

CAVELIER

ABOGADOS

EDIFICIO SISKI
CARRERA 4 No. 72 - 35
BOGOTÁ 8, COLOMBIA

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TELEFONO: (57) (1) 347-3611
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Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

227
224

SUPERINDUSTRIA Y COMERCIO
Radicación : 01038451 00000002 Folios: 19
Fecha (CMD): 2001-07-10 16:39:36
Trámite : 002 PATENTES D I REGISTRO/ 444 REPLASTA
Dependencia: 3020 DIVISION DE NUEVAS CREACIONES

Referencia : Expediente No.01.038.451

Solicitante : MONSANTO TECHNOLOGY LLC

Asunto : Solicitud de patente para: "COMPOSICIONES DE
POLIPÉPTIDOS TÓXICAS PARA INSECTOS
ANTONOMUS Y MÉTODO DE USO"

Fecha de la solicitud : 15 de mayo de 2001

TRAMITE:02 - EVENTO:01 - ACTUACION:444

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al oficio número 1717, notificado por fijación en lista de fecha del 7 de junio de 2001.

2. Mediante el oficio número 1717 se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Atiendo a las observaciones de la manera siguiente:

2.1. Documento de cesión de inventores. Presento junto con este escrito los documentos en que consta la cesión del derecho a la patente otorgado por los inventores al solicitante.

2.2 Poder. Presento con este memorial poder conferido por la sociedad MONSANTO TECHNOLOGY LLC, debidamente legalizado, y que me faculta para actuar en esta solicitud de patente.

Ruego a Usted reconocerme personería como apoderado de la solicitante.

2.3. Copia Certificada. Presento con este escrito copia certificada de la solicitud de patente estadounidense número 60/204,367 del 15 de mayo de 2000, en la cual se basa la reivindicación de la prioridad.

3. Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, atentamente solicito que se continúe su tramitación.

Bogotá, 11 0 JUL. 2001
Señora Jefe,



EMILIO FERRERO

T.P.A. No. 31.929

COLO 12746-801-001-3

ASN-ID-4

Apostill

(Convention de La Haye du 5 octobre 1961)

229
226

1. Country: United States of America

This public document

2. has been signed by MICHELLE LUDWIG

3. acting in the capacity of NOTARY PUBLIC

4. bears the seal/stamp of MICHELLE LUDWIG-NOTARY PUBLIC-STATE OF MISSOURI

Certified

5. at Jefferson City, Missouri

6. the 11th. day of JUNE, 2001.

7. by Matt Blunt, Secretary of State, State of Missouri

8. No. 63615

9. Seal/Stamp

10. Signature:

Matt Blunt

CESION

ASSIGNMENT

Yo, (nosotros), abajo firmado (s)

I (we), the undersigned
Barbara G. Isaac, Elysia K. Krieger, Anne-Marie
Light Mettus, Sakuntala Sivasupramaniam,
Farhad Moshiri

lomiciliado (s) en

of St. Charles, MO, Kirkwood, MO, Feasterville,
PA, Chesterfield, MO, Chesterfield, MO, USA,
respectively

declaro(amos) bajo juramento ser único(s) y
verdadero(s) inventor(es) de la invención:

state(s) under oath to be sole and true
inventor(s) of the invention:

POLYPEPTIDE COMPOSITIONS TOXIC TO
ANTHONOMUS INSECTS, AND METHODS OF USE

declaro(amos) así mismo que cedo(emos) y
transfiero(erimos), sin limitación, a favor de la
sociedad

state as well, that assign, without reservations or
limitations in favour of Monsanto Technology LLC

constituida y existente de conformidad con las
leyes del Estado de

a corporation constituted and existing in
conformity with the laws of the State of
Delaware

lomiciliada en

domiciled in St. Louis, MO USA

todos los derechos inherentes a dicha invención
que la citada Compañía queda facultada para
solicitar en su nombre la patente
correspondiente en la República de Colombia.

all rights inherent to said invention and that the
above mentioned company hereby is enabled to
apply, in its name, for the corresponding patent
in the Republic of Colombia.

Dado y firmado hoy/Given and signed this

en/in

Firma *Elysa Krieger* 5-23-01 Signature

AUTENTICACION NOTARIAL (INDIVIDUO)

NOTARIAL AUTHENTICATION (INDIVIDUAL)

A los ____ días del mes de ____ de 19__

On this 23rd day of May, 2001

ante mí, Notario Público de ____

before me, a Notary Public of St. Louis County, State of

compareció(eron) personalmente ____

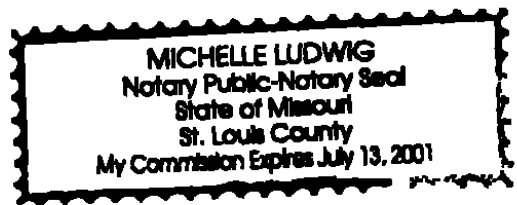
personally appeared Elysia K. Krieger

a quien(es) doy fe de conocer y me consta que es(son) la(s)
persona(s) firmante(s) del documento que precede, y quien(es)
tiene(n) capacidad legal para otorgarlo.

to me known, and known to me to be the person(s) w
executed that foregoing document, and who has(have) la
capacity to execute it.

____ Notario Público ____ Notary Public

Michelle Ludwig



NOTA: LA AUTENTICACION CONSULAR ES NECESARIA.

Ap stille

(Convention de La Haye du 5 octobre 1961)

254
228

1. Country: United States of America

This public document

2. has been signed by MICHELLE LUDWIG

3. acting in the capacity of NOTARY PUBLIC

4. bears the seal/stamp of MICHELLE LUDWIG-NOTARY PUBLIC-STATE OF MISSOURI

Certified

5. at Jefferson City, Missouri

6. the 11th. day of JUNE, 2001

7. by Matt Blunt, Secretary of State, State of Missouri

8. No. 63614

9. Seal/Stamp

10. Signature:

Matt Blunt

229 232

CESION

Yo, (nosotros), abajo firmado (s)

domiciliado (s) en

declaro(amos) bajo juramento ser único(s) y verdadero(s) inventor(es) de la invención:

declaro(amos) así mismo que cedo(emos) y transfiero(erimos), sin limitación, a favor de la sociedad

constituida y existente de conformidad con las leyes del Estado de

domiciliada en

todos los derechos inherentes a dicha invención y que la citada Compañía queda facultada para solicitar en su nombre la patente correspondiente en la República de Colombia.

ASSIGNMENT

I (we), the undersigned

Barbara G. Isaac, Elysia K. Krieger, Anne-Marie Light Mettus, Sakuntala Sivasupramaniam, Farhad Moshiri

of St. Charles, MO, Kirkwood, MO, Feasterville, PA, Chesterfield, MO, Chesterfield, MO, USA, respectively

state(s) under oath to be sole and true inventor(s) of the invention:

POLYPEPTIDE COMPOSITIONS TOXIC TO ANTHONOMUS INSECTS, AND METHODS OF USE

state as well, that assign, without reservations or limitations in favour of Monsanto Technology LLC

a corporation constituted and existing in conformity with the laws of the State of Delaware

domiciled in St. Louis, MO USA

all rights inherent to said invention and that the above mentioned company hereby is enabled to apply, in its name, for the corresponding patent in the Republic of Colombia.

Dado y firmado hoy/Given and signed this

en/in

Firma

Barbara Isaac 5/23/01
Signature

AUTENTICACION NOTARIAL (INDIVIDUO)

A los ____ días del mes de _____ de 19____
ante mí, Notario Público de _____
compareció(eron) personalmente _____

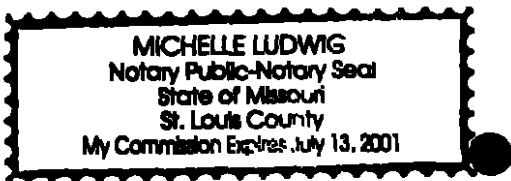
a quien(es) doy fe de conocer y me consta que es(son) la(s)
persona(s) firmante(s) del documento que precede, y quien(es)
tiene(n) capacidad legal para otorgarlo.

Notario Público — Notary Public Michelle Ludwig

NOTARIAL AUTHENTICATION (INDIVIDUAL)

On this 23rd day of May 2001
before me, a Notary Public of St. Louis County, State of mo
personally appeared Barbara G. Isaac

to me known, and known to me to be the person(s) who
executed that foregoing document, and who has(have) legal
capacity to execute it.



Apostill

(Convention de La Haye du 5 octobre 1961)

233
230

1. Country: United States of America

This public document

2. has been signed by MICHELLE LUDWIG

3. acting in the capacity of NOTARY PUBLIC

4. bears the seal/stamp of MICHELLE LUDWIG-NOTARY PUBLIC-STATE OF MISSOURI

Certified

5. at Jefferson City, Missouri

6. the 11th. day of JUNE, 2001

7. by Matt Blunt, Secretary of State, State of Missouri

8. No. 63613

9. Seal/Stamp

10. Signature:

Matt Blunt

CESION

Yo, (nosotros), abajo firmado (s)

domiciliado (s) en

declaro(amos) bajo juramento ser único(s) y verdadero(s) inventor(es) de la invención:

declaro(amos) así mismo que cedo(emos) y transfiero(erimos), sin limitación, a favor de la sociedad

constituida y existente de conformidad con las leyes del Estado de

domiciliada en

todos los derechos inherentes a dicha invención y que la citada Compañía queda facultada para solicitar en su nombre la patente correspondiente en la República de Colombia.

ASSIGNMENT

I (we), the undersigned

Barbara G. Isaac, Elysia K. Krieger, Anne-Marie Light Mettus, Sakuntala Sivasupramaniam, Farhad Moshiri

of St. Charles, MO, Kirkwood, MO, Feasterville, PA, Chesterfield, MO, Chesterfield, MO, USA, respectively

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domiciled in St. Louis, MO USA

all rights inherent to said invention and that the above mentioned company hereby is enabled to apply, in its name, for the corresponding patent in the Republic of Colombia.

Dado y firmado hoy/Given and signed this

en/in

Firma

Sakuntala Sivasupramaniam
Signature

May 22nd 2001

AUTENTICACION NOTARIAL (INDIVIDUO)

A los ____ días del mes de ____ de 19 ____

ante mí, Notario Público de ____

compareció(eron) personalmente ____

a quien(es) doy fe de conocer y me consta que es(son) la(s) persona(s) firmante(s) del documento que precede, y quien(es) tiene(n) capacidad legal para otorgarlo.

____ Notario Público ____ Notary Public

NOTARIAL AUTHENTICATION (INDIVIDUAL)

On this 22nd day of May 192001

before me, a Notary Public of St. Louis County, State of MO
personally appeared Sakuntala Sivasubramanian

to me known, and known to me to be the person(s) who executed that foregoing document, and who has(have) legal capacity to execute it.

