		74.6#
62LEU		13.4	62LEU		11.7	62LEU		10.0
63GLU		62.2#	63GLU		66.0#	63GLU		66.4#
64THR		51.2#	64THR		51.1#	64THR		49.0#
65ILE		46.1#	65ILE		41.5#	65ILE		38.4#
66PHE		27.0	66PHE		26.3	66PHE		29.6
67THR		52.5#	67THR		55.5#	67THR		62.2#
68THR*		31.6	68THR		35.8#	68THR		35.3
69THR		54.3#	69THR		51.1#	69THR		50.3#
70GLN*		31.0	70GLN		36.5#	70GLN		34.3
71VAL		53.4#	71VAL		53.2#	71VAL		53.1#
72LEU		7.9	72LEU		11.8	72LEU		8.1
73THR		40.5#	73THR		44.0#	73THR		47.0#
74ASN		0.6	74ASN		0.0	74ASN		0.0
75ASN		69.8#	75ASN		63.2#	75ASN		65.9#
76THR		36.7#	76THR		40.5#	76THR		44.0#
77ASP		80.2#	77ASP		63.0#	77ASP		62.1#
78LEU		62.6#	78LEU		62.5#	78VAL		63.8#
79GLN		74.4#	79GLN		54.7#	79GLN		43.5#
80GLN		33.3	80GLN		32.1	80GLN		40.1#
81SER		81.0#	81SER		68.6#	81SER		75.2#
82GLN		19.0	82GLN		23.9	82GLN		24.6
83THR		62.7#	83THR		63.6#	83THR		63.7#
84VAL		1.8	84VAL		0.9	84VAL		0.0
85SER		50.8#	85SER		55.7#	85SER		54.0#
86PHE		7.2	86PHE		5.8	86PHE		4.0
87ALA*		58.5#	87ALA		61.9#	87SER		68.0#
88LYS		30.5	88LYS		30.6	88LYS		32.6
89LYS		69.8#	89LYS		67.8#	89LYS		67.9#
90THR		19.9	90THR		23.1	90THR		16.7
91THR		54.1#	91THR		55.1#	91THR		48.1#
92THR		1.8	92THR		1.8	92THR		0.0
93THR ^{p2'}		40.3#	93THR		36.4#	93THR		29.4
94THR		0.0	94THR		0.6	94THR		0.2
95SER ^{p2*}		14.4	95SER		15.7	95SER		18.1
96THR		5.5	96THR		1.5	96THR		0.0

(A)			(B)			(C)		
TIC807_L11M (SEQ ID NO:185)			TIC807_M2 (SEQ ID NO:8)			TIC853 (SEQ ID NO:184)		
Position and amino acid residue	Calculated %SA per residue		Position and amino acid residue	Calculated %SA per residue		Position and amino acid residue	Estimated %SA per residue	
97SER ^{p2}	16.6		97SER	18.5		97SER	29.9	
98THR	8.2		98THR	5.9		98THR	1.9	
99THR	41.8#		99THR	46.4#		99THR	49.3#	
100ASN	37.7#		100ASN	34.1		100ASP	20.1	
101GLY	1.0		101GLY	1.9		101GLY	0.0	
102TRP	3.6		102TRP	10.4		102TRP	6.6	
103THR	8.1		103THR	8.1		103THR	3.9	
104GLU	9.7		104GLU	21.9		104GLU	14.8	
105GLY*	35.3		105GLY	46.8#		105GLY	31.4	
106GLY	57.0#		106GLY	68.6#		106GLY	61.8#	
107LYS	52.4#		107LYS	57.2#		107ARG	54.6#	
108ILE	61.8#		108ILE	63.5#		108ILE	67.1#	
109SER	43.5#		109SER	47.9#		109SER	47.7#	
110ASP	83.1#		110ASP	83.5#		110ASP	65.7#	
111THR	43.4#		111THR	41.3#		111THR	39.9#	
112LEU	26.7		112LEU	29.6		112LEU	31.3	
113GLU	53.8#		113GLU	64.1#		113GLU	62.5#	
114GLU	34.8		114GLU	30.9		114GLU	32.6	
115LYS	62.2#		115LYS	55.2#		115ASN	54.6#	
116VAL	6.4		116VAL	8.6		116VAL	10.4	
117SER*	46.6#		117SER	48.9#		117SER	51.2#	
118VAL	0.9		118VAL	2.5		118VAL	1.3	
119SER*	20.2		119SER	23.4		119SER	23.7	
120ILE	0.8		120ILE	0.3		120ILE	0.0	
121PRO	5.9		121PRO	10.7		121PRO	8.0	
122PHE	0.2		122PHE	1.4		122PHE	0.3	
123ILE	19.1		123ILE	20.8		123ILE	18.3	
124GLY	4.3		124GLY	6.2		124GLY	3.3	
125GLU*	59.7#		125GLU	56.2#		125ALA	57.8#	
126GLY	50.0#		126GLY	52.5#		126GLY	49.6#	
127GLY	47.2#		127GLY	56.7#		127GLY	38.6#	
128GLY*	34.7		128GLY	30.3		128ALA	23.0	
129LYS	68.8#		129LYS	73.9#		129LYS	78.4#	
130ASN	16.2		130ASN	14.6		130ASN	10.1	
131SER	78.2#		131SER	77.9#		131SER	80.3#	
132THR	9.8		132THR	10.3		132THR	12.3	
133THR*	45.7#		133THR	42.0#		133THR	44.3#	
134ILE*	1.1		134ILE	0.8		134ILE	0.0	
135GLU*	51.5#		135GLU	45.2#		135GLU	48.5#	
136ALA	0.0		136ALA	1.3		136ALA	2.4	
137ASN*	18.1		137ASN	15.5		137ASN	15.6	
138PHE*	1.9		138PHE	0.9		138VAL	2.5	
139ALA*	2.8		139ALA	6.3		139ALA	4.1	
140HIS	2.3		140HIS	2.1		140HIS	0.0	
141ASN	5.3		141ASN	6.5		141ASN	2.8	
142SER	5.4		142SER	4.4		142SER	6.6	
143SER	7.7		143SER	10.6		143SER	7.0	

(A)			(B)			(C)		
TIC807_L11M (SEQ ID NO:185)			TIC807_M2 (SEQ ID NO:8)			TIC853 (SEQ ID NO:184)		
Position amino residue	and acid	Calculated %SA per residue	Position amino residue	and acid	Calculated %SA per residue	Position amino residue	and acid	Estimated %SA per residue
144THR		23.5	144THR		17.3	144THR		16.6
145THR*		48.3#	145THR		52.7#	145THR		55.2#
146THR		50.2#	146THR		49.7#	146THR		53.6#
147PHE ^{p2*}		49.9#	147PHE		61.6#	147SER		51.7#
148GLN*		12.9	148GLN		17.8	148GLN		18.4
149GLN ^{p2*}		59.5#	149GLN		65.1#	149GLN		69.1#
150ALA*		6.9	150ALA		8.7	150ALA		9.1
151SER ^{p2*}		51.0#	151SER		51.7#	151SER		57.9#
152THR		9.9	152THR		8.7	152THR		12.3
153ASP*		83.5#	153ASP		84.5#	153GLU		63.3#
154ILE		11.2	154ILE		6.1	154ILE		6.3
155GLU*		49.5#	155GLU		63.9#	155GLU		49.7#
156TRP		1.7	156TRP		3.8	156TRP		1.8
157ASN*		59.1#	157ASN		59.1#	157ASN		53.4#
158ILE*		13.1	158ILE		5.9	158ILE		0.8
159SER*		60.2#	159SER		52.9#	159SER		52.2#
160GLN		29.2	160GLN		19.3	160GLN		9.3
161PRO		54.0#	161PRO		63.6#	161PRO		62.6#
162VAL		0.6	162VAL		4.0	162VAL		2.4
163LEU		53.8#	163LEU		56.6#	163LEU		64.5#
164VAL		0.0	164VAL		0.0	164VAL		0.0
165PRO		22.8	165PRO		22.1	165PRO		26.9
166PRO		30.7	166PRO		36.1#	166PRO		39.7#
167SER*		31.0	167ARG		32.8	167ARG		36.7#
168LYS		18.2	168LYS		18.5	168LYS		19.9
169GLN		17.4	169GLN		15.1	169GLN		10.7
170VAL		0.0	170VAL		0.0	170VAL		0.0
171VAL		13.2	171VAL		13.8	171VAL		12.2
172ALA		0.0	172ALA		0.0	172ALA		0.0
173THR		9.8	173THR		9.2	173THR		6.5
174LEU		1.3	174LEU		2.6	174LEU		0.2
175VAL*		17.2	175VAL		17.8	175VAL		13.4
176ILE		0.0	176ILE		0.0	176ILE		0.0
177MET*		7.0	177MET		7.7	177MET		17.3
178GLY		1.6	178GLY		0.5	178GLY		0.0
179GLY		15.9	179GLY		22.2	179GLY		16.5
180ASN ^{p2*}		60.0#	180ASN		60.1#	180ASP		44.9#
181PHE		0.7	181PHE		2.8	181PHE		1.8
182THR ^{p2*}		50.6#	182THR		44.3#	182THR		40.8#
183ILE		0.0	183ILE		1.1	183VAL		0.0
184PRO		36.6#	184PRO		34.2	184PRO		34.5
185MET		4.4	185MET		2.1	185MET		1.8
186ASP		52.4#	186ASP		23.5	186ASP		20.3
187LEU*		0.8	187LEU		0.0	187LEU		0.0
188MET		25.9	188MET		12.7	188ILE		24.9
189THR		1.4	189THR		2.9	189THR		0.5
190THR		26.1	190THR		26.2	190THR		24.1

(A)		(B)		(C)	
TIC807_L11M (SEQ ID NO:185)		TIC807_M2 (SEQ ID NO:8)		TIC853 (SEQ ID NO:184)	
Position and amino acid residue	Calculated %SA per residue	Position and amino acid residue	Calculated %SA per residue	Position and amino acid residue	Estimated %SA per residue
191ILE	4.0	191ILE	6.2	191ILE	1.8
192ASP	25.9	192ASP	29.2	192ASP	21.4
193SER ^{PI}	7.4	193SER	7.7	193SER	2.7
194THR ^{PI}	66.2#	194THR	60.2#	194THR	59.9#
195GLU ^{PI}	38.5#	195GLU	35.0	195GLN	35.5#
196HIS*	37.7#	-	100	-	100#
197TYR*	32.2	-	100#	-	100#
198SER*	35.5#	-	100#	-	100#
199HIS ^{PI*}	64.3#	196HIS	55.5#	196HIS	51.7#
200TYR ^{PI*}	85.3#	197TYR	56.2#	197PHE	45.8#
201SER ^{PI*}	50.3#	198SER	68.3#	198THR	64.5#
202GLY ^{PI}	32.8	199GLY	50.0#	199GLY	51.1#
203TYR ^{PI}	21.5	200TYR	22.6	200TYR	26.0
204PRO ^{PI}	1.4	201PRO	1.0	201PRO	1.0
205ILE ^{PI}	1.1	202ILE	0.3	202ILE	0.0
206LEU ^{PI}	1.8	203LEU	2.6	203LEU	0.0
207THR ^{PI}	0.0	204THR	0.0	204THR	0.0
208TRP ^{PI*}	38.8#	205TRP	35.6#	205TRP	22.5
209ILE ^{PI}	0.0	206ILE	0.0	206ILE	0.0
210SER ^{PI}	22.5	207SER	20.1	207GLU	17.0
211SER	3.1	208SER	3.4	208ASN	4.6
212PRO	56.5#	209PRO	58.4#	209PRO	56.2#
213ASP	68.0#	210ASP	55.2#	210GLU	60.5#
214ASN	65.5#	211ASN	66.4#	211HIS	64.4#
215SER	67.2#	212SER	74.1#	212ASN	74.2#
216TYR ^{PI}	42.5#	213TYR	39.8#	213VAL	29.4
217SER ^{PI*}	43.2#	214ASN	46.3#	214ARG	57.1#
218GLY ^{PI}	1.2	215GLY	4.1	215GLY	6.1
219PRO ^{PI*}	14.4	216PRO	14.7	216ARG	33.7
220PHE ^{PI}	0.0	217PHE	0.0	217PHE	0.0
221MET ^{PI}	15.2	218MET	16.1	218LEU	8.4
222SER ^{PI}	3.3	219SER	3.3	219SER	0.0
223TRP ^{PI*}	35.6#	220TRP	34.3	220TRP	42.5#
224TYR ^{PI}	13.5	221TYR	15.9	221PHE	11.3
225PHE ^{PI}	0.9	222PHE	1.4	222PHE	0.0
226ALA	15.9	223ALA	13.1	223ALA	7.8
227ASN	40.7#	224ASN	41.9#	224ASN	43.2#
228TRP	9.0	225TRP	8.9	225TRP	7.3
229PRO	56.3#	226PRO	61.5#	226PRO	65.5#
230ASN	67.6#	227ASN	67.3#	227ASN	67.3#
231LEU	21.1	228LEU	16.1	228LEU	16.1
232PRO	23.7	229PRO	23.0	229PRO	23.6
233SER	97.0#	230SER	95.8#	230SER	88.1#
234GLY	23.0	231GLY	19.5	231GLU	13.0
235PHE*	8.3	232PHE	9.0	232PHE	6.1
236GLY	26.1	233GLY	18.3	233GLY	28.5
237PRO	72.6#	234PRO	70.8#	234SER	81.9#