____ Michelle Ludwig

NOTA: LA AUTENTICACION CONSULAR ES NECESARIA.

Apostille

(Convention de La Haye du 5 octobre 1961)

235
232

1. Country: United States of America

This public document

2. has been signed by MICHELLE LUDWIG

3. acting in the capacity of NOTARY PUBLIC

4. bears the seal/stamp of MICHELLE LUDWIG-NOTARY PUBLIC-STATE OF MISSOURI

Certified

5. at Jefferson City, Missouri

6. the 11th. day of JUNE, 2001.

7. by Matt Blunt, Secretary of State, State of Missouri

8. No. 63612

9. Seal/Stamp

10. Signature:

Matt Blunt

236
233

CESION

Yo, (nosotros), abajo firmado (s)

domiciliado (s) en

declaro(amos) bajo juramento ser único(s) y
verdadero(s) inventor(es) de la invención:

declaro(amos) así mismo que cedo(emos) y
transfiero(erimos), sin limitación, a favor de la
sociedad

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leyes del Estado de

domiciliada en

todos los derechos inherentes a dicha invención
y que la citada Compañía queda facultada para
solicitar en su nombre la patente
correspondiente en la República de Colombia.

ASSIGNMENT

I (we), the undersigned

Barbara G. Isaac, Elysia K. Krieger, Anne-Marie
Light Mettus, Sakuntala Sivasupramaniam,
Farhad Moshiri

of St. Charles, MO, Kirkwood, MO, Feasterville,
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conformity with the laws of the State of
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domiciled in St. Louis, MO USA

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above mentioned company hereby is enabled to
apply, in its name, for the corresponding patent
in the Republic of Colombia.

Dado y firmado hoy/Given and signed this

en/in

Firma

Farhad Moshiri 5/18/01
Signature

AUTENTICACION NOTARIAL (INDIVIDUO)

A los ____ días del mes de ____ de 19____
ante mí, Notario Público de ____
compareció(eron) personalmente ____

a quien(es) doy fe de conocer y me consta que es(son) la(s)
persona(s) firmante(s) del documento que precede, y quien(es)
tiene(n) capacidad legal para otorgarlo.

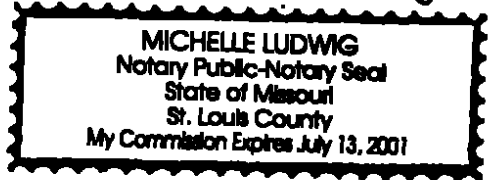
____ Notario Público ____ Notary Public

NOTARIAL AUTHENTICATION (INDIVIDUAL)

On this 18th day of May 2001
before me, a Notary Public of St. Louis County, State of MO
personally appeared Farhad Moshiri

to me known, and known to me to be the person(s) who
executed that foregoing document, and who has(have) legal
capacity to execute it.

Michelle Ludwig



234
234

APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. Country: United States of America
2. This public document has been signed by Dorothy Lynn Steiner
3. acting in the capacity of Notary Public, Montgomery County
4. bears the seal/stamp Dorothy Lynn Steiner, Notary Public, Commonwealth of Pennsylvania

Certified

5. at Harrisburg, Pennsylvania
6. The 13th day of June, 2001
7. by Kim Pizzigrilli, Secretary of the Commonwealth of Pennsylvania
8. No. 0113928
9. Seal/Stamp
10. Signature:



Kim Pizzigrilli

Kim Pizzigrilli

CESION

Yo, (nosotros), abajo firmado (s)

domiciliado (s) en

declaro(amos) bajo juramento ser único(s) y verdadero(s) inventor(es) de la invención:

declaro(amos) así mismo que cedo(emos) y transfiero(erimos), sin limitación, a favor de la sociedad

constituida y existente de conformidad con las leyes del Estado de

domiciliada en

todos los derechos inherentes a dicha invención y que la citada Compañía queda facultada para solicitar en su nombre la patente correspondiente en la República de Colombia.

ASSIGNMENT

I (we), the undersigned

Barbara G. Isaac, Elysia K. Krieger, Anne-Marie

Light Mettus, Sakuntala Sivasupramaniam,

Farhad Moshiri

of St. Charles, MO, Kirkwood, MO, Feasterville,

PA, Chesterfield, MO, Chesterfield, MO, USA,

respectively

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a corporation constituted and existing in conformity with the laws of the State of

Delaware

domiciled in St. Louis, MO USA

all rights inherent to said invention and that the above mentioned company hereby is enabled to apply, in its name, for the corresponding patent in the Republic of Colombia.

Dado y firmado hoy/Given and signed this 5/23/01

en/in Bucks Co., PA

Anne-Marie Light Mettus 5/23/01

Firma

Signature

AUTENTICACION NOTARIAL (INDIVIDUO)

A los ____ días del mes de ____ de 19____

ante mí, Notario Público de _____

compareció(eron) personalmente _____

a quien(es) doy fe de conocer y me consta que es(son) la(s)
 persona(s) firmante(s) del documento que precede, y quien(es)
 tiene(n) capacidad legal para otorgarlo.

____ Notario Público ____ Notary Public

NOTARIAL AUTHENTICATION (INDIVIDUAL)

On this 23RD day of MAY 2001before me, a Notary Public of PA, Bucks Co.personally appeared ANNE-MARIE LIGHT METTUS

to me known, and known to me to be the person(s) who
 executed that foregoing document, and who has(have) legal
 capacity to execute it.

Dorothy Lynn Steiner
 Notary Public

Notarial Seal
 Dorothy Lynn Steiner, Notary Public
 Lower Southampton Twp., Bucks County
 My Commission Expires Apr. 28, 2004
 Member, Pennsylvania Association of Notaries

NOTA: LA AUTENTICACION CONSULAR ES NECESARIA.

Apostille

(Convention de La Haye du 5 octobre 1961)

1. Country: United States of America

This public document

2. has been signed by JUDITH A. LORENZ

3. acting in the capacity of NOTARY PUBLIC

4. bears the seal/stamp of JUDITH A. LORENZ-NOTARY PUBLIC-STATE OF MISSOURI

Certified

5. at Jefferson City, Missouri

6. the 29th day of MAY, 2001

7. by Matt Blunt, Secretary of State, State of Missouri

8. No. 63158

9. Seal/Stamp

10. Signature:

Matt Blunt

Comm. 48 (12-2000)

TRADUCCION No.: 140601

MARIA ELVIRA MARTELO

Traductora e Interprete Oficial

Inglés - Español - Inglés

Res. No. 2879 Min. Justicia



CAVELIER

193

ABOGADOS
EDIFICIO SISKI
CARRERA 4a N° 72-35
BOGOTÁ 8 - COLOMBIA

TRADUCCION No.: 1406101
MARIA ELVIRA MARTELO
Traductora e Interprete Oficial
Inglés - Español - Inglés
Res. No. 2879 Min. Justicia

237 240
Representación, Ratificación y Poder con autenticación notarial y Apostille para patentes, modelos de utilidad diseños industriales, marcas de fábrica, nombres comerciales, enseñas, nombres de dominio, lemas comerciales, licencias.

Representation, ratification and Power-of-Attorney with notarial authentication and Apostille for patents, utility models, industrial designs, trademarks, commercial names, trade emblems, domain names, slogans, licenses.

REPRESENTACION, RATIFICACION Y PODER

REPRESENTATION, RATIFICATION AND POWER-OF-ATTORNEY

Yo (nosotros), abajo firmado (s)/I (we), the undersigned

MONSANTO TECHNOLOGY LLC

domiciliado (s) en/of 800 North Lindbergh

Boulevard, St. Louis, Missouri 63167,

United States of America,

por el presente ratificamos la solicitud de patente

No. 01038451

presentada por EMILIO FERRERO

como agente oficioso ante la Superintendencia de Industria y Comercio

en Bogotá, Colombia

el 15 de Mayo de 2001

y por el presente nombramos a EMILIO FERRERO, tpa. 31929; GONZALO PERDOMO, tpa. 831; LUZ CLEMENCIA DE PAEZ, tpa. 23555 y GERMAN CAVELIER, tpa. 832, domiciliados en la Carrera 4ª N° 72-35, Bogotá, Colombia, en obediencia a lo dispuesto en el parágrafo 1o del Artículo 543 del Código de Comercio, el primero como principal y los demás como suplentes, en su orden, como mis (nuestros) representantes en todos los asuntos de propiedad industrial con facultad para recibir notificaciones y nombrar apoderados judiciales o extrajudiciales. El primer representante suplente reemplazará al principal en caso de ausencia o impedimento temporal o definitivo de aquél. La misma regla se aplicará cuando el segundo representante suplente haya de reemplazar al primero, o el tercero al segundo. En tales casos, el representante principal, o el primero, o segundo suplente, en su caso, o ambos, declararán su intención de no ejercer la representación mediante declaración autenticada por Notario Público en Colombia, o por Notario u otro Funcionario Público, o Cónsul de Colombia, en el extranjero; y si se tratare de impedimento, con el certificado respectivo. Si alguno de los nombrados faltare definitivamente, el siguiente tomará su lugar. Cuando cesare la ausencia o impedimento del representante principal, o de su primero o segundo suplente, en su caso, podrán ejercer la representación, en su orden, uno u otros según el caso, asumiéndola por el solo hecho de ejercerla, cesando en ese momento el ejercicio de la representación por el primero, segundo o tercer suplente en su caso.

Además, confiero (conferimos) poder a los abogados arriba nombrados, para obtener la protección de mi (nuestra) propiedad industrial y contra la competencia desleal, a cuyo efecto autorizo (amos) a los dichos apoderados para que me (nos) representen ante cualesquiera entidades o personas, ejerzan o no jurisdicción, especialmente ante las del orden administrativo, contencioso administrativo y judicial, así como los tribunales de arbitramento, en todo lo concerniente a los siguientes asuntos: a) Obtención de patentes de invención, modelos de utilidad y

hereby ratify the patent application

No. 01038451

filed without a Power of Attorney by EMILIO FERRERO

before the Superintendency of Industry and Commerce

in Bogotá, Colombia

on May 15, 2001

and do hereby appoint EMILIO FERRERO, tpa. 31929; GONZALO PERDOMO, tpa. 831; LUZ CLEMENCIA DE PAEZ, tpa. 23555 and GERMAN CAVELIER, tpa. 832, domiciled at Carrera 4ª N° 72-35, Bogotá, Colombia, in due compliance with the terms of Paragraph 1st. of Article 543 of the Commercial Code, the first as principal and the others as alternates, in the order of their appointment, as our representatives for all industrial property matters with faculty to receive notifications and to appoint attorneys in and out of Court. The first alternate shall replace the principal in case of his absence or of temporal or definitive impediment. The same rule will apply when the second alternate representative is to replace the first one, or when the third is to replace the first or second alternates in their turn, as both of them, secondarily in such cases, the principal representative, or if both of them are absent, the third shall act as representatives through a duly authenticated declaration before a Notary Public in Colombia, or before a Notary or another Public Official or a Colombian Consul abroad; and in case of impediment, with the respective certificate shall suffice. If any of the appointed representatives is definitively absent, the following alternate shall replace him. When the absence or impediment of the principal representative, or of the first or second alternate in their turn ceases, the representation can be taken again in their sequence, by the principal, the first or second alternate depending on case, and the mere fact or its exercise will suffice. The exercise of the representation by the first, second or third alternates in their turn shall end at such time.