(A)			(B)			(C)		
TIC807_L11M (SEQ ID NO:185)			TIC807_M2 (SEQ ID NO:8)			TIC853 (SEQ ID NO:184)		
Position amino residue	and acid	Calculated %SA residue per	Position amino residue	and acid	Calculated %SA residue per	Position amino residue	and acid	Estimated %SA residue per
238LEU		27.7	235LEU		28.1	235LEU		25.9
239ASN*		33.2	236ASN		26.5	236ASN		42.2#
240SER		100#	237SER		100#	237SER		100#
241ASP*		62.2#	238ASP		61.7#	238ASP		55.2#
242ASN		15.1	239ASN		20.8	239ASN		21.7
243THR*		3.3	240THR		2.4	240THR		2.6
244VAL*		1.8	241VAL		3.1	241ILE		0.0
245THR*		19.0	242THR		23.8	242THR		30.1
246TYR*		8.6	243TYR		4.8	243TYR		0.4
247THR*		36.8#	244THR		40.8#	244LYS		58.8#
248GLY		2.5	245GLY		1.4	245GLY		0.0
249SER*		20.7	246SER		23.9	246SER		27.0
250VAL*		6.0	247VAL		1.4	247VAL		0.0
251VAL ^{p2*}		32.6	248VAL		30.0	248VAL		29.7
252SER*		0.0	249SER		0.0	249SER		0.0
253GLN ^{p2}		40.2#	250GLN		37.6#	250ARG		51.3#
254VAL		1.2	251VAL		1.5	251ILE		2.7
255SER ^{p2}		35.3	252SER		37.3#	252SER		43.7#
256ALA		6.1	253ALA		2.4	253ALA		2.1
257GLY		4.1	254GLY		6.1	254GLY		1.2
258VAL		0.3	255VAL		0.0	255VAL		0.0
259TYR		2.2	256TYR		1.1	256TYR		1.0
260ALA		0.7	257ALA		0.7	257ALA		0.0
261THR		9.2	258THR		9.3	258THR		5.0
262VAL		3.6	259VAL		1.5	259VAL		0.2
263ARG		26.6	260ARG		29.8	260ARG		26.9
264PHE		0.5	261PHE		3.8	261PHE		1.5
265ASP		6.9	262ASP		7.2	262ASP		10.8
266GLN		5.6	263GLN		5.8	263GLN		2.6
267TYR		16.1	264TYR		14.5	264TYR		12.4
268ASP		29.8	265ASP		31.4	265ALA		19.3
269ILE		25.4	266ILE		18.2	266ILE		14.5
270HIS		85.5#	267HIS		72.2#	267ASN		92.0#
271ASN		43.4#	268ASN		46.9#	268ASN		64.0#
272LEU		40.3#	269LEU		43.1#	269LEU		39.8#
273ARG*		86.3#	270ARG		63.1#	270ARG		84.4#
274THR*		52.0#	271THR		66.1#	271THR		76.8#
275ILE*		41.0#	272ILE		37.9#	272ILE		32.4
276GLU		47.9#	273GLU		50.1#	273GLU		53.0#
277LYS		49.8#	274LYS		47.2#	274LYS		70.2#
278THR		46.3#	275THR		51.2#	275THR		53.7#
279TRP		25.2	276TRP		25.0	276TRP		33.4
280TYR		35.5	277TYR		30.7	277TYR		21.3
281ALA		6.6	278ALA		7.9	278ALA		4.4
282ARG*		77.6#	279ARG		80.6#	279ARG		86.1#
283HIS		45.2#	280HIS		36.6#	280HIS		35.8#
284ALA		0.8	281ALA		0.8	281GLY		0.6

(A)		(B)		(C)	
TIC807_L11M (SEQ ID NO:185)		TIC807_M2 (SEQ ID NO:8)		TIC853 (SEQ ID NO:184)	
Position and amino acid residue	Calculated %SA per residue	Position and amino acid residue	Calculated %SA per residue	Position and amino acid residue	Estimated %SA per residue
285THR	14.7	282THR	8.6	282THR	2.0
286LEU	3.6	283LEU	5.9	283LEU	2.3
287HIS*	8.4	284HIS	16.5	284HIS	11.9
288ASN	40.4#	285ASN	43.9#	285ASN	38.7#
289GLY	61.0#	286GLY	53.5#	286GLY	61.1#
290LYS	61.7#	287LYS	61.6#	287LYS	73.8#
291LYS	68.4#	288LYS	66.2#	288LYS	51.9#
292ILE	19.2	289ILE	19.5	289ILE	21.0
293SER*	40.3#	290SER	47.9#	290SER	45.7#
294ILE	3.4	291ILE	4.8	291ILE	5.1
295ASN*	29.3	292ASN	21.9	292ASN	18.0
296ASN	38.2#	293ASN	40.4#	293ASN	37.4#
297VAL	1.3	294VAL	1.4	294VAL	0.7
298THR	10.1	295THR	9.5#	295THR	4.3
299GLU*	77.1#	296GLU	72.7#	296GLU	68.8#
300MET*	48.6#	297MET	46.4#	297MET	42.8#
301ALA	65.3#	298ALA	54.1#	298ALA	60.4#
302PRO	66.0#	299PRO	73.0#	299PRO	77.8#
303THR*	83.7#	300THR	85.8#	300THR	94.1#
304SER	77.4#	301SER	76.1#	301SER	84.9#
305PRO*	81.1#	302PRO	65.7#	302PRO	83.4#
306ILE*	78.1#	303ILE	81.6#	303ILE	91.3#
307LYS	81.9#	304LYS	99.3#	304GLU	100#
308THR*	89.4#	305THR	100#	305ARG	100#
309ASN	100#	306ASN	100#	306ASN	100#

P1 designates an amino acid in surface patch [1] of Figure 2.

P2 designates an amino acid in surface patch [2] of Figure 2.

* designates one of the 72 principally relevant amino acids described herein (see Figure 2).

Shown are residues of TIC807_L11M, TIC807_M2, and TIC853 aligned by Clustal W.

Numbers marked with # represent %SA of at least about 36%.

Receptor Binding

[0063] A surface patch ([1] of Figure 2) of residues having %SA values greater than 36% or within about 3 residues of a residue having %SA greater than 36% in a radius of about 9.2 – 12.2 Angstroms from the Cb atom of S95 of SEQ ID NO:2 (TIC807) was identified as a region comprising residues of a TIC807 protein that can be substituted to provide for eHTP's that exhibit enhanced Lygus inhibitory spectrum and/or improved Lygus inhibitory activity. This surface patch region may be associated with target insect receptor binding activity; and, includes residues T93, S95, S97, F147, Q149, S151, N180, T182, V251, Q253, and S255 of SEQ ID NO:2 (TIC807). eHTP's can include, but are not limited to, one or more

substitutions of surface patch 1 amino acid residues such as S95A, F147A, Q149E, and/or, V251A.

[0064] The combined engineering-testing-selecting approaches described herein identified residues located in surface patch 1 that can provide for eHTP's when substituted or otherwise modified. These residues may be important for productive binding of eHTP's to receptors in *Lygus* insect gut to provide for enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity when compared to TIC807. Modifications of the surface patch 1 amino acid residues that can provide for eHTP's include substitutions that provide aromatic groups and/or hydrogen-bonding groups which favoring binding to sugar groups found on glycosylated receptors of insects.

Membrane Binding

[0065] Certain amino acid residues located in beta-sheet regions of the protein were identified from the atomic structure of TIC807 and were substituted with aromatic residues. More specifically, amino acids L78, I123, H270, R273, I275 of the folded TIC807 beta sheet regions were substituted with Phenylalanine, Tyrosine, or Tryptophan. Aromatic amino acid substitutions of R273 and I275 were amongst those residues that provided for an enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity (See Table 4, data for SEQ ID NOs:32, 34, 68, 92, and 122). Amino acid side chains of residues in these positions may be likely to interact with the membrane of target insects.

Proteolytic activation sites

[0066] Glycine residues generally thought to be involved in proteolysis were substituted with Serines to alter proteolytic cleavage dynamics. The presence of a glycine residue in a loop region can impart more flexibility and therefore susceptibility to proteolysis, which can either increase insect inhibitory activity or decrease insect inhibitory activity. Residues in structurally identified loop regions were substituted with a glycine residue, and no improvements were observed. Positions in loops that were already glycines, (e.g. G18, G24, G27) were substituted with a serine, a small residue in an attempt to reduce proteolytic susceptibility, and no improvements were observed.

Combined Structure design approaches

[0067] The atomic structure of TIC807 (SEQ ID NO:2) was used to identify loop regions for library mutagenesis followed by testing of the engineered variants. A loop at amino acid positions 211-216 of SEQ ID NO:2 (TIC807) was library-mutagenized and tested. Consecutive loops in close proximity at amino acid positions 75-83, 161-167, and 267-276 of SEQ ID NO:2 (TIC807) was library-mutagenized and tested.

[0068] Analysis of the atomic structure of TIC807 suggests that a structural loop resides at residues 113-138 of SEQ ID NO:2, and variants were engineered to stabilize and destabilize the loop.

[0069] In another region spanning two beta-strands connected by a short loop, the two beta-strands exhibited an alternating pattern of hydrophobic and hydrophilic amino acid residues at positions 116 to 121 and at positions 133 to 138 relative to SEQ ID NO:2, characteristic of pore-forming loops. An expression library was engineered to modify both beta-strand segments replacing residues V116, V118, and I120 with respective combinations 116V/Y/L/H/F/D, 118V/Y/L/H/F/D, and 120I/D/F/H/L/N/V/Y for a total of 288 possible variants in the library. This procedure was repeated for: residues S117, S119, and P121 with respective combinations 117S/A/D/E/G/K/N/R/T, 119S/A/D/E/G/K/N/R/T, and 121P/S/T for 243 potential variants; residues I133, A135, and F137 with respective combinations 133I/D/F/N/V/Y, 135A/D/F/H/L/V/Y, and 137F/D/H/L/V/Y for 252 possible variants; and residues T134, E136, and N138 with respective combinations 134T/A/D/E/G/K/N/R/S, 136E/A/D/G/K/N/R/S/T, and 138N/A/D/G/S/T for 486 possible variants. An enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity was associated with certain of these substitutions as shown in Table 4.

Structure-Function Relationship

[0070] Altogether, more than 2000 clones (including mixed library clones) expressing variants of TIC807 were tested for enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity against *Lygus* spp. compared to TIC807. Semi-random modifications, directed modifications, and predictive structure-function modifications, including structure modeling, receptor binding potential, metal binding potential, oligomerization potential, uniformity of surface charge distribution, pore formation potential, ion channel function, and identification of surface exposed patches to with an objective of

identifying eHTP's with an enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity compared to TIC807. These clones were expressed for bioassay testing.

Example 3: Protein expression and purification of TIC807, including variants and fragments

[0071] Control protein TIC807 is a protein of 309 amino acids in length that can be expressed in crystalline form in *Bacillus thuringiensis* (*Bt*) or aggregate form in *E. coli*. Test variants thereof were recombinantly expressed in *Bt*. An expression characteristic of TIC807 and variants of TIC807 is the predominant crystalline and aggregate forms extracted from *Bt* and *E. coli* cells, respectively. To test for *Lygus* bioactivity, test and control samples were made suitable for *Lygus* bioassay by solubilizing samples in 25 mM Sodium Carbonate buffer and removing unsolubilized materials by centrifugation. The amount of protein in test and control samples were measured using total protein methods, e.g.s, a Bradford assay, an ELISA method, or similar. Gel electrophoresis was used to determine the purity and stock concentration of the solubilized recombinant protein. C-terminal HIS-tagged TIC807 protein was engineered to facilitate detection, purification, and quantification of large amounts of TIC807 control protein. C-terminal HIS-tagged TIC807 and un-tagged TIC807 test samples were separately assayed and confirmed to have equivalent activity against *Lygus* (see Examples 4, 5, and 6).