In addition, we grant a Power of Attorney in favor of the above-named attorneys-at-law so that they may obtain the protection of my (our) industrial property and against the unfair competition, for which purpose I (we) authorize the said attorneys to represent me (us) before any authorities or persons, with or without jurisdiction, specially those administrative, administrative contentious and judicial, as well as before Arbitration Court, in all matters concerning the following: a) Obtainment of patents, utility models and industrial designs; the registration of

14

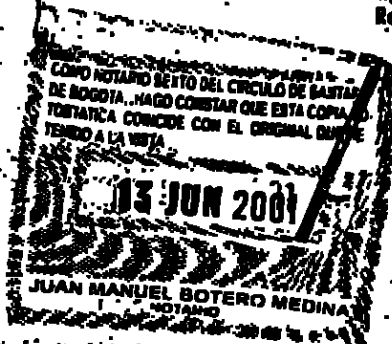
diseños industriales; el registro de marcas de fábrica, colectivas, defensivas, de servicio, lemas comerciales, nombres comerciales, enseñanzas, nombres de dominio, variedades vegetales y denominaciones de origen; su renovación, traspaso, cambio de nombre o domicilio, cancelación voluntaria; y el registro de licencias de uso de esos derechos; b) Las acciones de competencia desleal, protección del consumidor, oposiciones u observaciones, cancelación administrativa o judicial, nulidad, medidas cautelares, concesión de licencias, la tutela, la protección de secretos industriales, y en general los juicios civiles, penales, administrativos o contencioso-administrativos relacionados con estos derechos; acciones y procesos que promovamos o se nos promuevan. Y para que en todos los asuntos arriba determinados, puedan sustituir este mandato, transigir, conciliar, admitir los hechos del proceso, desistir, cancelar, recibir, renunciar, y ratificar los actos de agentes oficiosos.

trademarks, collective, defensive and service marks, slogans, commercial names, trade emblems, domain names, vegetal varieties and denominations of origin; its renewal, assignment, change of name, or domicile, voluntary cancellation; and the registration of licenses of use of the above rights; b) Actions of unfair competition, protection to the consumer, oppositions or observations, administrative or Court cancellation, nullity, infringement, granting of licenses, the action for writ mandamus, the protection of trade secrets, and in general the civil, penal, and administrative suits relating to those rights; actions and suits instituted by me (us) or against me (us). And that in all the above matters they may substitute this Power of Attorney, compromise, conciliate, admit the facts of the case, desist, cancel, receive, resign, and ratify acts done by representatives without a power of attorney.

Dado y firmado hoy/Given and signed this 21st day of May 2001

en/in St. Louis, MO
por/by Dennis R. Roemer, Jr.
[Signature]
Firma Signature

TRADUCCION No: 14,06/01
MARIA ELVIRA MARTELO
Traductora e Interprete Oficial
Inglés - Español - Ingles
Res. No. 2879 Min. Justicia



CERTIFICADO NOTARIAL (INDIVIDUO)

A los _____ días del mes de _____
de 200 _____ ante mí, Notario Público o Funcionario Autorizado
de _____
compareció(eron) personalmente _____

a quien(es) doy fe de conocer y me consta que es(son) la(s)
persona(s) firmante(s) del documento que precede, y quien(es)
tiene(n) capacidad legal para otorgarlo

Notario Público o Funcionario Autorizado - Notary Public or Attesting Official

CERTIFICADO NOTARIAL (SOCIEDAD)

Hoy 18 de Mayo de 2001
Compareció(eron) ante mí

Dennis R. Hoerner, Jr.
quien(es) conozco personalmente y que firma(n) este
documento en representación de la Sociedad/compañía
denominada MONSANTO TECHNOLOGY LLC

Certifico:

1. Que he visto los siguientes documentos:*

Acción de miembro Unico, firmada
en Saint Louis, Missouri el
15/03/01

2. Que dichos documentos prueban lo siguiente:

2.1. Que MONSANTO TECHNOLOGY LLC

es una Sociedad/Compañía legalmente constituida y existente
de acuerdo con las leyes de 1 Estado de Delaware,
Estados Unidos de América,

2.2. Que el domicilio de dicha Sociedad/Compañía es
800 North Lindbergh Boulevard, St.
Louis, Missouri 63167, Estados Unidos
de América,

2.3. Que el objeto u objetos para los cuales se otorga el presente
poder están comprendidos dentro del objeto social de la
mencionada Sociedad/Compañía.

2.4. Que el(los) Sr.(Sres.)
Dennis R. Hoerner Jr. Presidente
está(n) autorizado(s) para otorgar el presente Poder.

Ciudad St. Louis

Estado Missouri E.U.A.

Hoy 18 de Mayo de 2001

Notario Público o Funcionario Autorizado - Notary Public or Attesting Official

TRADUCCION No.: 14.06/01

MARIA ELVIRA MARTELO
* Los documentos deben identificarse con fecha y origen
Traductora e Intérprete Oficial
Inglés - Español - Inglés
Res. No. 2879 Min. Justicia

CAVELIER ABOGAD S
WPP00E01-193 (MINUTA 12.1.1.2.9 USA)

NOTARIAL CERTIFICATE (INDIVIDUAL)

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On this _____ day of _____
200 _____ before me, a Notary Public or Attesting Official of _____
personally appeared _____

to me known, and known to me to be the person(s) who
executed that foregoing document, and who has(have) legal
capacity to execute it.

NOTARIAL CERTIFICATE (CORPORATION)

On this 18th day of May 2001
appeared before me

Dennis R. Hoerner Jr.
who(m) I know personally and who(m) signed this document as
representative(s) of the Corporation/Company named
MONSANTO TECHNOLOGY LLC

I certify:

1. That I have seen the following documents:*

Action of Sole Member, signed in
St. Louis, Missouri on March 15, 2001

2. That the above documents duly prove the following:

2.1. That MONSANTO TECHNOLOGY LLC

is a Corporation/Company legally constituted and in existence
in accordance with the laws of the State of Delaware,
United States of America,

2.2. That the domicile of said Corporation/Company is
800 North Lindbergh Boulevard, St.
Louis, Missouri 63167, United States
of America.

2.3. That the purpose or purposes for which the present Power
of Attorney is granted are comprised within the corporation
purposes set forth in the articles of incorporation and the
Corporation/Company.

2.4. That Mr.(Messrs.) Dennis R. Hoerner Jr. President
is(are) authorized to grant the present Power of Attorney.

City St. Louis

State Missouri US

This 18th day of May 2001

KATHA A. LORENZ
Notary Public - State of Missouri
St. Louis County

* Documents must be identified with date of identification Expires November 20, 2008

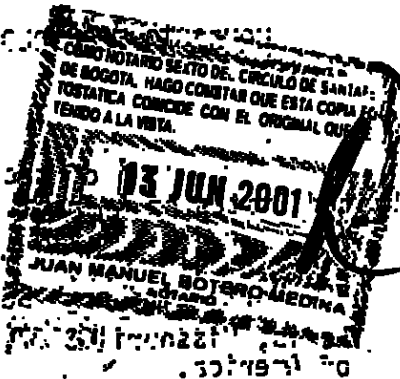
APOSTILLE

(Convention de La Haye du 5 octobre 1961)

1. Country:
This public document
2. has been signed by
3. acting in the capacity of
4. bears the seal/stamp of

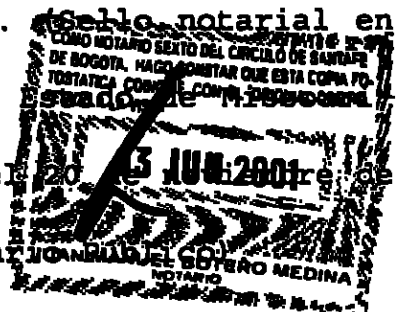
Certified

5. at 6. the 1310
7. by
8. No.
9. Seal/stamp 10. Signature



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(Firma) Judith A. Lorenz, Notario Público. (Sello notarial en tinta) JUDITH A. LORENZ, Notario Público, Estado de Missouri, Condado de St. Louis, Mi comisión expira el 20 JUN 2003. (Sello seco de Judith A. Lorenz, Notario Público, Condado de St. Louis, Missouri, Mi comisión expira el 20 JUN 2003.)



1. País: Estados Unidos de América

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Este documento público

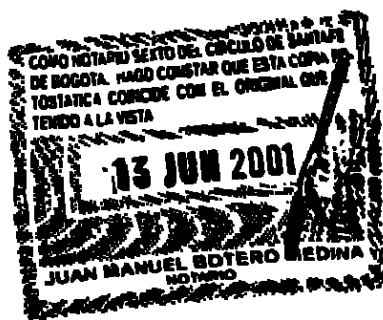
2. Ha sido firmado por JUDITH A. LORENZ
3. Actuando en su capacidad de NOTARIO PÚBLICO
4. Lleva el sello/timbre de JUDITH A. LORENZ, NOTARIO PÚBLICO,
ESTADO DE MISSOURI
- CERTIFICADO
5. En Jefferson City, Missouri.
6. el 29 de mayo de 2001
7. Por Matt Blunt, Secretario de Estado, Estado de Missouri.
8. No. 63158.
9. Sello: Sello del Secretario de Estado, Missouri.
10. Firma: (Firma) Matt Blunt.

Es traducción fiel y completa de la cual se deja copia en esta oficina para su confrontación.

WPC 12746-801-001-3

ID 3

JUN 12/01



Maria E. Martelo
TRADUCCION No.: 1406 b1
MARIA ELVIRA MARTELO
Traductora e Interprete Oficial
Inglés - Español - Inglés
Res. No. 2879 Min. Justicia

MINISTERIO DE RELACIONES
EXTERIORES
Quarile
33457
CUYA FIRMA APARECE EN EL
DOCUMENTO DESAMPEÑA
LAS FUNCIONES INDICADAS

NO SE ASUME
RESPONSABILIDAD DEL TEXTO
2001 JUN 12
JEFE DE LEGATACIONES
SANTAFE DE BOGOTA D.C.

COMO NOTARIO SEXTO DE CIRCULO DE SANTAFE DE BOGOTA, HAGO CONSTAR QUE LAS ANTERIORES FIRMA(S) PUESTA(S) POR

[Signature]

IDENTIFICADOS CON C.C. No.(s)

ES(SON) AUTENTICA(S)

SANTAFE DE BOGOTA, 12 JUN 2001



COMO ACTARIO SEXTO DEL CIRCULO DE SANTAFE DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA CONCORDA CON EL ORIGINAL QUE HE TENIDO A LA VISTA

13 JUN 2001

JUAN MANUEL BOTERO MEDINA
NOTARIO

con
firmado
por
el
notario

PA 412853

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THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

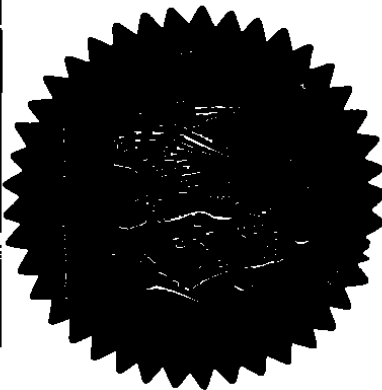
United States Patent and Trademark Office

May 18, 2001

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/204,367

FILING DATE: May 15, 2000



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

L. Edelen

L. EDELEN

Certifying Officer

05-16-00

A/P 245
Prov 242
Jc759 U.S. PTO
60/204367
05/15/0005/15/00
Jc759 U.S. PTO
60/204367
05/15/00
Pat 1-127 Re 528

FORM 23-1

23-3

Practitioner's Docket No. 38-21(51932)A

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Isaac et al.

For: Polypeptide Compositions Toxic to Anthonomus Insects,
and Methods of Use
Box Provisional Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

COVER SHEET FOR FILING PROVISIONAL APPLICATION
(37 C.F.R. § 1.51(c)(1))

WARNING: "A provisional application must also include the cover sheet required by § 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this section." 37 C.F.R. § 1.536(i)(7).

NOTE: "A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application." Notice of December 8, 1994, 59 FR 63951, at 63953.

"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

NOTE: "A provisional application is not entitled to the right of priority under 35 U.S.C. 119 or 365(a) or § 1.55, or to the benefit of an earlier filing date under 35 U.S.C. 120, 121 or 365(c) or § 1.78 of any other application. No claim for priority under § 1.78(b)(2) may be made in a design application based on a provisional application. No request under § 1.293 for a statutory invention registration may be filed in a provisional application. The requirements of §§ 1.821 through 1.825 regarding application disclosures containing nucleotide and/or amino acid sequences are not mandatory for provisional applications." 37 C.F.R. § 1.536(j)(3).

NOTE: "No information disclosure statement may be filed in a provisional application." 37 C.F.R. § 1.51(i).
"Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 FR 63991, at 63994.

CERTIFICATION UNDER 37 C.F.R. 1.10^{*}
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on May 15, 2000 (date), in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10, Mailing Label Number EM57239027805 addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Timothy K. Ball

(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

WARNING: Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(f).

"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 68,438, at 68,442.

(Cover Sheet for Filing Provisional Application [23-1]—page 1 of 3)

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NOTE: "No amendment other than to make the provisional application comply with the patent statute and all applicable regulations may be made to the provisional application after the filing date of the provisional application." 37 C.F.R. § 1.53(f).

WARNING: A provisional application may be abandoned by operation of 35 U.S.C. 111(b)(5) on a Saturday, Sunday, or Federal holiday within the District of Columbia, in which case, a nonprovisional application claiming benefit of the provisional application under 35 U.S.C. 119(b) must be filed no later than the preceding day that is not a Saturday, Sunday, or Federal holiday within the District of Columbia. Notice of April 14, 1995, 60 Fed. Reg. 20,185 at 20,202.

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 C.F.R. § 1.51(c)(1)(i).

- 1. The following comprises the information required by 37 C.F.R. § 1.51(c)(1):
- 2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(c)(1)(ii)):

NOTE: "If the correct inventor or inventors are not named on filing a provisional application without a cover sheet under § 1.18(c)(1), the later submission of a cover sheet under § 1.18(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship." 37 C.F.R. § 1.48(b)(2).

NOTE: "The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in § 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(b) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(b)(2) application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,185, at 20,202.

The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.48(c) states that "If multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that § 1.48(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C. 111(b) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111(a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(b). Notice of April 14, 1995, 60 Fed. Reg. 20,185, at 20,202.

See 37 C.F.R. § 1.52.

1.	Barbara	G.	ISAAC.
	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME
2.	Elysia	K.	Joyce
	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME
3.	Anne-Marie	Light	Mettus
	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME

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3. Residence address(es) of the inventor(s), as numbered above (37 C.F.R. § 1.51(c)(1)(ii)):

1. St. Charles, Missouri
2. Kirkwood, Missouri
3. Feasterville, Pennsylvania

4. The title of the invention is (37 C.F.R. § 1.51(c)(1)(iii)):

Polypeptide Compositions Toxic to Anthonomus Insects,
and Methods of Use

5. The name, registration, customer and telephone numbers of the practitioner (if applicable) is (37 C.F.R. § 1.51(c)(1)(iv)):

Name of practitioner: Timothy K. Ball
Reg. No. 42,287 Tel. (636) 737-7387
Customer No. _____

(complete the following, if applicable)

☐ A power of attorney accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(c)(1)(v)):

Docket No.: 38-21 (51932)A

7. The correspondence address for this application is (37 C.F.R. § 1.51(c)(1)(vi)):

Gail Wuellner, Docket Department, Pharmacia Corporation
800 North Lindbergh, Mail Zone E2NA, St. Louis, MO 63167

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(c)(1)(vii))

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

☒ No.

☐ Yes.

The name of the U.S. Government agency and the Government contract number are: _____

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9. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. §§ 1.51(c)(2)-(3):

Specification: No. of pages 86
Drawings: No. of sheets 0

B. Additional documents:

☒ Claims: No. of claims 5

Note: See 37 C.F.R. § 1.51.

- ☐ Power of attorney
☐ Small entity statement
☐ Assignment
☒ Other Sequence Listing

NOTE: Provisional applications may be filed in a language other than English as set forth in existing § 1.52(c). However, an English language translation is necessary for security screening purposes. Therefore, the PTO will require the English language translation and payment of the fee mandated in § 1.52(c) in the provisional application. Failure to timely submit the translation in response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 111(b) application is filed without providing the English language translation in the provisional application, the English language translation will be required to be supplied in every 35 U.S.C. 111(b) application claiming priority of the non-English language provisional application. Notice of April 14, 1995, 60 Fed. Reg. 20,185, at 20,208.

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(d), is \$150.00, for other than a small entity, and \$75.00, for a small entity.

- ☐ Applicant is a small entity.

NOTE: "A . . . statement in compliance with existing § 1.27 is required to be filed in each provisional application in which it is desired to pay reduced fees." Notice of April 14, 1995, 60 Fed. Reg. 20,185, at 20,187.

11. Small entity statement

- ☐ The statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is(are) attached.

12. Fee payment

- ☒ Fee payment in the amount of \$ 150.00 is being made at this time.
☐ No filing fee is to be paid at this time. (This and the surcharge required by 37 C.F.R. 1.16(f) can be paid subsequently).

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13. Method of fee payment

☐ Check in the amount of \$ _____☒ Charge Account No. 13-4125 in the amount of \$ 150.00

A duplicate of this Cover Sheet is attached.

Date: _____

Tel: (. .)

Signature of submitter

OR


Signature of practitioner

Timothy K. Ball

(type or print name of practitioner)

Pharmacia Corporation
700 Chesterfield Parkway North

P.O. Address

Mail Zone BB4F
Chesterfield, Missouri 63198Date: 5-15-00

Reg. No.: 42,287

Tel: (636) 737-7387

Customer No.:

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STRENGTH 02-87

Approved by our District: 10/1/88 CDR DAN LADD

Patent and Trademark Office U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no person is required to respond to a collection of information unless it displays a valid OMB control number.

Docket Number 38-21(5193)		Type a plus sign (+) inside this box +
INVENTOR(S)/APPLICANT(S)		
Given Name (First and middle if used)	Family or Surname	Residence (City and other State or Foreign Country)
Farhad Sakuntala	Moshiri Sivasupramaniam	Chesterfield, Missouri Chesterfield, Missouri

Number 1 of 1

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**PROVISIONAL APPLICATION IN THE UNITED STATES PATENT OFFICE
FOR
POLYPEPTIDE COMPOSITIONS TOXIC TO ANTHEONOMUS INSECTS, AND METHODS OF USE**

**BY
BARBARA G. ISAAC,
ELYSIA K. JOYCE,
ANNE-MARIE LIGHT METTUS,
FARHAD MOSHIRI,
AND
SAKUNTALA SIVASUPRAMANIAM**

005150-29210303

CERTIFICATE OF EXPRESS MAILING NUMBER: EM572390278US DATE OF DEPOSIT: May 15, 2000 I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEES" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, DC 20231.  Timothy K. Ball
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1.0 BACKGROUND OF THE INVENTION

1.1 FIELD OF THE INVENTION

5 The present invention relates generally to the fields of molecular biology. Methods and compositions comprising DNA sequences, and polypeptides derived from *Bacillus thuringiensis* for use in insecticidal formulations and the development of transgenic insect-resistant plants are provided. Novel nucleic acids obtained from *Bacillus thuringiensis* that encode coleopteran-toxic polypeptides are disclosed. Various methods for making and using these nucleic acids, synthetically modified DNA sequences encoding tC851 polypeptides, and native and synthetic polypeptide compositions are also disclosed. The use of DNA sequences as diagnostic probes and templates for protein synthesis, and the use of polypeptides, fusion proteins, antibodies, and peptide fragments in various insecticidal, immunological, and diagnostic applications are also disclosed, as are methods of making and using nucleic acid sequences in the development of transgenic plant cells comprising the polynucleotides.