[0072] Site-directed amino acid substitutions were made to TIC807_M13 (SEQ ID NO:34) to elevate expression of a soluble form. Inventors postulate that more readily soluble variants of the proteins of the present invention can facilitate expression and purification, e.g., expressed in *E. coli* host cells; and can increase insect inhibitory efficacy when expressed in plant host cells. Recombinant DNA constructs encoding TIC807_M13 (SEQ ID NO:34) were engineered three different ways to reflect three different variants: Relative to TIC807_M13, the modifications were for Variant #1: I58K and P59K, for Variant #2: S198K and G199K, and for Variant #3: S246R, V248E, and Q250R. Relative to TIC807 (SEQ ID NO:2), the modifications can be alternatively described as follows for Variant #1: I58K and P59K, for Variant #2: S201K and G202K, and for Variant #3: S249R, V251E, and Q253R; this positional difference is congruent due to a contiguous triple deletion of SEQ ID NO:2 (TIC807) in residue range 196-201 that is reflected in TIC807_M13 (SEQ ID NO:34). The four engineered recombinant DNA constructs were each cloned and expressed in *E. coli*. The soluble fraction from the four *E. coli* preparations were evaluated by coomassie-stained SDS-PAGE, which showed that TIC807_M13 (SEQ ID NO:34) was not detectable in the soluble

fraction; but, in contrast, Variant #s 1, 2, and 3 were soluble. Similar amino acid substitutions either singly or in combination are made to proteins of the present invention to elevate their solubility in non-Bt or plant host cells. Recombinant DNA constructs were engineered to encode for and express TIC807_M13 variant #3 (renamed TIC807_M14; nucleotide SEQ ID NO:203 and amino acid SEQ ID NO:204). Prepared *E. coli* lysate was clarified, and the recombinant protein purified and enriched-for on a series of columns, including ion-exchange and gel filtration methods. Pooled protein fractions were quantified and determined to be active against *Lygus* insects (See Example 4, Table 4B).

[0073] Proteins of the present invention, including but not limited to proteins having the amino acid sequence as set forth as SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, or SEQ ID NO:36, are engineered to elevate expression of a soluble form when expressed in a host cell, e.g., expressed in Bt, *E. coli*, or in a plant cell or in a compartment of a plant cell. Engineering includes substituting a lysine amino acid residue at one or more of the following positions 58, 59, 198, 199, 201, or 202; or, a Glutamic acid at one or more of the following positions 198, 248, or 301; or, an Arginine at one or more of the following positions 246, 250, or 253.

[0074] The C-terminal region protrudes away from the monomeric core of the protein (See Figure 2). A recombinant DNA construct was engineered to encode for and express a protein having the amino acid sequence of SEQ ID NO:202, which is a protein fragment (amino acids 1 to 301) of TIC807_M8 (SEQ ID NO:16); and, the expressed protein was purified, quantified, and determined active against *Lygus* insects (See Example 4, Table 4B). Recombinant DNA constructs were designed to encode for and express TIC807 fragments exhibiting varying truncations off of the C-terminus end of the proteins of the present invention at the respective TIC807 positions A281, G289, S293, A301, and S304. Protein fragments are engineered to encode for and express proteins having the amino acid sequences set forth as SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, and SEQ ID NO:36; and, the expressed protein fragments are used as test samples against *Lygus* insects.

Example 4: Hemipteran activity of Engineered proteins

[0075] This example illustrates eHTP's to have improved insecticidal activity or enhanced insecticidal specificity against Hemipteran insects when provided in the diet of Hemipteran insects, including but not limited to members of the Heteroptera miridae, including the genus *Lygus*, e.g., *Lygus hesperus* and *Lygus lineolaris*, and the family Cicadellidae, including the genus *Amrasca*, e.g. *Amrasca devastans*, and *Empoasca*, e.g. *Empoasca fabae*. This example

with Table 4B illustrates the feeding assay used to determine the enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity of a *Bt* expressed recombinant proteins of the present invention against both *Lygus hesperus* and *Lygus lineolaris*. Proteins expressed in recombinant bacterium host cells were solubilized in carbonate buffer and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE); and, protein concentrations determined by densitometry using bovine serum albumin (BSA) as a standard. Protein stock (2X) prepared this way were mixed with diet for feeding assays.

[0076] Feeding assays with the Hemipteran species *Lygus hesperus* and *Lygus lineolaris* were based on a 96 well micro-titer plate format with *Lygus* diet encapsulated between stretched Parafilm[®] and Mylar sheets. Artificial diet was obtained from Bio-Serv[®] (Bio-Serv[®] Diet F9644B, Frenchtown, NJ). Autoclaved, boiling water (518 mL) was combined with 156.3 grams of Bio-Serv[®] diet F9644B in a surface-sterilized blender. The contents of four surface-sterilized chicken eggs were added and the mixture blended until smooth, then adjusted to one liter total volume and allowed to cool to room temperature, this being the 2X diet. Test samples were prepared by mixing in a 1:1 ratio of 2X diet and 2X sample. A sheet of Parafilm[®] (Pechiney Plastic Packing, Chicago, IL) was placed over a vacuum manifold designed for 96-well format (Analytical Research Systems, Gainesville, FL) and a vacuum of approximately -20 millimeters mercury was applied, sufficient to cause extrusion of the Parafilm[®] into the wells. Twenty to forty microliters of test sample were added to the Parafilm[®] extrusions. A sheet of Mylar film (Clear Lam Packaging, Inc., Elk Grove Village, IL) was placed over the sample filled Parafilm[®] extrusions and sealed with a tacking iron (Bienfang Sealector II, Hunt Corporation, Philadelphia, PA), thus forming diet filled Parafilm[®] sachets. These Parafilm[®] sachets were positioned over a flat-bottom 96-well plate containing *Lygus* eggs suspended in a dilute agarose solution. Upon hatching, *Lygus* nymphs feed on the diet by piercing the diet filled Parafilm[®] sachets. Alternatively, newly hatched *Lygus* nymphs instead of eggs were manually infested into each well. Stunting and mortality scores were determined on day 5 and compared to controls. Data were analyzed using JMP4 statistical software. For each protein at a test concentration, three populations of eight nymphs were subjected to this bioassay, and mortality scores reported in Table 4B.

[0077] For LC50 determinations listed in Table 1 and Table 4B, proteins were presented to newly hatched *Lygus* nymphs at 8-10 concentrations and the nymphs allowed to feed for 5 days before scoring for mortality over the dose range. For each concentration, three populations of eight nymphs were subjected to this bioassay, and all LC50 determinations in Table 1 and Table 4B were repeated at least once.

[0078] For LC50 estimations, proteins were presented to newly hatched *Lygus lineolaris* nymphs at 4 concentrations and the nymphs allowed to feed for 5 days before scoring for mortality over the dose range. *Lygus lineolaris* LC50 estimations were performed on TIC807 and TIC807_M2 because significantly large amounts of these proteins in excess of 1000 µg/mL have not been possible to provide in *Lygus* diet in order to complete the high range of toxicity dose response to *Lygus lineolaris*; and therefore, an LC50 value was not determined for TIC807 or TIC807_M2. Instead, a 4-dose LC50 estimation in the low range was performed, and reported in Table 1 and Table 4B. The estimated *Lygus lineolaris* LC50 for TIC807_M14 is 4.4 µg/mL. For each concentration, three populations of eight nymphs were subjected to this bioassay.

[0079] This example with Tables 4A and 4B illustrate the feeding assay used to determine the enhanced inhibitory spectrum and/or improved inhibitory activity of a *Bt* expressed recombinant protein disclosed herein against *Amrasca devastans*. TIC807 variants with improved insecticidal activity or enhanced insecticidal specificity against *Lygus hesperus* and *Lygus lineolaris* exhibit improved insecticidal activity against *Amrasca devastans*.

[0080] TIC807, and TIC807-M13 were dissolved in 25mM sodium carbonate buffer, pH 10. *Amrasca devastans* eggs were collected on Okra leaf and incubated in a petriplate containing 2% agar. Upon hatching the neonates were used for bioassays using the diluted (1:5) *Lygus* diet. The proteins and diet were mixed at equal proportion (bringing final concentration of protein to 500 µg/mL) and dispensed into test arena. Untreated control was prepared by mixing the buffer with the diet. Individual neonates were infested into the test arena, the assays were incubated at 25°C, 60% RH. Twenty neonate nymphs were tested for each concentration, protein and in 2 replicates. A control was maintained with 25mM Sodium Carbonate buffer, pH 10, in 1:5 diluted *Lygus* diet. Mortality of the insects was determined on the fifth day. Mortality values were calculated by the following formula: (% mortality in treatment - % mortality in control)/(100 - % mortality in control) x 100. Table 4A tabulates *Amrasca* activity for TIC807 and TIC807_M13 at 5 different concentrations.

Table 4A. TIC807 and TIC807_M13 Percent Mortality Directed to *Amrasca* species

SEQ ID NO:	Protein Name	Mortality (%)				
		500 µg/mL	166.66 µg/mL	55.55 µg/mL	18.51 µg/mL	6.17 µg/mL
2	TIC807	100 %	55.88 %	17.64 %	0	0
34	TIC807_M13	100 %	88.23 %	73.52 %	44.11 %	26.47 %

[0081] LC50 values were determined for TIC807 and TIC807_M13 in a separate test. SEQ ID NO:2 (TIC807) exhibited a LC50 value of 116.79 µg/mL and LC90 of 437.27 µg/mL. SEQ ID NO:34 (TIC807_M13) exhibited a LC50 value of 7.59 µg/mL and LC90 value of 239.8 µg/mL.

[0082] A feeding assay as described for *Amrasca devastans* is used to test eHTP's for improved insecticidal activity and/or enhanced insecticidal specificity against *Empoasca fabae*. TIC807 variants with improved insecticidal activity or enhanced insecticidal specificity against *Lygus hesperus* and *Lygus lineolaris* exhibit improved insecticidal activity against *Empoasca fabae*.

[0083] The LC50 values of Cry51Aa1 (SEQ ID NO:182), for TIC807 (SEQ ID NO:2), TIC807_M2 (SEQ ID NO:8), TIC807_M10 (SEQ ID NO:30) and TIC807-M13 (SEQ ID NO:34) against *Lygus hesperus* and *Lygus lineolaris* were determined in one testset. TIC807_M2, TIC807_M10 and TIC807_M12 exhibit improved LC50 values compared to Cry51Aa1.

[0084] It should be apparent to those skilled in the art that variations to this procedure can exist that should not affect results.

Table 4B. Iterative engineering-testing-selecting of eHTP's against *Lygus* spp. resulted in 267 proteins with enhanced *Lygus* inhibitory spectrum and/or improved *Lygus* inhibitory activity against *Lygus* spp. compared to TIC807.

Amino acid differences compared to TIC807 parent protein					Lygus hesperus				Lygus ilneolaris			
SEQ ID NO:	Protein Name	Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino difference(s) in the first patch	Amino acid difference(s) in the second patch	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein *	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein *		
2	TIC807	Parent	Parent	Parent	73	1	0	>223	1	0		
6	TIC807_M1	F147A	F147A	None	23	3	ND	100	at least 2	ND		
8	TIC807_M2	F46S, Y54H, S167R, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201	6.0	12	39	>223	-	14		
10	TIC807_M3	F46S, Y54H, T93A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	2.9	25	43	ND	-	ND		
12	TIC807_M4	F46S, Y54H, S95A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	2.4	30	20	ND	-	ND		
14	TIC807_M5	F46S, Y54H, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	1.1	66	34	ND	-	ND		
16	TIC807_M8	F46S, Y54H, S95A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A, F147A	S217N, then a contiguous triple deletion in residue range 196-201	0.8	91	ND	223	at least 1	ND		
18	TIC807_M6	F46S, Y54H, T93A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A, F147A	S217N, then a contiguous triple deletion in residue range 196-201	1.5	50	ND	ND	-	ND		
20	TIC807_M7	F46S, Y54H, Q149E, S167R, S217N, then a contiguous triple deletion in residue range 196-201	Q149E	S217N, then a contiguous triple deletion in residue range 196-201	1.4	52	62	ND	-	49		
28	TIC807_M9	F46S, Y54H, S95A, F147A, S167R, P219R, then a contiguous triple deletion in residue range 196-201	S95A, F147A	P219R, then a contiguous triple deletion in residue range 196-201	9.9	7	ND	8.3	at least 27	ND		

SEQ ID NO.	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino acid difference(s) relative to SEQ ID NO:2 (TIC807) deletion in residue range 196-201	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch range 196-201		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
30	TIC807 M10	F46S, Y54H, S95A, F147A, S167R, P219R, V251A, then a contiguous triple deletion in residue range 196-201	S95A, F147A, V251A	P219R, then a contiguous triple deletion in residue range 196-201		0.6	122	ND		4.8	at least 46	ND	
32	TIC807 M11	F46S, Y54H, S95A, F147A, S167R, P219R, R273W, then a contiguous triple deletion in residue range 196-201	S95A, F147A	P219R, then a contiguous triple deletion in residue range 196-201		1.4	54	ND		5.9	at least 38	ND	
34	TIC807 M13	F46S, Y54H, S95A, F147S, Q149E, S167R, P219R, R273W, then a contiguous triple deletion in residue range 196-201	S95A, F147A, Q149E	P219R, then a contiguous triple deletion in residue range 196-201		0.3	243	ND		0.85	at least 262	ND	
36	TIC807 M12	F46S, Y54H, S95A, F147A, S167R, P219R, N239A, V251A, then a contiguous triple deletion in residue range 196-201	S95A, F147A, V251A	P219R, then a contiguous triple deletion in residue range 196-201		0.4	182	ND		1.2	at least 186	ND	
37	TIC807 37	TIC807 HYS deletion	none	a contiguous triple deletion in HYSHYS residues (positions 196-201)		22.4	3	ND		ND	-	ND	
38	TIC807 38	F46S, Y54H, F138V, S167R, S217N, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	41		ND	-	44	
39	TIC807 39	F46S, Y54H, S167R, S217N, H287F, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	57		ND	-	31	
40	TIC807 40	F46S, I52M, Y54H, S167R, S217N, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	52		ND	-	36	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
		deletion in residue range 196-201											
41	TIC807_41	N12D, F46S, Y54H, S167R, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	52		ND	-	31	
42	TIC807_42	F46S, Y54H, S167R, N180D, S217N, then a contiguous triple deletion in residue range 196-201	N180D	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	63		ND	-	50	
43	TIC807_43	F46S, Y54H, S167R, then a contiguous triple deletion in residue range 196-201	none	a contiguous triple deletion in HYSHYS residues (positions 196-201)		ND	-	46		ND	-	87	
44	TIC807_44	F46S, Y54H, S167R, S217N, P219R, then a contiguous triple deletion in residue range 196-201	none	S217N, P219R, then a contiguous triple deletion in residue range 196-201		ND	-	30		ND	-	94	
45	TIC807_45	F46S, Y54H, S159T, S167R, S217N, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	67		ND	-	21	
46	TIC807_46	F46S, Y54H, S167R, S217N, T247K, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	52		ND	-	31	
47	TIC807_47	F46S, Y54H, S167R, S217N, V244L, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	59		ND	-	31	
48	TIC807_48	F46S, Y54H, S167R, S217N, V244L, T247K, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	58		ND	-	34	
49	TIC807_49	F46S, Y54H, S167R, S217N, W223Y, then a	none	S217N, W223Y, then a contiguous		ND	-	17		ND	-	13	

Amino acid differences compared to TIC807 parent protein					Lygus hesperus			Lygus lineolaris		
SEQ ID NO:	Protein Name	Amino acid difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch	LCSO value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LCSO value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
		contiguous triple deletion in residue range 196-201		triple deletion in residue range 196-201						
50	TIC807_50	F46S, Y54H, S167R, S217N, Y246F, then a contiguous triple deletion in residue range 196-201	none	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	48	ND	-	19
51	TIC807_51	F46S, Y54H, F147A, G128A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	77	ND	-	ND
52	TIC807_52	F46S, Y54H, F147A, S167R, S217N, M300A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	73	ND	-	ND
53	TIC807_53	F46S, Y54H, F147A, S167R, S217N, S293A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	73	ND	-	ND
54	TIC807_54	F46S, Y54H, F147A, S167R, S217N, H287A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	67	ND	-	ND
55	TIC807_55	F46S, Y54H, F147A, S167R, S217N, T274A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	65	ND	-	ND
56	TIC807_56	F46S, Y54H, F147A, S167R, S217N, R282A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	64	ND	-	ND
57	TIC807_57	F46S, Y54H, T93A, S167R, S217N, T308A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	63	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
58	TIC807_58	F46S, Y54H, Q70A, T93A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	61		ND	-	ND	
59	TIC807_59	F46S, Y54H, E125A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	61		ND	-	ND	
60	TIC807_60	F46S, Y54H, F147A, S167R, S217N, T247A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	61		ND	-	ND	
61	TIC807_61	F46S, Y54H, T93A, S167R, S217N, P305A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	59		ND	-	ND	
62	TIC807_62	F46S, Y54H, F147A, S167R, S217N, I306A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	57		ND	-	ND	
63	TIC807_63	F46S, Y54H, T93A, S167R, S217N, R282A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	56		ND	-	ND	
64	TIC807_64	F46S, Y54H, T93A, S167R, S217N, T308A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	55		ND	-	ND	
65	TIC807_65	F46S, Y54H, T93A, S167R, S217N, M300A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	55		ND	-	ND	
66	TIC807_66	F46S, Y54H, T93A, S167R, S217N, H287A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	53		ND	-	ND	

Amino acid differences compared to TIC807 parent protein					<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
SEQ ID NO:	Protein Name	Amino acid difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*		
67	TIC807_67	F46S, Y54H, S95A, S167R, S217N, M300A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	52	ND	-	ND		
68	TIC807_68	F46S, Y54H, F147A, S167R, S217N, I275A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	52	ND	-	ND		
69	TIC807_69	F46S, Y54H, S95A, S167R, S217N, T247A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	51	ND	-	ND		
70	TIC807_70	F46S, Y54H, F147A, S167R, V175A, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	50	ND	-	ND		
71	TIC807_71	F46S, Y54H, F147A, S159A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	50	ND	-	ND		
72	TIC807_72	F46S, Y54H, S95A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A, F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	48	ND	-	ND		
73	TIC807_73	F46S, Y54H, F147A, S167R, L187A, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	47	ND	-	ND		
74	TIC807_74	F46S, Y54H, T93A, S167R, T182A, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	47	ND	-	ND		
75	TIC807_75	F46S, Y54H, F147A, S217N, then a	F147A	S217N, then a	ND	-	46	ND	-	ND		

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
		S167R, S217N, T245A, then a contiguous triple deletion in residue range 196-201											
76	TIC807_76	F46S, Y54H, T93A, S167R, S217N, S249A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	46		ND	-	ND	
77	TIC807_77	F46S, Y54H, T93A, Q149A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A, Q149A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	45		ND	-	ND	
78	TIC807_78	F46S, Y54H, T93A, S151A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A, S151A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	45		ND	-	ND	
79	TIC807_79	F46S, Y54H, Q70A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	45		ND	-	ND	
80	TIC807_80	F46S, Y54H, S95A, Q148A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	44		ND	-	ND	
81	TIC807_81	F46S, Y54H, T93A, S167R, S217N, T274A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	44		ND	-	ND	
82	TIC807_82	F46S, Y54H, T93A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	43		ND	-	ND	
83	TIC807_83	F46S, Y54H, T93A, S167R, M177A, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	42		ND	-	ND	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein					Lygus hesperus				Lygus lineolaris			
		Amino difference(s) relative to SEQ ID NO:2 (TIC807) 196-201	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
84	TIC807_84	F46S, Y54H, S95A, S167R, S217N, V250A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	42	ND	-	ND	-	ND		
85	TIC807_85	F46S, Y54H, T93A, E155A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	42	ND	-	ND	-	ND		
86	TIC807_86	F46S, Y54H, I134A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	41	ND	-	ND	-	ND		
87	TIC807_87	F46S, Y54H, T93A, S167R, S217N, T245A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	41	ND	-	ND	-	ND		
88	TIC807_88	F46S, Y54H, T93A, S167R, S217N, then a contiguous triple deletion in residue range 196-201 followed by a S198A	T93A	S217N, then a contiguous triple deletion in residue range 196-201 followed by S198A	ND	-	40	ND	-	ND	-	ND		
89	TIC807_89	F46S, Y54H, F147A, S167R, S217N, N295A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	40	ND	-	ND	-	ND		
90	TIC807_90	F46S, Y54H, A87S, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	38	ND	-	ND	-	ND		
91	TIC807_91	F46S, Y54H, F147A, S167R, S217N, S249A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	38	ND	-	ND	-	ND		
92	TIC807_92	F46S, Y54H, S95A, S217N, then a	S95A	S217N, then a	ND	-	37	ND	-	ND	-	ND		

SEQ ID NO:	Amino acid differences compared to TIC807 parent protein					<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
	Protein Name	Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
		S167R, S217N, I275A, then a contiguous triple deletion in residue range 196-201		contiguous triple deletion in residue range 196-201									
93	TIC807_93	F46S, Y54H, T93A, A139S, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	37		ND	-	ND	
94	TIC807_94	F46S, Y54H, F147A, S167R, S217N, P305A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	36		ND	-	ND	
95	TIC807_95	F46S, Y54H, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201 followed by a S198A	F147A	S217N, then a contiguous triple deletion in residue range 196-201 followed by S198A		ND	-	36		ND	-	ND	
96	TIC807_96	F46S, Y54H, F147A, S167R, S217N, S252A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	36		ND	-	ND	
97	TIC807_97	F46S, Y54H, T93A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A, F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	36		ND	-	ND	
98	TIC807_98	F46S, Y54H, F147A, S167R, S217N, V250A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	36		ND	-	ND	
99	TIC807_99	F46S, Y54H, F147A, S167R, S217N, T243A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	35		ND	-	ND	
100	TIC807_100	F46S, Y54H, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	35		ND	-	ND	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
		deletion in residue range 196-201 followed by a Y197A											
101	TIC807_101	F46S, Y54H, S95A, S167R, S217N, N295A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	35		ND	-	ND	
102	TIC807_102	F46S, Y54H, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	34		ND	-	ND	
103	TIC807_103	F46S, Y54H, F147A, S167R, S217N, E299A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	32		ND	-	ND	
104	TIC807_104	F46S, Y54H, S95A, S167R, S217N, R282A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	32		ND	-	ND	
105	TIC807_105	F46S, Y54H, T93A, S167R, S217N, I306A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	32		ND	-	ND	
106	TIC807_106	F46S, Y54H, S95A, S167R, S217N, S249A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	30		ND	-	ND	
107	TIC807_107	F46S, Y54H, A87S, T93A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	30		ND	-	ND	
108	TIC807_108	F46S, Y54H, T93A, S159A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	29		ND	-	ND	