15 1.2 DESCRIPTION OF THE RELATED ART

20 Environmentally-sensitive methods for controlling or eradicating insect infestation are desirable in many instances, in particular when crops of commercial interest are at issue. The most widely used environmentally-sensitive insecticidal formulations developed in recent years have been composed of microbial pesticides derived from the bacterium *Bacillus thuringiensis*. *B. thuringiensis* is well known in the art, and is characterized morphologically as a Gram-positive bacterium that produces crystal proteins or inclusion bodies which are aggregations of proteins specifically toxic to certain orders and species of insects. Many different strains of *B. thuringiensis* have been shown to produce insecticidal crystal proteins. Compositions including *B. thuringiensis* strains which produce insecticidal proteins have been commercially-available and used as environmentally-acceptable insecticides because they are quite toxic to the specific target insect, but are harmless to plants and other non-targeted organisms.

25 There are several toxin categories established based on primary structure information and the degree of toxin similarities to another. Over the past decade research on the structure and function of *B. thuringiensis* toxins has covered all of the major toxin categories, and while these toxins differ in specific structure and function, general similarities in the structure and function are assumed. Based on the accumulated knowledge of *B. thuringiensis* toxins, a generalized mode of action for *B. thuringiensis* toxins has been created and includes: ingestion by the insect, solubilization in the insect midgut (a

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combination stomach and small intestine), resistance to digestive enzymes sometimes with partial digestion actually "activating" the toxin, binding to the midgut cells, formation of a pore in the insect cells and the disruption of cellular homeostasis (English and Slatin, 1992).

Many of the δ -endotoxins are related to various degrees by similarities in their amino acid sequences. Historically, the proteins and the genes which encode them were classified based largely upon their spectrum of insecticidal activity. The review by Schnepf et al. (Microbiol. Mol. Biol. Rev. (1998) 62:775-806) discusses the genes and proteins that were identified in *B. thuringiensis* prior to 1998, and sets forth the most recent nomenclature and classification scheme as applied to *B. thuringiensis* insecticidal genes and proteins. Using older nomenclature classification schemes, *cry1* genes were deemed to encode lepidopteran-toxic Cry1 proteins, *cry2* genes were deemed to encode Cry2 proteins toxic to both lepidopterans and dipterans, *cry3* genes were deemed to encode coleopteran-toxic Cry3 proteins, and *cry4* genes were deemed to encode dipteran-toxic Cry4 proteins. However, new nomenclature systematically classifies the Cry proteins based upon amino acid sequence homology rather than upon insect target specificities. The classification scheme for many known toxins, not including allelic variations in individual proteins, including dendograms and full *Bacillus thuringiensis* toxin lists is summarized and regularly updated at http://epunix.biola.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html

Most of the nearly 200 Bt crystal protein toxins presently known have some degree of lepidopteran activity associated with them. The large majority of *Bacillus thuringiensis* insecticidal proteins which have been identified do not have coleopteran controlling activity. Therefore, it is particularly important at least for commercial purposes to identify additional coleopteran specific insecticidal proteins.

Cry3 proteins generally display coleopteran activity, however, these generally have limited host range specificity and are not significantly toxic to target pests unless ingested in very high doses. The cloning and expression of the *cry3Bb* gene has been described (Donovan et al., 1992). This gene codes for a protein of 74 kDa with activity against Coleopteran insects, particularly the Colorado potato beetle (CPB) and the southern corn root worm (SCRW). Improved Cry3Bb proteins have been engineered which display increased toxicity at the same or lower doses than the wild type protein (US Patent Serial No. 6,023,013; Feb. 8, 2000).

A *B. thuringiensis* strain, PS201T6, was reported to have activity against WCRW (*Diabrotica virgifera virgifera*) (U. S. Patent No. 5,436,002). This strain also had activity against *Musca domestica*, *Aedes aegypti*, and *Liriomyza trifoli*. The *vip1A* gene, which

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produces a vegetative, soluble, insecticidal protein, has been cloned and sequenced (Intl. Pat. Appl. Pub. No. WO 96/10083, 1996). This gene produces a protein of approximately 80 kDa with activity against both WCRW and Northern Corn Root Worm (NCRW). Another toxin protein with activity against coleopteran insects, including WCRW, is CryIIa, an 81-kDa polypeptide, the gene encoding which has been cloned and sequenced (Intl. Pat. Appl. Pub. No. WO 90/13651, 1990).

2.0 SUMMARY OF THE INVENTION

The polypeptide of the present invention and the novel DNA sequences that encode the protein represent a new *B. thuringiensis* crystal protein and gene, and share only insubstantial sequence homology with any previously identified coleopteran inhibitory endotoxins described in the prior art. Similarly, the *B. thuringiensis* strains of the present invention comprise novel gene sequences that express a polypeptide having insecticidal activity against coleopteran insects, the cotton boll weevil (*Anthonomus grandis* Boheman) in particular.

Disclosed and claimed herein is an isolated *Bacillus thuringiensis* δ -endotoxin polypeptide comprising SEQ ID NO:8. The inventors have identified an insecticidally-active polypeptide comprising the 632 amino acid long sequence of SEQ ID NO:8 which displays insecticidal activity against coleopteran insects. For example, the inventors have shown that a δ -endotoxin polypeptide comprising the sequence of SEQ ID NO:8 has insecticidal activity against boll weevil larvae (BWV), but not against western corn rootworm larvae.

The polypeptide of SEQ ID NO:8 is encoded by a nucleic acid segment comprising at least the open reading frame as shown in SEQ ID NO:7 from nucleotide position 28 through nucleotide position 1923. The invention also discloses compositions and insecticidal formulations that comprise such a polypeptide. Such composition may be a cell extract, cell suspension, cell homogenate, cell lysate, cell supernatant, cell filtrate, or cell pellet of a bacteria cell that comprises a polynucleotide that encodes such a polypeptide. Exemplary bacterial cells that produce such a polypeptide include *Bacillus thuringiensis* EG4135 and EG4268, deposited with NRRL respectively on April 28, 2000. The composition as described in detail below may be formulated as a powder, dust, pellet, granule, spray, emulsion, colloid, solution, or such like, and may be preparable by such conventional means as desiccation, lyophilization, homogenization, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of cells comprising the polypeptide. Preferably such compositions are obtainable from cultures of *Bacillus thuringiensis* EG4135 and EG4268 cells. In all such compositions that contain at least one such insecticidal

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polypeptide, the polypeptide may be present in a concentration of from about 0.001% to about 99% by weight.

5 An exemplary insecticidal polypeptide formulation may be prepared by a process comprising the steps of culturing *Bacillus thuringiensis* EG4135 and EG4268 cells under conditions effective to produce the insecticidal polypeptide; and obtaining the insecticidal polypeptide so produced.

10 For example, the invention discloses and claims a method of preparing a δ -endotoxin polypeptide having insecticidal activity against a coleopteran insect. The method generally involves isolating from a culture of *Bacillus thuringiensis* EG4135 and EG4268 cells that have been grown under appropriate conditions, the δ -endotoxin polypeptide produced by the cells. Such polypeptides may be isolated from the cell culture or supernatant or from spore suspensions derived from the cell culture and used in the native form, or may be otherwise purified or concentrated as appropriate for the particular application.

15 A method of controlling a coleopteran insect population is also provided by the invention. The method generally involves contacting the population with an insecticidally-effective amount of a polypeptide comprising the amino acid sequence of SEQ ID NO:8. Such methods may be used to kill or reduce the numbers of coleopteran insects in a given area, or may be prophylactically applied to an environmental area to prevent infestation by a susceptible insect. Preferably the insect ingests, or is contacted with, an insecticidally-effective amount of the polypeptide.

20 Additionally, the invention provides a purified antibody that specifically binds to the insecticidal polypeptide. Also provided are methods of preparing such an antibody, and methods for using the antibody to isolate, identify, characterize, and/or purify polypeptides to which such an antibody specifically binds. Immunological kits and immunodetection methods useful in the identification of such polypeptides and peptide fragments and/or epitopes thereof are provided in detail herein, and also represent important aspects of the present invention.

25 Such antibodies may be used to detect the presence of such polypeptides in a sample, or may be used as described hereinbelow in a variety of immunological methods. An exemplary method for detecting a δ -endotoxin polypeptide in a biological sample generally involves obtaining a biological sample suspected of containing a δ -endotoxin polypeptide; contacting the sample with an antibody that specifically binds to the polypeptide, under conditions effective to allow the formation of complexes; and detecting the complexes so formed.

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For such methods, the invention also provides an immunodetection kit. Such a kit generally contains, in suitable container means, an antibody that binds to the δ -endotoxin polypeptide, and at least a first immunodetection reagent. Optionally, the kit may provide additional reagents or instructions for using the antibody in the detection of δ -endotoxin polypeptides in a sample.

Preparation of such antibodies may be achieved using the disclosed polypeptide as an antigen in an animal as described below. Antigenic epitopes, shorter peptides, peptide fusions, carrier-linked peptide fragments, and the like may also be generated from a whole or a portion of the polypeptide sequence disclosed in SEQ ID NO:8. Particularly preferred peptides are those that comprise at least 10 contiguous amino acids from the sequence disclosed in SEQ ID NO:8.

In another embodiment, the present invention also provides nucleic acid segments that comprise a selected nucleotide sequence region that comprises the polynucleotide sequence of SEQ ID NO:7. In preferred embodiments, this selected nucleotide sequence region comprises a gene that encodes a polypeptide comprising at least SEQ ID NO:8.

Another aspect of the invention relates to a biologically-pure culture of a wild-type *B. thuringiensis* bacterium selected from the strains EG4135 and EG4268, deposited on April 28, 2000 with the Agricultural Research Culture Collection, Northern Regional Research Laboratory (NRRL), Peoria, Illinois. Also deposited was strain sIC8501 which is an *E. coli* DH5a containing plasmid pIC17501 which contains at least the native *B. thuringiensis* strain EG4135 tIC851 coding sequence. These strains were deposited under the terms of the Budapest Treaty, and viability statements pursuant to International Receipt Form BP/4 were obtained. *B. thuringiensis* strains EG4135 and EG4268 are naturally-occurring strains that contain at least one sequence region encoding the 632 amino acid long polypeptide sequence in SEQ ID NO:8.

A further embodiment of the invention relates to a vector comprising a sequence region that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:8, a recombinant host cell transformed with such a recombinant vector, and biologically-pure cultures of recombinant bacteria transformed with a polynucleotide sequence that encodes the polypeptide disclosed in SEQ ID NO:8. Exemplary vectors, recombinant host cells, transgenic cell lines, and transgenic plants comprising at least a first sequence region that encodes a polypeptide comprising the sequence of SEQ ID NO:8 are described in detail herein.

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5 The present invention also provides transformed host cells, embryonic plant tissue, plant calli, plantlets, and transgenic plants that comprise a selected sequence region that encodes the insecticidal polypeptide. Such cells are preferably prokaryotic or eukaryotic cells such as bacterial, fungal, or plant cells, with exemplary bacterial cells including *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus cereus*, *Escherichia*, *Salmonella*, *Agrobacterium* or *Pseudomonas* cells.