SEQ ID NO:	Amino acid differences compared to TIC807 parent protein					<i>Lygus hesperus</i>			<i>Lygus lineolaris</i>		
	Protein Name	Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
109	TIC807_109	F46S, Y54H, F147A, S167R, S217N, T303A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	28	ND	-	ND
110	TIC807_110	F46S, Y54H, T93A, Q148A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	27	ND	-	ND
111	TIC807_111	F46S, Y54H, S95A, S167R, V175A, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	27	ND	-	ND
112	TIC807_112	F46S, Y54H, F147A, S167R, S217N, D241A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	27	ND	-	ND
113	TIC807_113	F46S, Y54H, S95A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A, F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	26	ND	-	ND
114	TIC807_114	F46S, Y54H, F147A, S167R, M177A, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	26	ND	-	ND
115	TIC807_115	F46S, Y54H, F147A, S167R, S217N, M300A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	26	ND	-	ND
116	TIC807_116	F46S, Y54H, S95A, S167R, S217N, P305A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	26	ND	-	ND
117	TIC807_117	F46S, Y54H, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	25	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				Lygus hesperus			Lygus lineolaris		
		Amino acid difference(s) relative to SEQ ID NO:2 (TIC807) deletion in residue range 196-201 followed by H196A	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch range 196-201 followed by H196A	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein *	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein *	
118	TIC807_118	F46S, Y54H, A139S, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	25	ND	-	ND	
119	TIC807_119	F46S, Y54H, T93A, S167R, S217N, N295A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	25	ND	-	ND	
120	TIC807_120	F46S, Y54H, T93A, T145A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	24	ND	-	ND	
121	TIC807_121	F46S, Y54H, S117A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	24	ND	-	ND	
122	TIC807_122	F46S, Y54H, T93A, S167R, S217N, I275A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	23	ND	-	ND	
123	TIC807_123	F46S, Y54H, S95A, S167R, S217N, H287A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	23	ND	-	ND	
124	TIC807_124	F46S, Y54H, S95A, G105A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	21	ND	-	ND	
125	TIC807_125	F46S, Y54H, S95A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201	ND	-	20	ND	-	ND	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				Lygus hesperus			Lygus lineolaris		
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
126	TIC807_126	F46S, Y54H, S95A, I134A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	20	ND	-	ND
127	TIC807_127	F46S, Y54H, T93A, S167R, S217N, M300A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	20	ND	-	ND
128	TIC807_128	F46S, Y54H, T93A, S167R, S217N, T303A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	18	ND	-	ND
129	TIC807_129	F46S, Y54H, T93A, A150S, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	18	ND	-	ND
130	TIC807_130	F46S, Y54H, S95A, E155A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	18	ND	-	ND
131	TIC807_131	F46S, Y54H, T93A, T145A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	17	ND	-	ND
132	TIC807_132	F46S, Y54H, S95A, S167R, W208A, S217N, then a contiguous triple deletion in residue range 196-201	S95A	W208A, S217N, then a contiguous triple deletion in residue range 196-201		ND	-	17	ND	-	ND
133	TIC807_133	F46S, Y54H, S95A, S167R, T182A, S217N, then a contiguous triple deletion in residue range 196-201	S95A, T182A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	17	ND	-	ND
134	TIC807_134	F46S, Y54H, T93A, S167R, S217N, T243A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	17	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino acid difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
135	TIC807_135	F46S, Y54H, S95A, S167R, S217N, I306A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	16		ND	-	ND	
136	TIC807_136	F46S, Y54H, S95A, S117A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	16		ND	-	ND	
137	TIC807_137	F46S, Y54H, T93A, S119A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	15		ND	-	ND	
138	TIC807_138	F46S, Y54H, T68A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	15		ND	-	ND	
139	TIC807_139	F46S, Y54H, G105A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	15		ND	-	ND	
140	TIC807_140	F46S, Y54H, S95A, E125A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	15		ND	-	ND	
141	TIC807_141	F46S, Y54H, T93A, E155A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	14		ND	-	ND	
142	TIC807_142	F46S, Y54H, T93A, S167R, S217N, P305A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	14		ND	-	ND	
143	TIC807_143	F46S, Y54H, S95A, S217N, then a	S95A	S217N, then a		ND	-	13		ND	-	ND	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein					Lygus hesperus			Lygus lineolaris		
		Amino acid difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch	Amino acid difference(s) in the third patch	Amino acid difference(s) in the fourth patch	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
		S167R, M177A, S217N, then a contiguous triple deletion in residue range 196-201			contiguous triple deletion in residue range 196-201							
144	TIC807_144	F46S, Y54H, T93A, S167R, S217N, then a contiguous triple deletion in residue range 196-201 followed by H196A	T93A	S217N, then a contiguous triple deletion in residue range 196-201 followed by H196A		ND	-	13	ND	-	ND	
145	TIC807_145	F46S, Y54H, T93A, D153A, S167R, S217N, then a contiguous triple deletion in residue range 196-201-T93A-D153A	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	11	ND	-	ND	
146	TIC807_146	F46S, Y54H, F147A, S167R, S217N, W208A, then a contiguous triple deletion in residue range 196-201	F147A			ND	-	10	ND	-	ND	
147	TIC807_147	F46S, Y54H, T93A, S167R, S217N, I306A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	9	ND	-	ND	
148	TIC807_148	F46S, Y54H, S95A, S167R, S217N, then a contiguous triple deletion in residue range 196-201 followed by H196A	S95A	S217N, then a contiguous triple deletion in residue range 196-201 followed by H196A		ND	-	9	ND	-	ND	
149	TIC807_149	F46S, Y54H, S95A, Q149A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A, Q149A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	9	ND	-	ND	
150	TIC807_150	F46S, Y54H, S95A, S167R, S217N, S293A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	8	ND	-	ND	
151	TIC807_151	F46S, Y54H, S95A, A150S, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	8	ND	-	ND	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				Lygus hesperus			Lygus lineolaris		
		Amino acid difference(s) relative to SEQ ID NO:2 (TIC807) then a contiguous triple deletion in residue range 196-201	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	Told increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	Told increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
152	TIC807_152	F46S, Y54H, S119A, F147A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	7	ND	-	ND
153	TIC807_153	F46S, Y54H, S95A, S167A, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	7	ND	-	ND
154	TIC807_154	F46S, Y54H, F147A, I158A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	7	ND	-	ND
155	TIC807_155	F46S, Y54H, S95A, S167R, S217N, M300A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	7	ND	-	ND
156	TIC807_156	F46S, Y54H, F147A, T182A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	F147A, T182A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	6	ND	-	ND
157	TIC807_157	F46S, Y54H, F147A, S167R, S217N, F235A, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	5	ND	-	ND
158	TIC807_158	F46S, Y54H, F147A, S167A, S217N, then a contiguous triple deletion in residue range 196-201	F147A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	5	ND	-	ND
159	TIC807_159	F46S, Y54H, T93A, S167R, S217N, D241A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	5	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>			<i>Lygus lineolaris</i>		
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
160	TIC807_160	F46S, Y54H, S95A, S167R, S217N, T274A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	4	ND	-	ND
161	TIC807_161	F46S, Y54H, T93A, Q148A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	4	ND	-	ND
162	TIC807_162	F46S, Y54H, S95A, S167R, S217N, D241A, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	4	ND	-	ND
163	TIC807_163	F46S, Y54H, S95A, E155A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	S95A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	4	ND	-	ND
164	TIC807_164	F46S, Y54H, T93A, S167R, L187A, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	4	ND	-	ND
165	TIC807_165	F46S, Y54H, T93A, S167R, S217N, T303A, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	4	ND	-	ND
166	TIC807_166	F46S, Y54H, T93A, E155A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	3	ND	-	ND
167	TIC807_167	F46S, Y54H, T93A, D153A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	3	ND	-	ND
168	TIC807_168	F46S, Y54H, T93A, I134A, S167R, S217N, then a contiguous triple deletion in residue range 196-201	T93A	S217N, then a contiguous triple deletion in residue range 196-201		ND	-	3	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein					<i>Lygus hesperus</i>			<i>Lygus lineolaris</i>		
		Amino acid difference(s) relative to SEQ ID NO:2 (TIC807) deletion in residue range 196-201	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch range 196-201			LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
169	TIC807_169	N137T	none	None			ND	-	44*	ND	-	ND
169	TIC807_169	E135S	none	None			ND	-	20*	ND	-	ND
169	TIC807_169	N137T, E135S	none	None			ND	-	34*	ND	-	ND
169	TIC807_169	E135T, N137D	none	None			ND	-	16*	ND	-	ND
169	TIC807_169	T133E	none	None			ND	-	60*	ND	-	ND
169	TIC807_169	E135A, N137G	none	None			ND	-	50*	ND	-	ND
170	TIC807_170	E125C	none	None			ND	-	12	ND	-	ND
170	TIC807_170	E125H	none	None			ND	-	38	ND	-	ND
170	TIC807_170	E125R	none	None			ND	-	14	ND	-	ND
170	TIC807_170	E125F	none	None			ND	-	33	ND	-	ND
170	TIC807_170	E125S	none	None			ND	-	24	ND	-	ND
170	TIC807_170	E125Q	none	None			ND	-	21	ND	-	ND
170	TIC807_170	E125K	none	None			ND	-	20	ND	-	ND
170	TIC807_170	E125T	none	None			ND	-	33	ND	-	ND
170	TIC807_170	E125N	none	None			ND	-	19	ND	-	ND
170	TIC807_170	E125A	none	None			ND	-	41	ND	-	ND
170	TIC807_170	E125L	none	None			ND	-	13	ND	-	ND
170	TIC807_170	E125V	none	None			ND	-	14	ND	-	ND
170	TIC807_170	E125M	none	None			ND	-	13	ND	-	ND
170	TIC807_170	E125D	none	None			ND	-	15	ND	-	ND
170	TIC807_170	E125Y	none	None			ND	-	38	ND	-	ND
171	TIC807_171	T133E	none	None			ND	-	23	ND	-	ND
171	TIC807_171	T133Y	none	None			ND	-	17	ND	-	ND
171	TIC807_171	T133W	none	None			ND	-	13	ND	-	ND
172	TIC807_172	I134V	none	None			ND	-	18	ND	-	ND
172	TIC807_172	I134L	none	None			ND	-	10	ND	-	ND
172	TIC807_172	I134F	none	None			ND	-	18	ND	-	ND
172	TIC807_172	I134K	none	None			ND	-	13	ND	-	ND
172	TIC807_172	I134C	none	None			ND	-	30	ND	-	ND
172	TIC807_172	I134M	none	None			ND	-	33	ND	-	ND
173	TIC807_173	E135V	none	None			ND	-	13	ND	-	ND
173	TIC807_173	E135W	none	None			ND	-	13	ND	-	ND
173	TIC807_173	E135T	none	None			ND	-	39	ND	-	ND
174	TIC807_174	N137H	none	None			ND	-	42	ND	-	ND
174	TIC807_174	N137Y	none	None			ND	-	17	ND	-	ND
174	TIC807_174	N137T	none	None			ND	-	31	ND	-	ND
174	TIC807_174	N137E	none	None			ND	-	32	ND	-	ND
174	TIC807_174	N137S	none	None			ND	-	24	ND	-	ND
174	TIC807_174	N137A	none	None			ND	-	24	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein					Lygus hesperus			Lygus lineolaris		
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch			LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
174	TIC807 174	N137Q	none	None			ND	-	21	ND	-	ND
174	TIC807 174	N137G	none	None			ND	-	18	ND	-	ND
174	TIC807 174	N137I	none	None			ND	-	10	ND	-	ND
174	TIC807 174	N137W	none	None			ND	-	17	ND	-	ND
174	TIC807 174	N137K	none	None			ND	-	66	ND	-	ND
174	TIC807 174	N137C	none	None			ND	-	19	ND	-	ND
174	TIC807 174	N137M	none	None			ND	-	41	ND	-	ND
174	TIC807 174	N137D	none	None			ND	-	69	ND	-	ND
174	TIC807 174	N137F	none	None			ND	-	13	ND	-	ND
174	TIC807 174	N137R	none	None			ND	-	37	ND	-	ND
175	TIC807 175	F147V	F147V	None			ND	-	87	ND	-	ND
175	TIC807 175	F147T	F147T	None			ND	-	68	ND	-	ND
175	TIC807 175	F147C	F147C	None			ND	-	74	ND	-	ND
175	TIC807 175	F147L	F147L	None			ND	-	62	ND	-	ND
175	TIC807 175	F147D	F147D	None			ND	-	51	ND	-	ND
175	TIC807 175	F147A	F147A	None			ND	-	57	ND	-	ND
175	TIC807 175	F147G	F147G	None			ND	-	56	ND	-	ND
175	TIC807 175	F147E	F147E	None			ND	-	50	ND	-	ND
175	TIC807 175	F147I	F147I	None			ND	-	69	ND	-	ND
175	TIC807 175	F147Y	F147Y	None			ND	-	67	ND	-	ND
175	TIC807 175	F147M	F147M	None			ND	-	64	ND	-	ND
175	TIC807 175	F147N	F147N	None			ND	-	64	ND	-	ND
175	TIC807 175	F147Q	F147Q	None			ND	-	50	ND	-	ND
175	TIC807 175	F147H	F147H	None			ND	-	60	ND	-	ND
175	TIC807 175	F147R	F147R	None			ND	-	20	ND	-	ND
175	TIC807 175	F147W	F147W	None			ND	-	82	ND	-	ND
175	TIC807 175	F147P	F147P	None			ND	-	7	ND	-	ND
176	TIC807 176	Q149D	Q149D	None			ND	-	92	ND	-	ND
176	TIC807 176	Q149E	Q149E	None			ND	-	89	ND	-	ND
176	TIC807 176	Q149C	Q149C	None			ND	-	87	ND	-	ND
176	TIC807 176	Q149A	Q149A	None			ND	-	76	ND	-	ND
176	TIC807 176	Q149F	Q149F	None			ND	-	54	ND	-	ND
177	TIC807 177	A150S	none	None			ND	-	34	ND	-	ND
177	TIC807 177	A150L	none	None			ND	-	24	ND	-	ND
177	TIC807 177	A150V	none	None			ND	-	25	ND	-	ND
177	TIC807 177	A150G	none	None			ND	-	28	ND	-	ND
177	TIC807 177	A150D	none	None			ND	-	19	ND	-	ND
177	TIC807 177	A150W	none	None			ND	-	13	ND	-	ND
177	TIC807 177	A150E	none	None			ND	-	24	ND	-	ND
177	TIC807 177	A150N	none	None			ND	-	18	ND	-	ND
177	TIC807 177	A150Y	none	None			ND	-	11	ND	-	ND