10 The plants and plant host cells are preferably monocotyledonous or dicotyledonous plant cells such as corn, wheat, soybean, oat, cotton, rice, rye, sorghum, sugarcane, tomato, tobacco, kapok, flax, potato, barley, turf grass, pasture grass, berry, fruit, legume, vegetable, ornamental plant, shrub, cactus, succulent, and tree cell.

15 Transgenic plants of the present invention preferably have incorporated into their genome or transformed into their chloroplast or plastid genomes a selected polynucleotide (or "transgene"), that comprises at least a first sequence region that encodes the insecticidal polypeptide of SEQ ID NO:8. Transgenic plants are also meant to comprise progeny (descendant, offspring, etc.) of any generation of such a transgenic plant. A seed of any generation of all such transgenic insect-resistant plants wherein said seed comprises a DNA sequence encoding the polypeptide of the present invention is also an important aspect of the invention.

20 Insect resistant, crossed fertile transgenic plants comprising a transgene that encodes the polypeptide of SEQ ID NO:8 may be prepared by a method that generally involves obtaining a fertile transgenic plant that contains a chromosomally incorporated transgene encoding the insecticidal polypeptide of SEQ ID NO:8; operably linked to a promoter active in the plant; crossing the fertile transgenic plant with a second plant lacking the transgene to obtain a third plant comprising the transgene; and backcrossing the third
25 plant to obtain a backcrossed fertile plant. In such cases, the transgene may be inherited through a male parent or through a female parent. The second plant may be an inbred, and the third plant may be a hybrid.

30 Likewise, an insect resistant hybrid, transgenic plant may be prepared by a method that generally involves crossing a first and a second inbred plant, wherein one or both of the first and second inbred plants comprises a chromosomally incorporated transgene that encodes the polypeptide of SEQ ID NO:8 operably linked to a plant expressible promoter that expresses the transgene. In illustrative embodiments, the first and second inbred plants may be monocot plants selected from the group consisting of: corn, wheat, rice, barley, oats, rye, sorghum, turfgrass and sugarcane.

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In related embodiment, the invention also provides a method of preparing an insect resistant plant. The method generally involves contacting a recipient plant cell with a DNA composition comprising at least a first transgene that encodes the polypeptide of SEQ ID NO:8 under conditions permitting the uptake of the DNA composition; selecting a recipient cell comprising a chromosomally incorporated transgene that encodes the polypeptide; regenerating a plant from the selected cell; and identifying a fertile transgenic plant that has enhanced insect resistance relative to the corresponding non-transformed plant.

A method of producing transgenic seed generally involves obtaining a fertile transgenic plant comprising a chromosomally integrated transgene that encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:8, operably linked to a promoter that expresses the transgene in a plant; and growing the plant under appropriate conditions to produce the transgenic seed.

A method of producing progeny of any generation of an insect resistance-enhanced fertile transgenic plant is also provided by the invention. The method generally involves collecting transgenic seed from a transgenic plant comprising a chromosomally integrated transgene that encodes the polypeptide of SEQ ID NO:8, operably linked to a promoter that expresses the transgene in the plant; planting the collected transgenic seed; and growing the progeny transgenic plants from the seed.

These methods for creating transgenic plants, progeny and seed may involve contacting the plant cell with the DNA composition using one of the processes well-known for plant cell transformation such as microprojectile bombardment, electroporation or *Agrobacterium*-mediated transformation.

An exemplary method disclosed herein provides for protecting a plant from cotton boll weevil infestation comprising providing to a boll weevil in its diet a plant transformed to express a protein toxic to said weevil wherein said protein is expressed in sufficient amounts to control boll weevil infestation and wherein said protein is selected from the group consisting of Cry22Aa, ET70, and tIC851. In a further embodiment of this method, a plant expressing two or more of these proteins for the purpose of reducing boll weevil infestation is contemplated, in particular for reducing the development of races of boll weevils resistant to any of these proteins.

These and other embodiments of the present invention will be apparent to those of skill in the art from the following examples and claims, having benefit of the teachings of the Specification herein.

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2.1 tIC851 POLYNUCLEOTIDE SEQUENCES

The present invention provides polynucleotide sequences that can be isolated from *Bacillus thuringiensis* strains, that are free from total genomic DNA, and that encode the novel insecticidal polypeptides and peptide fragments disclosed herein. The polynucleotides encoding these peptides and polypeptides may encode active insecticidal proteins, or peptide fragments, polypeptide subunits, functional domains, or the like of one or more tIC851 or tIC851-related crystal proteins, such as the polypeptide disclosed in SEQ ID NO:8. In addition the invention encompasses nucleic acid sequences which may be synthesized entirely *in vitro* using methods that are well-known to those of skill in the art which encode the novel tIC851 polypeptide, peptides, peptide fragments, subunits, or functional domains disclosed herein.

As used herein, the term "nucleic acid sequence" or "polynucleotide" refers to a nucleic acid molecule that has been isolated free of the total genomic DNA or otherwise of a particular species. Therefore, a nucleic acid sequence or polynucleotide encoding an endotoxin polypeptide refers to a nucleic acid molecule that comprises at least a first crystal protein-encoding sequence yet is isolated away from, or purified free from, total genomic DNA of the species from which the nucleic acid sequence is obtained, which in the instant case is the genome of the Gram-positive bacterial genus, *Bacillus*, and in particular, the species of *Bacillus* known as *B. thuringiensis*. Included within the term "nucleic acid sequence", are polynucleotide sequences and smaller fragments of such sequences, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids, phage, virions, baculoviruses, artificial chromosomes, viruses, and the like. Accordingly, polynucleotide sequences that have between about 70% and about 80%, or more preferably between about 81% and about 90%, or even more preferably between about 91% and about 99% nucleic acid sequence identity or functional equivalence to the polynucleotide sequence of SEQ ID NO:7 will be sequences that are "essentially as set forth in SEQ ID NO:7." Highly preferred sequences are those which are preferably from about 91% to about 100% identical or functionally equivalent to the nucleotide sequence of SEQ ID NO:7. Other preferred sequences that encode tIC851- or tIC851-related sequences are those which are from about 81% to about 90% identical or functionally equivalent to the polynucleotide sequence set forth in SEQ ID NO:7. Likewise, sequences that are from about 71% to about 80% identical or functionally equivalent to the polynucleotide sequence set forth in SEQ ID NO:7 are also contemplated to be useful in the practice of the present invention.

Similarly, a polynucleotide comprising an isolated, purified, or selected gene or sequence region refers to a polynucleotide which may include in addition to peptide encoding

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sequences, certain other elements such as, regulatory sequences, isolated substantially away from other naturally occurring genes or protein-encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein-, or polypeptide-encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, operator sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides. In certain embodiments, a nucleic acid segment will comprise at least a first gene that encodes a polypeptide comprising the sequence of SEQ ID NO:8.

To permit expression of the gene, and translation of the mRNA into mature polypeptide, the nucleic acid sequence preferably also comprises at least a first promoter operably linked to the gene to express the insecticidal polypeptide in a host cell transformed with this nucleic acid sequence. The promoter may be an endogenous promoter, or alternatively, a heterologous promoter selected for its ability to promote expression of the gene in one or more particular cell types. For example, in the creation of transgenic plants and plant cells comprising a *lC851* gene, the heterologous promoter of choice is one that is plant-expressible, and in many instances, may preferably be a plant-expressible promoter that is tissue- or cell cycle-specific. The selection of plant-expressible promoters is well-known to those skilled in the art of plant transformation, and exemplary suitable promoters are described herein. In certain embodiments, the plant-expressible promoter may be selected from the group consisting of corn sucrose synthetase 1, corn alcohol dehydrogenase 1, corn light harvesting complex, corn heat shock protein, pea small subunit RuBP carboxylase, Ti plasmid mannopine synthase, Ti plasmid nopaline synthase, petunia chalcone isomerase, bean glycine rich protein 1, Potato patatin, lectin, CaMV 35S, and the S-E9 small subunit RuBP carboxylase promoter.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding a bacterial crystal protein, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or operon coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes, recombinant genes, synthetic linkers, or coding regions later added to the segment by the hand of man.

It will also be understood that this invention is not limited to the particular nucleic acid sequences which encode peptides of the present invention, or which encode the amino acid sequence of SEQ ID NO:8, including the DNA sequence which is particularly disclosed in SEQ ID NO:7. Recombinant vectors and isolated DNA segments may therefore

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variously include the polypeptide-coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include these peptide-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

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The DNA sequences of the present invention encompass biologically-functional, equivalent peptides. Such sequences may arise as a consequence of codon degeneracy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally-equivalent proteins or peptides may be created *via* the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. If desired, one may also prepare fusion proteins and peptides, *e.g.*, where the peptide-coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (*e.g.*, proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively). Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding portion of the DNA sequence, whether encoding a full-length insecticidal protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a gene encoding peptides of the present invention, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein. In many cases, the promoter may be the native tIC851 promoter, or alternatively, a heterologous promoter, such as those of bacterial origin (including promoters from other crystal proteins), fungal origin, viral, phage or phagemid origin (including promoters such as CaMV35, and its derivatives, T3, T7, λ, and φ promoters and the like), or plant origin (including constitutive, inducible, and/or tissue-specific promoters and the like).

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In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA sequence under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with a DNA sequence encoding a crystal protein or peptide in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral,

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5 eukaryotic, or plant cell. Naturally, it will be important to employ a promoter that effectively
directs the expression of the DNA segment in the cell type, organism, or even animal, chosen
for expression. The use of promoter and cell type combinations for protein expression is
generally known to those of skill in the art of molecular biology, for example, see Sambrook
10 *et al.*, 1989. The promoters employed may be constitutive, or inducible, and can be used
under the appropriate conditions to direct high level expression of the introduced DNA
sequence, such as is advantageous in the large-scale production of recombinant proteins or
peptides. Appropriate promoter systems contemplated for use in high-level expression
include, but are not limited to, the *Pichia* expression vector system (Pharmacia LKB
15 Biotechnology).

In yet another aspect, the present invention provides methods for producing a
transgenic plant that expresses a selected nucleic acid sequence comprising a sequence
region that encodes the novel endotoxin polypeptides of the present invention. The process
of producing transgenic plants is well-known in the art. In general, the method comprises
20 transforming a suitable plant host cell with a DNA sequence that contains a promoter
operatively linked to a coding region that encodes one or more tIC851 polypeptides. Such a
coding region is generally operatively linked to at least a first transcription-terminating
region, whereby the promoter is capable of driving the transcription of the coding region in
the cell, and hence providing the cell the ability to produce the polypeptide *in vivo*.
25 Alternatively, in instances where it is desirable to control, regulate, or decrease the amount of
a particular recombinant crystal protein expressed in a particular transgenic cell, the
invention also provides for the expression of crystal protein antisense mRNA. The use of
antisense mRNA as a means of controlling or decreasing the amount of a given protein of
interest in a cell is well-known in the art.

Another aspect of the invention comprises transgenic plants which express a
gene, gene sequence, or sequence region that encodes at least one or more of the novel
polypeptide compositions disclosed herein. As used herein, the term "transgenic plant" is
intended to refer to a plant that has incorporated DNA sequences, including but not limited to
30 genes which are perhaps not normally present, DNA sequences not normally transcribed into
RNA or translated into a protein ("expressed"), or any other genes or DNA sequences which
one desires to introduce into the non-transformed plant, such as genes which may normally
be present in the non-transformed plant but which one desires to either genetically engineer
or to have altered expression.

It is contemplated that in some instances the genome of a transgenic plant of the
35 present invention will have been augmented through the stable introduction of one or more

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transgenes, either native, synthetically modified, or mutated, that encodes an insecticidal polypeptide that is identical to, or highly homologous to the polypeptide disclosed in SEQ ID NO:8. In some instances, more than one transgene will be incorporated into the genome of the transformed host plant cell. Such is the case when more than one crystal protein-encoding DNA sequence is incorporated into the genome of such a plant. In certain situations, it may be desirable to have one, two, three, four, or even more *B. thuringiensis* crystal proteins (either native or recombinantly-engineered) incorporated and stably expressed in the transformed transgenic plant. Alternatively, a second transgene may be introduced into the plant cell to confer additional phenotypic traits to the plant. Such transgenes may confer resistance to one or more insects, bacteria, fungi, viruses, nematodes, or other pathogens.

A preferred gene which may be introduced includes, for example, a crystal protein-encoding DNA sequence from bacterial origin, and particularly one or more of those described herein which are obtained from *Bacillus* spp. Highly preferred nucleic acid sequences are those obtained from *B. thuringiensis*, or any of those sequences which have been genetically engineered to decrease or increase the insecticidal activity of the crystal protein in such a transformed host cell.

Means for transforming a plant cell and the preparation of plant cells, and regeneration of a transgenic cell line from a transformed cell, cell culture, embryo, or callus tissue are well-known in the art, and are discussed herein. Vectors, (including plasmids, cosmids, phage, phagemids, baculovirus, viruses, virions, BACs [bacterial artificial chromosomes], YACs [yeast artificial chromosomes]) comprising at least a first nucleic acid segment encoding an insecticidal polypeptide for use in transforming such cells will, of course, generally comprise either the operons, genes, or gene-derived sequences of the present invention, either native, or synthetically-derived, and particularly those encoding the disclosed crystal proteins. These nucleic acid constructs can further include structures such as promoters, enhancers, polylinkers, introns, terminators, or even gene sequences which have positively- or negatively-regulating activity upon the cloned δ -endotoxin gene as desired. The DNA sequence or gene may encode either a native or modified crystal protein, which will be expressed in the resultant recombinant cells, and/or which will confer to a transgenic plant comprising such a segment, an improved phenotype (in this case, increased resistance to insect attack, infestation, or colonization).

The preparation of a transgenic plant that comprises at least one polynucleotide sequence encoding a δ IC851 or δ IC851-derived polypeptide for the purpose of increasing or enhancing the resistance of such a plant to attack by a target insect represents an important

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aspect of the invention. In particular, the inventors describe herein the preparation of insect-resistant monocotyledonous or dicotyledonous plants, by incorporating into such a plant, a transgenic DNA sequence encoding at least one tIC851 polypeptide toxic to a coleopteran insect.

5 In a related aspect, the present invention also encompasses a seed produced by the transformed plant, a progeny from such seed, and a seed produced by the progeny of the original transgenic plant, produced in accordance with the above process. Such progeny and seeds will have a crystal protein-encoding transgene stably incorporated into their genome, and such progeny plants will inherit the traits afforded by the introduction of a stable
10 transgene in Mendelian fashion. All such transgenic plants having incorporated into their genome transgenic DNA sequences encoding one or more tIC851 crystal proteins or polypeptides are aspects of this invention. As well-known to those of skill in the art, a progeny of a plant is understood to mean any offspring or any descendant from such a plant.

15 2.3 DEFINITIONS

The following words and phrases have the meanings set forth below.

A, an: In keeping with long-standing patent tradition, "a" or "an" used throughout this disclosure is intended to mean "one or more."

20 Comprising, comprises: In keeping with long-standing patent tradition, "comprising" and "comprises" used throughout this disclosure is intended to mean "including, but not limited to."

Expression: The combination of intracellular processes, including at least transcription and often the subsequent translation of mRNA of a coding DNA molecule such as a structural gene to produce a polypeptide.

25 Promoter: A recognition site on a DNA sequence or group of DNA sequences that provide an expression control element for a structural gene or sequence to be transcribed and to which an RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene or sequence to be transcribed.

30 Regeneration: The process of growing a plant from a plant cell (e.g., plant protoplast or explant).

Structural gene: A DNA sequence that encodes a messenger RNA which can be transcribed to produce a polypeptide.

Transformation: A process of introducing an exogenous DNA sequence (e.g., a vector, a recombinant DNA molecule) into a cell, protoplast, or organelle within a cell, in

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which that exogenous DNA is incorporated into DNA native to the cell, or is capable of autonomous replication within the cell.

Transformed cell: A cell whose genotype has been altered by the introduction of an exogenous DNA sequence into that cell.

5 **Transgenic cell:** Any cell derived from or regenerated from a transformed cell. Exemplary transgenic cells include plant calli derived from a transformed plant call and particular cells such as leaf, root, stem, e.g., somatic cells, or reproductive (germ) cells obtained from a transgenic plant.

10 **Transgenic plant:** A plant or a progeny of any generation of the plant that was derived from a transformed plant cell or protoplast, wherein the plant nucleic acids contains an exogenous selected nucleic acid sequence region not originally present in a native, non-transgenic plant of the same variety. The terms "transgenic plant" and "transformed plant" have sometimes been used in the art as synonymous terms to define a plant whose native DNA has been altered to contain a heterologous DNA molecule.
15 However, it is thought more scientifically correct to refer to a regenerated plant or callus obtained from a transformed plant cell or protoplast cells as being a transgenic plant. Preferably, transgenic plants of the present invention include those plants that comprise at least a first selected polynucleotide that encodes an insecticidal polypeptide. This selected polynucleotide is preferably a δ -endotoxin coding region (or gene) operably linked to at least
20 a first promoter that expresses the coding region to produce the insecticidal polypeptide in the transgenic plant. Preferably, the transgenic plants of the present invention that produce the encoded polypeptide demonstrate a phenotype of improved resistance to target insect pests. Such transgenic plants, their progeny, descendants, and seed from any such generation are preferably insect resistant plants.

25 **Vector:** A nucleic acid molecule capable of replication in a host cell and/or to which another nucleic acid sequence can be operably linked so as to bring about replication of the attached segment. Plasmids, phage, phagemids, and cosmids are all exemplary vectors. In many embodiments, vectors are used as a vehicle to introduce one or more selected polynucleotides into a host cell, thereby generating a "transformed" or
30 "recombinant" host cell.

3.0 BRIEF DESCRIPTION OF THE DRAWINGS

· The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood

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5 **Figure 2 illustrates an amino acid sequence alignment of the related proteins CryET70 and Cry22Aa, as well as the bestfit alignment of tUC851.**

10 The present invention provides a novel δ -endotoxin, designated tIC851, which is highly toxic to the cotton boll weevil, *Anthonomus grandis* Boheman. This protein has an amino acid sequence which is substantially unrelated to other δ -endotoxins that are toxic to coleopteran insects. The identification of Cry22Aa and CryET70 represented a new class of insecticidal crystal proteins. Unlike other WCRW toxic insecticidal crystal proteins from *B.* *thuringiensis*, CryET70 does not have significant toxicity to SCRW or CPB. The only known protein that is related to CryET70 is Cry22Aa, an insecticidal crystal protein that is reported to be toxic only to hymenopteran insects (GenBank Accession No. I34547). The inventors herein disclose a novel *Bacillus thuringiensis* δ -endotoxin displaying only insubstantial similarity to either CryET70 or to Cry22Aa, and displaying substantial differences in insecticidal spectrum and activity when compared to both of these proteins. 20 The inventors also disclose that both CryET70 and Cry22Aa have significant toxicity to larvae of the cotton boll weevil.

25 Almost all field crops, plants, and commercial farming areas are susceptible to attack by one or more insect pests. Particularly problematic coleopteran pests are identified in Table 1.

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TABLE I
TAXONOMY OF COLEOPTERAN PESTS IN THE SUBORDERS ARCHOSTEMATA AND POLYPHAGA

Infraorder &/or Superfamily	Family	Subfamily	Tribe	Genus	Species
	Cupedidae (reticulated beetles)			<i>Priocma</i>	<i>P. serrata</i>
Bostrichiformia	Dermestidae (skin and larder beetles)			<i>Attagenus</i>	<i>A. pellio</i>
Chrysomeliformia	Cerambycidae (long- horned beetles)			<i>Agapanthia</i>	<i>Agapanthia</i> sp.
		Lepturinae		<i>Leptura</i>	<i>Leptura</i> sp. (flower long-horned beetle)
				<i>Rhagium</i>	<i>Rhagium</i> sp.
				<i>Megacyllene</i>	<i>M. robiniae</i>
		Prioninae		<i>Derobrachus</i>	<i>D. geminatus</i>
				<i>Tetraopes</i>	<i>T. tetropthalmus</i>
	Chrysomelidae (leaf beetles)	Chlamisinae		<i>Ecema</i>	<i>E. neglecta</i>
		Chrysomelinae	Chrysomelini	<i>Chrysomela</i>	<i>C. tremula</i> , <i>Chrysomela</i> sp.
				<i>Oreina</i>	<i>O. cacaliae</i>
			Doryphorini	<i>Chrysolina</i>	<i>Chrysolina</i> sp.
				<i>Leptinotarsa</i>	<i>L. decemlineata</i> (Colorado potato beetle)

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Table 1 (Continued)