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>				<i>Lygus lineolaris</i>			
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino difference(s) in the first patch	Amino difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*	
177	TIC807_177	A150F	none	None		ND	-	11		ND	-	ND	
177	TIC807_177	A150P	none	None		ND	-	11		ND	-	ND	
177	TIC807_177	A150K	none	None		ND	-	17		ND	-	ND	
177	TIC807_177	A150T	none	None		ND	-	17		ND	-	ND	
177	TIC807_177	A150Q	none	None		ND	-	11		ND	-	ND	
177	TIC807_177	A150R	none	None		ND	-	11		ND	-	ND	
178	TIC807_178	E155C	none	None		ND	-	82		ND	-	ND	
178	TIC807_178	E155T	none	None		ND	-	36		ND	-	ND	
178	TIC807_178	E155K	none	None		ND	-	28		ND	-	ND	
178	TIC807_178	E155D	none	None		ND	-	22		ND	-	ND	
178	TIC807_178	E155H	none	None		ND	-	22		ND	-	ND	
178	TIC807_178	E155Y	none	None		ND	-	16		ND	-	ND	
178	TIC807_178	E155Q	none	None		ND	-	16		ND	-	ND	
178	TIC807_178	E155L	none	None		ND	-	15		ND	-	ND	
178	TIC807_178	E155N	none	None		ND	-	14		ND	-	ND	
178	TIC807_178	E155T	none	None		ND	-	13		ND	-	ND	
178	TIC807_178	E155A	none	None		ND	-	11		ND	-	ND	
178	TIC807_178	E155F	none	None		ND	-	7		ND	-	ND	
178	TIC807_178	E155R	none	None		ND	-	6		ND	-	ND	
178	TIC807_178	E155M	none	None		ND	-	6		ND	-	ND	
178	TIC807_178	E155P	none	None		ND	-	5		ND	-	ND	
178	TIC807_178	E155W	none	None		ND	-	5		ND	-	ND	
178	TIC807_178	E155S	none	None		ND	-	4		ND	-	ND	
178	TIC807_178	E155V	none	None		ND	-	4		ND	-	ND	
179	TIC807_179	N157C	none	None		ND	-	86		ND	-	ND	
179	TIC807_179	N157D	none	None		ND	-	64		ND	-	ND	
179	TIC807_179	N157W	none	None		ND	-	52		ND	-	ND	
179	TIC807_179	N157Y	none	None		ND	-	39		ND	-	ND	
179	TIC807_179	N157M	none	None		ND	-	22		ND	-	ND	
179	TIC807_179	N157A	none	None		ND	-	22		ND	-	ND	
179	TIC807_179	N157F	none	None		ND	-	20		ND	-	ND	
179	TIC807_179	N157V	none	None		ND	-	18		ND	-	ND	
179	TIC807_179	N157L	none	None		ND	-	18		ND	-	ND	
179	TIC807_179	N157P	none	None		ND	-	18		ND	-	ND	
179	TIC807_179	N157E	none	None		ND	-	17		ND	-	ND	
179	TIC807_179	N157T	none	None		ND	-	8		ND	-	ND	
179	TIC807_179	N157G	none	None		ND	-	7		ND	-	ND	
179	TIC807_179	N157I	none	None		ND	-	7		ND	-	ND	
179	TIC807_179	N157R	none	None		ND	-	6		ND	-	ND	
202	TIC807_M8_d5C	F46S, Y54H, S95A, F147A, S167R, S217N,	S95A, F147A	S217N, then a contiguous triple		ND	-	92*		ND	-	ND	

SEQ ID NO:	Protein Name	Amino acid differences compared to TIC807 parent protein				<i>Lygus hesperus</i>			<i>Lygus lineolaris</i>		
		Amino difference(s) relative to SEQ ID NO:2 (TIC807)	Amino acid difference(s) in the first patch	Amino acid difference(s) in the second patch		LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 1-3 µg/mL protein*	LC50 value (µg/mL)	fold increased toxicity (LC50)	% Mortality at about 100 µg/mL protein*
		then a five amino acid deletion at 305-309, then a contiguous triple deletion in residue range 196-201									
		F46S, Y54H, S95A, F147S, Q149E, S167R, P219R, S249R, V251E, Q253R, R273W, then a contiguous triple deletion in residue range 196-201	S95A, Q149E, Q253R	P219R, then a contiguous triple deletion in residue range 196-201							
204	TIC807 M14					ND	-	-	ND	-	60*

ND = Not Determined; * tested at about 5 µg/mL.

Example 5: Insect inhibitory activities of protein members of the present invention

[0085] Proteins of the present invention, such as but not limited to TIC807_M1 (SEQ ID NO:6), TIC807_M2 (SEQ ID NO:8), TIC807_M3 (SEQ ID NO:10), TIC807_M4 (SEQ ID NO:12), TIC807_M5 (SEQ ID NO:14), TIC807_M6 (SEQ ID NO:18), TIC807_M7 (SEQ ID NO:20), TIC807_M8 (SEQ ID NO:16), TIC807_M9 (SEQ ID NO:28), TIC807_M11 (SEQ ID NO:32), TIC807_M13 (SEQ ID NO:34), and TIC807_M12 (SEQ ID NO:36), are prepared and tested for bioactivity against pests of plants other than from *Lygus*.

[0086] Proteins TIC807_M10 (SEQ ID NO:30), TIC807_M11 (SEQ ID NO:32), TIC807_M12 (SEQ ID NO:36), and TIC807_M13 (SEQ ID NO:34) were prepared and tested for bioactivity against pests from the order Lepidoptera, Coleoptera, Heteroptera, and Homoptera. Protein TIC807_M5 (SEQ ID NO:14) was prepared and tested for bioactivity against Coleopteran pests. Bioassays were conducted to evaluate the effects of these proteins on insects as shown in Table 5. Feeding assays were conducted on an artificial diet containing the insecticidal protein. The insecticidal protein was prepared as described in example 3 and topically applied using an insect-specific artificial diet, depending on the insect being tested. The toxin was suspended in a buffer and applied at a rate of 500 µg/mL of sample per well, and in the case of TIC807_M5 of 1000 µg/mL, and then allowed to dry. Mean stunting scores and population mortalities were determined on three populations of 8 insects per insect species tested. Results were expressed as positive (+) for insect reactions such as stunting and mortality that were statistically significant compared to the untreated control. Results were expressed as negative (-) if the insects were similar to the UTC, that is, feeding diet to which the above buffer only has been applied.

Table 5. eHTP's demonstrate additional insect inhibitory activities against pests other than *Lygus* spp.

Protein	µg/mL	CPB	WCR	ECB	SWCB	CEW	FAW	SGSB	NBSB	GPA
UTC	0	-	-	-	-	-	-	-	-	-
TIC807_M5	1000	+	-	ND	ND	ND	ND	ND	ND	ND
TIC807_M10	500	-	-	-	-	-	-	-	-	-
TIC807_M11	500	+	-	-	-	-	-	-	-	-
TIC807_M12	500	-	-	-	-	-	-	-	-	-
TIC807_M13	500	+	-	-	-	-	-	-	-	-

UTC = UnTreated Control; ND = Not Determined

CPB = Colorado potato beetle (*Leptinotarsa decemlineata*); WCR = western corn rootworm (*Diabrotica virgifera*); ECB = European corn borer (*Ostrinia nubilalis*); southwestern corn borer (*Diatraea grandiosella*); CEW = corn earworm (*Helicoverpa zea*); FAW = Fall armyworm (*Spodoptera*

frugiperda); SGSB = southern green stink bug (*Nezara viridula*); NBSB = neotropical brown stink bug (*Euschistus heros*); GPA = Green peach aphid (*Myzus persicae*).

[0087] The proteins of the present invention are also tested for bioactivity against a pest from the phylum Nematoda.

Example 6: Plants expressing proteins of the present invention exhibit insect inhibitory activity

[0088] This example illustrates expression of proteins of the present invention in plants, and demonstrates that cotton plants expressing proteins of the present invention exhibit insect inhibitory activity.

[0089] Polynucleotide segments for use in expression of the proteins of the present invention in plants are made according to the methods set forth in US Patent No. 7,741,118. For example, toxin proteins having the amino acid sequence as set forth in SEQ ID NO:4 (TIC807_4), SEQ ID NO:6 (TIC807_M1), SEQ ID NO:8 (TIC807_M2), SEQ ID NO:10 (TIC807_M3), SEQ ID NO:12 (TIC807_M4), SEQ ID NO:14 (TIC807_M5), SEQ ID NO:16 (TIC807_M8), SEQ ID NO:18 (TIC807_M6), SEQ ID NO:20 (TIC807_M7), SEQ ID NO:22 (TIC807_22), SEQ ID NO:24 (TIC807_24), SEQ ID NO:26 (TIC807_26), SEQ ID NO:28 (TIC807_M9), SEQ ID NO:30 (TIC807_M10), SEQ ID NO:32 (TIC807_M11), and SEQ ID NO:34 (TIC807_M13), are expressed from polynucleotide segments designed for use in plants and encoding the proteins of the present invention, including the polynucleotide sequences as set forth in SEQ ID NO:186, SEQ ID NO:187, SEQ ID NO:188, SEQ ID NO:189, SEQ ID NO:190, SEQ ID NO:191, SEQ ID NO:192, SEQ ID NO:193, SEQ ID NO:194, SEQ ID NO:195, SEQ ID NO:196, SEQ ID NO:197, SEQ ID NO:198, SEQ ID NO:199, SEQ ID NO:200, and SEQ ID NO:201, respectively.

[0090] It is intended that polynucleotide segments (or polynucleotide molecules) encoding each of the variant proteins or insect inhibitory fragments thereof, be used alone or in combination with each other, or in combination with other insect inhibitory proteins or insect inhibitory agents such as dsRNA mediated gene suppression molecules. Such combinations designed to work in synergistic or compatible mechanism with the proteins of the present invention. The intention of these combinations is to achieve plants and plant cells protected from pest, particularly insect pest, infestation. The specific variant proteins within the scope of the invention include the proteins corresponding to SEQ ID NOs listed in Table 4B and described throughout the application as filed.

[0091] Polynucleotide segments from SEQ ID NO:188 (encodes for TIC807_M2, SEQ ID NO:8) and from SEQ ID NO:192 (encodes for TIC807_M8, SEQ ID NO:16) were each recombinantly engineered into expression constructs for cotton transformation.

[0092] Transgenic cotton plants (recombinant cotton plants) were produced and tested for efficacy. Regenerated (R0) transgenic plants were selected that were low in copy number and high in expression of the respective variant protein, as determined by various quantitative and semi-quantitative methods, e.g. PCR, ELISAs and Westerns. Expression levels in R0 cotton leaf tissue typically ranged from 0.5 to 500 ppm fresh weight. R0 plants expressing high levels of protein were transferred to soil and selfed. Thirty seed from each of the selfed R0 plants were planted and progeny homozygous for the transgene were grown to flowering. Eleven to 18 plants per 4 to 5 events per each construct of this example were tested for efficacy against *Lygus* (Tables 6A, 6B, and 6C). The untransformed cotton cultivar, plants from the pooled negative segregate population (progeny not containing the transgene), and plants expressing TIC807 parent protein served as negative controls. A branch of a flowering stage cotton plant was enclosed in a mesh bag made from breathable plastic 'pollination' sleeves (Vilutis and Co. Inc., Frankfort, IL), and multiple branches set up in similar fashion. Each mesh bag was secured at the stem using a twist tie. About 4-6 *Lygus hesperus* nymphs (<24 hours post-hatch) were placed into a 1.4 ml conical tube (Matrix Technologies Corp., NH). The branch inside a mesh bag was infested with nymphs by sliding the uncapped conical tube into the mesh bag. Insects were allowed to feed for a period of 10-11 days before all surviving insects in the mesh bag were collected on dry ice. Survivors were weighed to obtain a gross mass. Percent mortality and mean survivor mass were calculated. Missing insects were included in the mortality percent mortality calculation. As shown in Tables 6A, 6B, and 6C, cotton plants expressing the variant proteins TIC807_M2 and TIC807_M8 significantly impacted the growth and development of *Lygus hesperus* nymphs. Based on these results, these plants, seed, expression constructs were advanced for further development.

Table 6A. Mean % mortality determined from flowering stage *Lygus* feeding assays with cotton plants expressing the variant TIC807 proteins TIC807_M2 and TIC807_M8.

Plant Event ID	Protein	N	Mean % Mortality	Std Dev	SEM	Lo 95%	Up 95%	Mortality t group
64	TIC807_M8	18	88.889	19.967	4.706	68.959	88.818	A
49	TIC807_M8	18	75.556	22.288	5.253	64.472	86.639	A
91	TIC807_M2	18	74.444	20.356	4.798	64.321	84.567	A
20	TIC807_M8	18	73.333	19.403	4.573	63.685	82.982	A
15	TIC807_M8	18	66.667	25.668	6.050	53.902	79.431	AB
58	TIC807_M2	18	65.556	19.166	4.517	56.025	75.086	AB
48	TIC807_M2	18	64.444	21.206	4.998	53.899	74.990	AB
19	TIC807_M2	18	53.333	25.668	6.050	40.569	66.098	BC

Plant Event ID	Protein	N	Mean % Mortality	Std Dev	SEM	Lo 95%	Up 95%	Mortality t group
68	TIC807 M2	18	47.778	25.795	6.080	34.950	60.605	C
Negative		24	41.667	22.001	4.491	32.376	50.957	C

Table 6B. Mean Instar determined from flowering stage Lygus feeding assays with cotton plants expressing the variant TIC807 proteins TIC807 M2 and TIC807 M8.

Plant Event ID	Construct	N	Mean Instar	Std Dev	SEM	Lo 95%	Up 95%	Instar t group
64	TIC807 M8	11	3.936	0.552	0.166	3.266	4.007	C
68	TIC807 M2	16	3.949	0.803	0.201	3.521	4.377	BC
48	TIC807 M2	16	4.042	0.604	0.151	3.720	4.364	BC
58	TIC807 M2	17	4.069	0.802	0.194	3.657	4.481	BC
15	TIC807 M8	15	4.094	0.747	0.193	3.681	4.508	BC
19	TIC807 M2	17	4.100	0.698	0.169	3.741	4.459	BC
91	TIC807 M2	12	4.125	0.829	0.239	3.598	4.652	ABC
49	TIC807 M8	12	4.133	0.762	0.220	3.655	4.623	ABC
20	TIC807 M8	14	4.298	0.918	0.245	3.768	4.828	AB
Negative		24	4.599	0.774	0.158	4.273	4.926	A

Table 6C. Mean Survival Mass determined from flowering stage Lygus feeding assays with cotton plants expressing the variant TIC807 proteins TIC807 M2 and TIC807 M8.

Plant Event ID	Construct	N	Mean Survival Mass	Std Dev	SEM	Lo 95%	Up 95%	Survivor Mass t group
64	TIC807 M8	11	2.315	1.489	0.449	1.314	3.315	C
68	TIC807 M2	16	3.548	1.325	0.331	2.843	4.254	B
58	TIC807 M2	17	3.561	1.348	0.327	2.868	4.255	B
48	TIC807 M2	16	3.580	1.436	0.359	2.831	4.362	B
91	TIC807 M2	12	3.775	1.775	0.512	2.647	4.902	AB
49	TIC807 M8	12	3.837	2.135	0.616	2.481	5.193	AB
19	TIC807 M2	17	3.908	1.467	0.356	3.154	4.662	AB
20	TIC807 M8	14	3.918	1.950	0.521	2.792	5.044	AB
15	TIC807 M8	15	3.937	1.906	0.492	2.881	4.993	AB
Negative		24	4.735	1.179	0.241	4.237	5.233	A

Std Dev = standard deviation

SEM = Standard error on the mean

Lo 95% = Lower limit at 95% confidence interval

Up 95% = Upper limit at 95% confidence interval

T grouping = Using a least significant difference test, F value = 101.1756, df = 15, 44, Pr < 0.0001

[0093] In another example, cotton plants from five transgenic events expressing TIC807_M11 were tested in a field trial having natural *Lygus* infestation pressures. These plants demonstrated field efficacy compared to the non-transgenic recipient line (DP393 germplasm used for transformation). The average number of *Lygus lineolaris* insects on five plants per event was significantly lower than the average number of *Lygus lineolaris* insects on plants from the non-transgenic control. Seed cotton yield from plants from the five events was statistically comparable to seed cotton yield of the non-transgenic control, e.g. season-long square retention.

[0094] In another similar field trial, cotton plants from seven transgenic events expressing TIC807_M10 demonstrated field efficacy compared to the non-transgenic control. The average number of *Lygus lineolaris* insects on five plants per event was significantly lower than the average number of *Lygus lineolaris* insects on plants from the non-transgenic

control. Seed cotton yield from plants from three of the seven events was statistically higher than to seed cotton yield of the non-transgenic control.

[0095] In another example, cotton plants from thirty-four transgenic events expressing TIC807_M13 demonstrated growth chamber efficacy compared to the non-transgenic control. Mesh bags were placed around the whole cotton plants at flowering stage (instead of just around single branches described earlier in this example). Five plants per event were evaluated and the average number of *Lygus lineolaris* insects recovered (nymphs to adults to 2nd generation Lygus) per plant was significantly lower than the average number of *Lygus lineolaris* insects per non-transgenic plant.

[0096] Similar experiments are performed with plants expressing proteins listed in Table 1 and in Tables 4A and 4B.

Example 7: Tissue from alfalfa plants expressing proteins of the present invention exhibit insect inhibitory activity

[0097] This example illustrates expression of proteins of the present invention in alfalfa plants, and demonstrates that tissue from alfalfa plants expressing proteins of the present invention exhibit insect inhibitory activity.

[0098] Polynucleotide segment from SEQ ID NO:192 (encodes for TIC807_M8, SEQ ID NO:16) was recombinantly engineered into three differently configured expression constructs for alfalfa transformation. For purposes of data reporting, the three recombinant constructs are coded [ER], [ES], and [ET].

[0099] Transgenic alfalfa plants (recombinant alfalfa plants) were recovered from transformants that were outcrossed and then selfed. Recombinant alfalfa plants were selected that were low in copy number and high in TIC807 expression as determined by RT-PCR and semi-quantitative Western methods, respectively. Alfalfa plant tissue from ten separate events were pooled, lyophilized, ground, and resuspended in stock buffer, 25 mM NaCarb, pH10.5. Plant tissue from Alfalfa having no TIC807_M8 expressing transgene was prepared for use as control. Stock preparations were serially diluted 100, 300, and 900 fold for incorporation into Lygus diet. Using the feeding assay method of Example 4, mortality and stunting scores were determined on day 5 and compared to controls (See Tables 7A and 7B; data were analyzed using JMP4 statistical software). For each test sample and each dilution, three populations of eight nymphs were subjected to this bioassay. Stunting scores correspond to visual mass ratings where 0 = no difference to negative control, 1 = about 25%

less mass, 2 = about 50% less mass, and 3 = about 75% less mass. The average of the stunting scores for each population of eight nymphs is reported.

Table 7A. Mean % mortality determined from *Lygus* feeding assays with diet incorporated with tissue from alfalfa plants expressing the variant TIC807 protein TIC807_M8.

Construct	Sample Source	Dilution fold	Mean Stunting Score	Std Dev	SEM	Lower 95%	Upper 95%	t Grouping
[ER] TIC807_M8	Pooled Alfalfa tissue from 10 events per construct	100	2.00	0.00	0.00	2.00	2.00	CD
		300	0.00	0.00	0.00	0.00	0.00	E
		900	0.00	0.00	0.00	0.00	0.00	E
[ES] TIC807_M8		100	3.00	0.00	0.00	3.00	3.00	A
		300	2.33	0.58	0.33	0.90	3.77	BC
		900	0.00	0.00	0.00	0.00	0.00	E
[ET] TIC807_M8		100	2.67	0.58	0.33	1.23	4.10	AB
		300	1.67	0.58	0.33	0.23	3.10	D
		900	0.00	0.00	0.00	0.00	0.00	E
None	Control Alfalfa	100	2.00	0.00	0.00	2.00	2.00	CD
		300	0.00	0.00	0.00	0.00	0.00	E
		900	0.00	0.00	0.00	0.00	0.00	E
No Alfalfa incorporated in the diet		0	0.00	0.00	0.00	0.00	0.00	E

Table 7B. Mean stunting determined from *Lygus* feeding assays with diet incorporated with tissue from alfalfa plants expressing the variant TIC807 protein TIC807_M8.

Construct	Sample Source	Dilution fold	Mean Percent mortality	Std Dev	SEM	Lower 95%	Upper 95%	t Grouping
[ER] TIC807_M8	Pooled Alfalfa tissue from 10 events per construct	100	4.17	7.22	4.17	-13.76	22.09	CD
		300	0.00	0.00	0.00	0.00	0.00	CD
		900	13.10	12.54	7.24	-18.06	44.25	CD
[ES] TIC807_M8		100	56.55	6.27	3.62	40.97	72.12	AB
		300	41.67	19.09	11.02	-5.77	89.10	B
		900	0.00	0.00	0.00	0.00	0.00	CD
[ET] TIC807_M8		100	64.88	19.91	11.50	15.42	114.34	A
		300	16.67	19.09	11.02	-30.77	64.10	C
		900	12.50	12.50	7.22	-18.55	43.55	CD
None	Control Alfalfa	100	12.50	12.50	7.22	-18.55	43.55	CD
		300	0.00	0.00	0.00	0.00	0.00	CD
		900	8.33	14.43	8.33	-27.52	44.19	CD
No Alfalfa incorporated in the diet		0	2.50	7.01	1.81	-1.38	6.38	D

Example 8: Plants co-expressing an eHTP and a second insect inhibitory protein exhibiting *Lygus* species inhibitory activity

[00100] Protein samples were prepared containing various mixtures of TIC1415 and TIC807_M13 and tested in bioassay. The TIC1415 protein and other *Lygus* inhibitory proteins are described in PCT Patent Application Publication No. WO 2012/139004. Sample mixtures were fed to *Lygus lineolaris* using bioactivity assay. TIC1415 protein alone and TIC807_M13 alone were also prepared as positive controls. Buffer was used as negative

control. Samples from all three types of preparations exhibited mortality against *Lygus lineolaris* and survivors were stunted. Mortality and stunting scores were significant compared to bioactivity scores of insects fed with buffer (see Table 8A). The data suggests that there are no antagonistic effects. Additional bioassay tests are performed on mixtures to demonstrate synergistic and/or additive effects.

Table 8A. Bioassay data for protein mix: TIC1415 combined with TIC807_M13

SAMPLE	TIC1415 (µg/mL)	TIC807_M13 (µg/mL)	Mean† Population mortality	T Grouping on mort	Mean† stunting‡ score	T Grouping on stunting
TIC1415 + TIC807_M13	4.35	1	21.79	AB*	0.60	AB*
TIC1415 + TIC807_M13	2.175	1	20.36	B*	0.60	AB*
TIC1415 + TIC807_M13	1.0875	1	12.50	BC	0.60	AB*
TIC1415 + TIC807_M13	4.35	0.5	32.50	A*	0.80	A*
TIC1415 + TIC807_M13	1.75	0.265	7.86	CD	0.40	ABC
TIC1415 + TIC807_M13	0.875	0.265	0.00	D	0.00	C
TIC1415 + TIC807_M13	0.4375	0.265	5.36	CD	0.00	C
TIC1415 + TIC807_M13	4.35	0.25	13.21	BC	0.40	ABC
TIC1415 + TIC807_M13	1.75	0.1325	0.00	D	0.00	C
TIC1415 + TIC807_M13	1.75	0.06625	0.00	D	0.00	C
TIC1415	4.35	0	12.50	BC	0.40	ABC
TIC1415	1.75	0	7.86	CD	0.00	C
TIC807_M13	0	1	0.00	D	0.20	BC
TIC807_M13	0	0.265	2.50	CD	0.00	C
Buffer (negative) control	0	0	0.00	D	0.00	C

†Average (mean) of 5 populations of 8 nymphs per population.

‡ Stunting scores correspond to visual mass ratings where 0 = no difference to negative control, 1 = about 25% less mass, 2 = about 50% less mass, and 3 = about 75% less mass. The average of the stunting scores for each population of eight nymphs is reported.

* At 95% confidence interval.

[00101] Cotton plants comprising events with transgenic DNA were designed to co-express respective proteins TIC1415 and TIC807_M13. Such plants were evaluated in a caged whole

plant assay infested with *Lygus lineolaris*. Five plants each from ten events were caged and infested with 2 pairs of male and female *L. lineolaris* per plant. The assay was incubated in a growth chamber under normal environmental conditions for cotton plant development for 21 days. DP393 negative control plants were grown in similar manner. At the end of the 3 week period, *Lygus* of various stages of development were counted. The mean number per plant of *Lygus hesperus* insects at each stage in development were calculated (see Table 8B).

Table 8B. In-planta data for for protein mix: TIC1415 combined with TIC807_M13

Construct	Event	N	Mean 3rd Instar or <	Mean 4th Instar Nymphs	Mean 5th Instar Nymphs	Mean Live 2nd Gen. Adults	Mean Total 2nd Gen. Lygus	SEM	Tukey Grouping
12	021	5	0.00	0.00	0.00	0.00	0.00	0.00	B
	625	5	0.20	0.20	0.20	0.00	0.60	0.24	B
	830	5	2.20	0.20	0.00	0.00	2.40	1.12	AB
	890	5	4.40	0.00	0.20	0.00	4.60	2.62	AB
	521	5	4.60	0.60	0.00	0.00	5.20	4.27	AB
	980	5	3.40	1.20	1.20	0.00	5.80	4.86	AB
13	426	5	0.00	0.00	0.00	0.00	0.00	0.00	B
	611	5	0.60	0.00	0.00	0.00	0.60	0.60	B
	999	5	0.40	0.00	0.40	0.00	0.80	0.37	B
	356	5	6.20	0.00	0.40	0.00	6.60	4.73	AB
Inbred	DP393 (Negative)	10	7.00	2.50	0.80	0.00	10.30	3.75	A

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. An insect inhibitory recombinant polypeptide comprising a sequence having at least 95% amino acid identity to SEQ ID NO: 2 (TIC807), wherein said insect inhibitory recombinant polypeptide exhibits about 15 fold or greater Hemipteran inhibitory activity against a Hemipteran pest species relative to the Hemipteran inhibitory activity of SEQ ID NO: 2, and wherein said insect inhibitory recombinant polypeptide comprises:
 - a) at least one modification at an amino acid residue selected from the group consisting of Val10, Asn12, Ile14, Asn22, Asn23, Gly24, Ile25, Gln26, Gly27, Phe30, Gln38, Ile39, Asp40, Thr41, Ile43, Ile52, Thr93, Ser95, Ser97, Phe138, Phe147, Gln149, Ser151, Ser159, Asn180, Thr182, Ser193, Thr194, Glu195, His196, Tyr197, Ser198, His199, Tyr200, Ser201, Gly202, Tyr203, Pro204, Ile205, Leu206, Thr207, Trp208, Ile209, Ser210, Tyr216, Gly218, Pro219, Met221, Ser222, Trp223, Tyr224, Phe225, Asn239, Val244, Tyr246, Thr247, Val251, Gln253, Ser255, and His287 relative to SEQ ID NO:2; and
 - (b) a contiguous triple deletion in residue range 196-201 relative to SEQ ID NO:2.
2. The insect inhibitory recombinant polypeptide of claim 1, wherein said insect inhibitory recombinant polypeptide exhibits from about 20 fold to about 260 fold increased Hemipteran inhibitory activity against a Hemipteran pest species relative to the Hemipteran inhibitory activity of SEQ ID NO: 2.
3. The insect inhibitory recombinant polypeptide of claim 2, wherein said insect inhibitory recombinant polypeptide exhibits about 25 fold or greater Hemipteran inhibitory activity against a Hemipteran pest species relative to the Hemipteran inhibitory activity of SEQ ID NO: 2 (TIC807).
4. The insect inhibitory recombinant polypeptide of claim 2, wherein said insect inhibitory recombinant polypeptide exhibits about 50 fold or greater Hemipteran inhibitory activity against a Hemipteran pest species relative to the Hemipteran inhibitory activity of SEQ ID NO: 2 (TIC807).
5. The insect inhibitory recombinant polypeptide of claim 1, wherein said insect inhibitory recombinant polypeptide comprises at least one mutation in SEQ ID NO:2 selected from the group consisting of N12D, I52M, T93A, S95A, F138V, F147S, F147A, Q149E, Q149A, S151A, S159T, N180D, T182A, H196A, Y197A, S198A, W208A, P219R, W223Y, Y246F, T247K, V251A, and H287F.

6. The insect inhibitory recombinant polypeptide of claim 1, wherein said insect inhibitory recombinant polypeptide comprises at least two mutations in SEQ ID NO:2 selected from the group consisting of T93A, S95A, F147S, F147A, Q149E, Q149A, S151A, N180D, T182A, H196A, Y197A, S198A, W208A, S217N, P219R, W223Y, V251A.
7. The insect inhibitory recombinant polypeptide of claim 1, wherein said insect inhibitory recombinant polypeptide comprises at least three mutations in SEQ ID NO:2 selected from the group consisting of T93A, S95A, F147S, F147A, Q149E, Q149A, S151A, N180D, T182A, H196A, S198A, Y197A, W208A, S217N, P219R, W223Y, V251A.
8. The insect inhibitory recombinant polypeptide of claim 1, wherein said insect inhibitory recombinant polypeptide comprises at least four mutations in SEQ ID NO:2 selected from the group consisting of T93A, S95A, F147S, F147A, Q149E, Q149A, S151A, N180D, T182A, H196A, Y197A, S198A, W208A, S217N, P219R, W223Y, V251A.
9. The insect inhibitory recombinant polypeptide of claim 1, wherein said Hemipteran pest species is selected from the group consisting of a *Lygus* sp., a *Emrasca* sp., a *Amrasca* sp, and a combination thereof; and wherein said Hemipteran pest species is selected from the group consisting of *Lygus hesperus*, *Lygus lineolaris*, *Amrasca devastans*, and a combination thereof.
10. The insect inhibitory recombinant polypeptide of claim 1, comprising an enhanced inhibitory spectrum against *Lygus*, *Empoasca*, *Amrasca*, or a combination thereof.
11. The insect inhibitory recombinant polypeptide of claim 1, wherein said insect inhibitory recombinant polypeptide exhibits an enhanced *Lygus* inhibitory spectrum relative to TIC807 in that said insect inhibitory recombinant polypeptide provides increased inhibition of *Lygus lineolaris*.

FIGURE 1

FIG. 1A

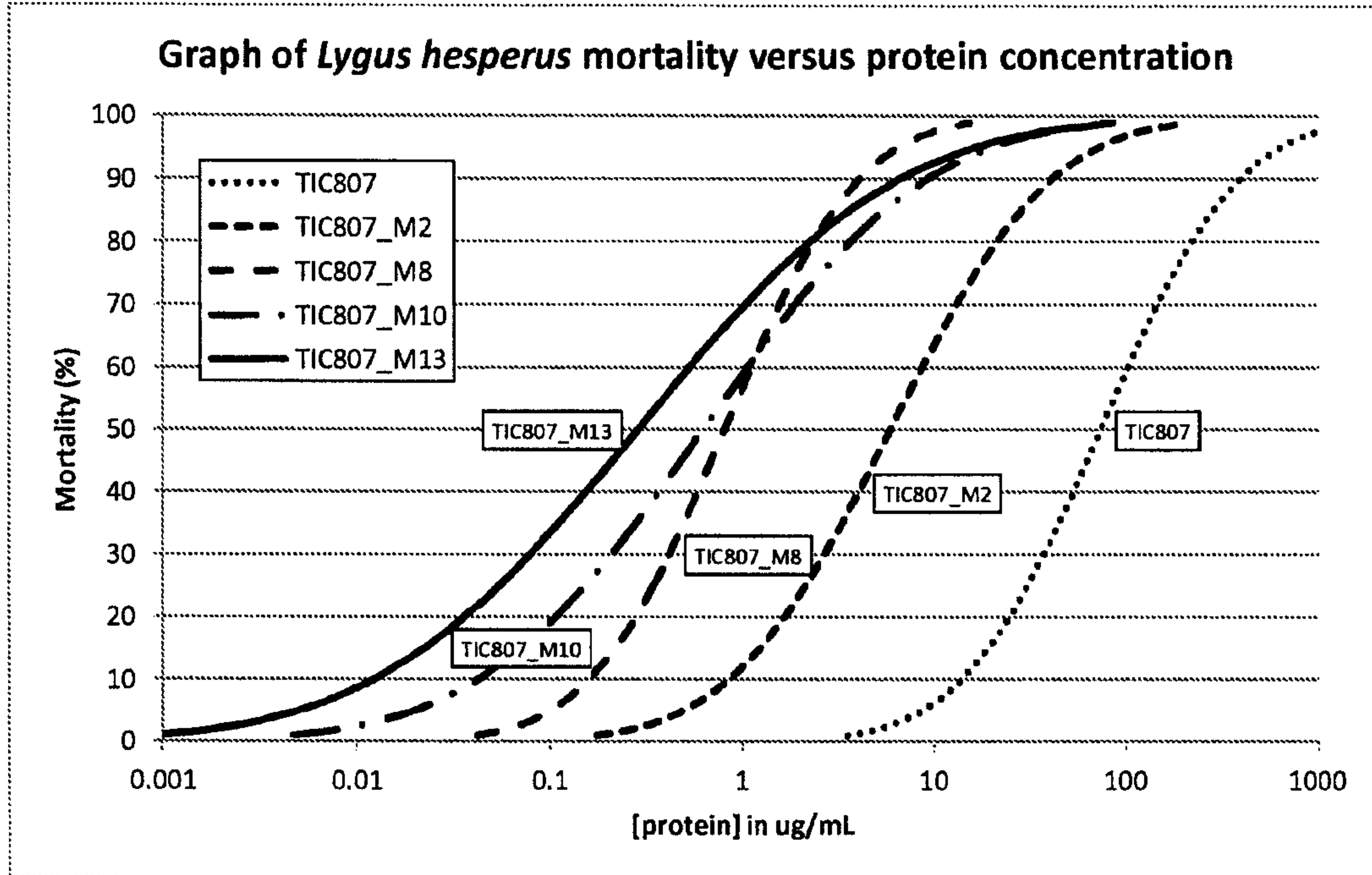


FIG. 1B

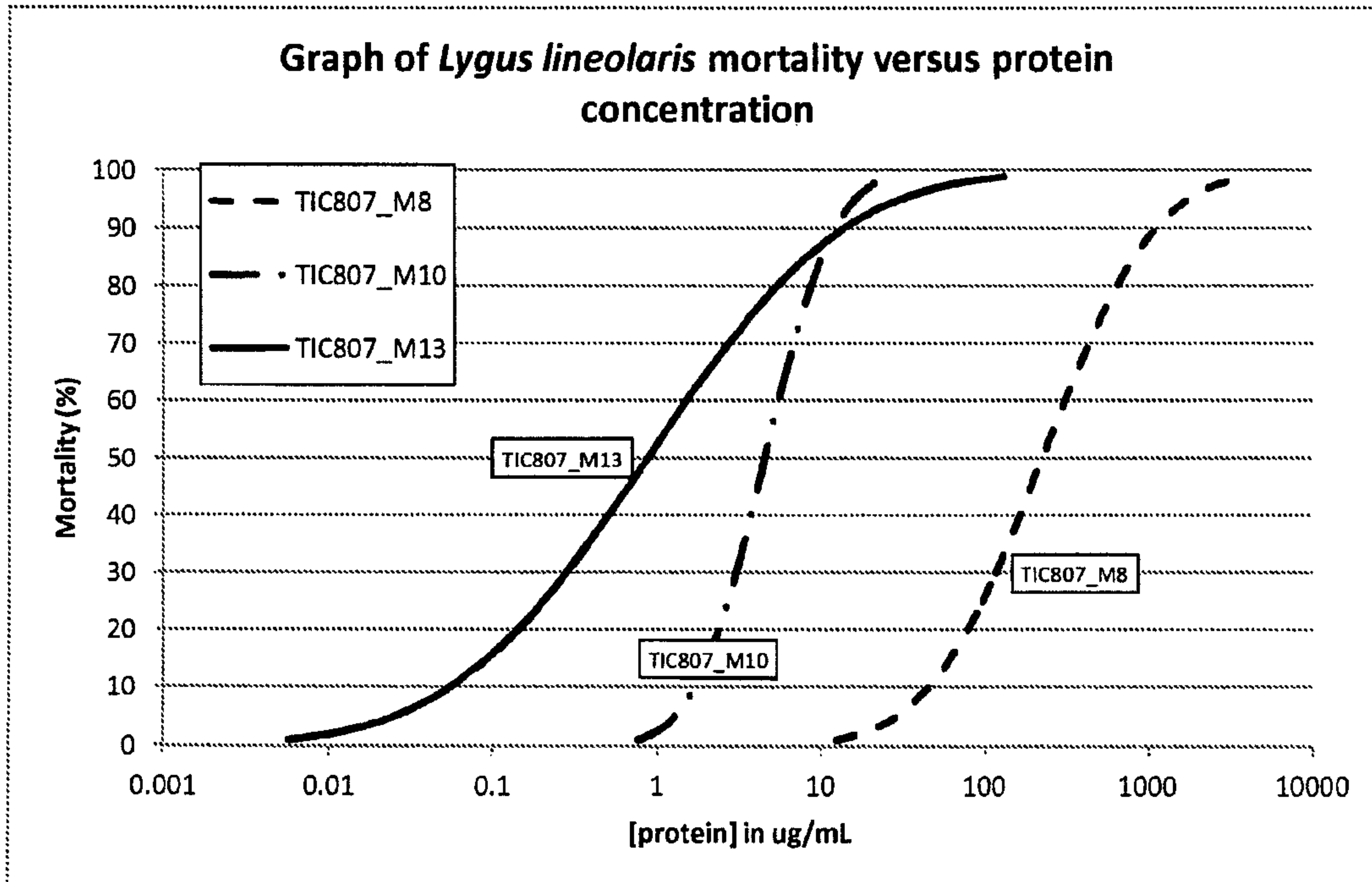


FIGURE 2



FIGURE 3