Infraorder	Family	Subfamily	Tribe	Genus	Species
			Goniocetini	<i>Goniocetena</i>	<i>G. fornicata</i> , <i>G. holdhausi</i> , <i>G. intermedia</i> , <i>G. interposita</i> , <i>G. kamikawai</i> , <i>G. linnasana</i> , <i>G. nigroplagiata</i> , <i>G. occidentalis</i> , <i>G. olivacea</i> , <i>G. pallida</i> , <i>G. quin-quepunctata</i> , <i>G. rubripennis</i> , <i>G. rufipes</i> , <i>G. tredecim-maculata</i> , <i>G. variabilis</i> , <i>G. viminalis</i>
			Timarchini	<i>Timarcha</i>	<i>Timarcha</i> sp.
		Criocerinae		<i>Oulema</i>	<i>Oulema</i> sp.
		Galerucinae	Galerucini	<i>Monaxia</i>	<i>M. inornata</i> , <i>Monaxia</i> sp.
				<i>Ophraella</i>	<i>O. arctica</i> , <i>O. artemisiata</i> , <i>O. bilineata</i> , <i>O. communis</i> , <i>O. conferta</i> , <i>O. cribrata</i> , <i>O. notata</i> , <i>O. notulata</i> , <i>O. nuda</i> , <i>O. pilosa</i> , <i>O. saxatilis</i> , <i>O. slobodkini</i>
			Luperini	<i>Ceratomyza</i>	<i>C. trifurcata</i>
				<i>Diabrotica</i>	<i>D. barberi</i> (northern corn rootworm), <i>D. undecimpunctata</i> (southern corn rootworm), <i>D. virgifera</i> (western corn rootworm)
		unclassified Chrysomelidae		<i>Lachnalia</i>	<i>Lachnalia</i> sp.
				<i>Epitrix</i>	<i>E. cucumeris</i> (Harris) (potato flea beetle), <i>E. fuscata</i> (eggplant flea beetle)
	Curculionidae (weevils)	Curculioninae		<i>Anthonomus</i>	<i>A. grandis</i> (boll weevil)
		Entiminae	Neospectini	<i>Araucaria</i>	<i>A. confinis</i> , <i>A. globoculus</i> , <i>A. intermedius</i> , <i>A. planticola</i> , <i>A. tessellatus</i>
				<i>Otiorynchus</i>	<i>Otiorynchus</i> sp.

Table 1 (Continued)

Infraorder	Family	Subfamily	Tribe	Genus	Species
			Phylloblini	<i>Diaprepes</i>	<i>D. abbreviata</i>
				<i>Phyllobius</i>	<i>Phyllobius</i> sp.
				<i>Galapaganus</i>	<i>G. galapagoensis</i>
		Hyperinae		<i>Hypera</i>	<i>H. brunneipennis</i> (Egyptian alfalfa weevil), <i>H. postica</i> (alfalfa weevil), <i>H. punctata</i> (clover leaf weevil)
		Molytinae		<i>Pissodes</i>	<i>P. affinis</i> , <i>P. nemorensis</i> , <i>P. schwarzi</i> , <i>P. strobi</i> , <i>P. terminalis</i>
		Rhynchophorinae	Sitophilini	<i>Sitophilus</i>	<i>S. granarius</i> (granary weevil), <i>S. zeamais</i> (maize weevil)
	Nemonychidae			<i>Lebanorhinus</i>	<i>L. succinus</i>
	Scolytidae			<i>Ips</i>	<i>I. acuminatus</i> , <i>I. amitinus</i> , <i>I. cambres</i> , <i>I. duplicatus</i> , <i>I. mansfeldi</i> , <i>I. sexdentatus</i> , <i>I. typographus</i>
				<i>Orthotomicus</i>	<i>O. erosus</i>
				<i>Tomicus</i>	<i>T. minor</i>
Cucujiformia	Coccinellidae (ladybird beetles)			<i>Epilachna</i>	<i>E. borealis</i> (squash ladybird beetle), <i>E. varivittata</i> (Mexican bean beetle)
	Cucujidae (flat bark beetles)			<i>Cryptolestes</i>	<i>C. ferrugineus</i>
				<i>Oryzaephilus</i> (grain beetles)	<i>O. surinamensis</i> (saw-toothed grain beetle)
	Lagriidae (long-joined beetles)			<i>Lagria</i>	<i>Lagria</i> sp.
	Meloidae (blister beetles)			<i>Epicauta</i>	<i>E. fimbriata</i>
				<i>Meloe</i>	<i>M. proscarabaeus</i>
	Rhipiphoridae			<i>Rhipiphorus</i>	<i>R. fasciatus</i>

Table 1 (Continued)

Infraorder	Family	Subfamily	Tribe	Genus	Species
	Tenebrionidae (darkling ground beetles)			<i>Alphitobius</i>	<i>A. diaperinus</i> (lesser mealworm)
				<i>Elegeter</i>	<i>H. amaroides</i> , <i>H. brevicollis</i> , <i>H. costipennis</i> , <i>H. fernandesi</i> , <i>H. glaber</i> , <i>H. gomarensis</i> , <i>H. gran-canariensis</i> , <i>H. impressus</i> , <i>H. intercedens</i> , <i>H. lateralis</i> , <i>H. plicifrons</i> , <i>H. politus</i> , <i>H. subrotundatus</i> , <i>H. tenui-punctatus</i> , <i>H. transversus</i> , <i>H. webbianus</i>
				<i>Misolampus</i>	<i>M. goudoti</i>
				<i>Palorus</i>	<i>P. ficalia</i> , <i>P. raineburgi</i> (small-eyed flour beetle), <i>P. subdepressus</i> (depressed flour beetle)
				<i>Pimelia</i>	<i>P. basica</i> , <i>P. canariensis</i> , <i>P. criba</i> , <i>P. elevata</i> , <i>P. estevaei</i> , <i>P. fernan-dazlopezi</i> , <i>P. grandis</i> , <i>P. granulicollis</i> , <i>P. integra</i> , <i>P. interfecta</i> , <i>P. laevigata</i> , <i>P. lusaria</i> , <i>P. radula</i> , <i>P. sparsa</i> , <i>P. variolosa</i>
				<i>Tenebrio</i>	<i>T. molitor</i> (yellow mealworm), <i>T. obscurus</i> (dark mealworm)
				<i>Tentyria</i>	<i>T. schaumii</i>
				<i>Tribolium</i>	<i>T. brevicornis</i> , <i>T. castaneum</i> (red flour beetle), <i>T. confusum</i> (confused flour beetle), <i>T. freemani</i> , <i>T. madens</i>
				<i>Zophobas</i>	<i>Z. atratus</i> <i>Z. rugipes</i>

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Table 1 (Continued)

Infraorder	Family	Subfamily	Tribe	Genus	Species
Elateriformis - Superfamily Elateroidea				<i>Octinodes</i>	<i>Octinodes</i> sp.
				<i>Pyrophorus</i>	<i>P. plagio-phthalamus</i>
Scarabaeiformis	Lucanidae (Stag beetles)			<i>Dorcus</i>	<i>D. parallelo-pipedus</i>
				<i>Lucanus</i>	<i>L. curvis</i>
	Scarabaeidae (lamellicorn beetles)			<i>Allomyrina</i>	<i>A. dichotoma</i>
		Cetoniinae (flower beetle)		<i>Pachnoda</i>	<i>P. marginata</i>
		Dynastinae		<i>Xyloryctes</i>	<i>X. faunus</i>
		Geotrupinae (earth- boring dung beetles)		<i>Geotrupes</i>	<i>G. stercorosus</i>
		Melolonthinae (chafers)		<i>Costelytra</i>	<i>C. zealandica</i>
				<i>Holotrichia</i>	<i>H. diomphalia</i>
				<i>Melolontha</i>	<i>M. melolontha</i> (cockchafer)
				<i>Odontria</i>	<i>O. striata</i>
					<i>O. variegata</i>
				<i>Prodontria</i>	<i>P. bicolorata</i> , <i>P. capita</i> , <i>P. lewisi</i> , <i>P. tarsis</i> , <i>P. modesta</i> , <i>P. pinguis</i> , <i>P. praeclatella</i> , <i>P. truncata</i> , <i>Prodontria</i> sp.
				<i>Scythrodus</i>	<i>S. squalidus</i>
		Rutelinae (shining leaf chafers)		<i>Popillia</i>	<i>P. japonica</i> (Japanese beetle)

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Table 1 (Continued)

Infraorder	Family	Subfamily	Tribe	Genus	Species
		Scarabaeinae		<i>Copris</i>	<i>C. lunaris</i> (black dung beetle)
				<i>Scarabaeus</i>	<i>Scarabaeus</i> sp. (scarab)
Staphyliniformia	Hydrophilidae			<i>Cercyon</i>	<i>Cercyon</i> sp.
	Silphidae			<i>Nicrophorus</i>	<i>N. americanus</i> , <i>N. marginatus</i> , <i>N. orbicollis</i> , <i>N. tomentosus</i>
	Staphylinidae (rove beetles)			<i>Carpellinus</i>	<i>Carpellinus</i> sp.
				<i>Quedius</i>	<i>Q. mesomelinus</i>
				<i>Tachyporus</i>	<i>Tachyporus</i> sp.
				<i>Xantholinus</i>	<i>Xantholinus</i> sp.

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4.3 PROBES AND PRIMERS

In another aspect, DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene sequences of the selected polynucleotides disclosed herein. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of a selected crystal protein-encoding gene sequence, e.g., a sequence such as that shown in SEQ ID NO:8 (*UC851*), SEQ ID NO:10 (*Cry22Aa*), and SEQ ID NO:2 (*CryET70*). The ability of such DNAs and nucleic acid probes to specifically hybridize to a crystal protein-encoding gene sequence lends them particular utility in a variety of embodiments. Most importantly, the probes may be used in a variety of assays for detecting the presence of complementary sequences in a given sample.

In certain embodiments, it is advantageous to use oligonucleotide primers. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or mutating a defined segment of a crystal protein gene from *A. thuringiensis* using thermal amplification technology. Sequences of related crystal protein genes from other species may also be amplified using such primers.

To provide certain of the advantages in accordance with the present invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes sequences that are complementary to at least an about 23 to about 40 or so long nucleotide stretch of a crystal protein-encoding sequence, such as that shown in SEQ ID NO:7 (*UC851*), SEQ ID NO:9 (*Cry22Aa*), or SEQ ID NO:1 (*CryET70*). A size of at least about 14 or 15 or so nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than about 23 or so bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of about 14 to about 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patents 4,683,195, and 4,683,202, specifically incorporated herein by reference, or by excising selected DNA fragments from recombinant plasmids containing appropriate inserts and suitable restriction sites.

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4.4 EXPRESSION VECTORS

5 The present invention contemplates a polynucleotide of the present invention comprised within one or more expression vectors. Thus, in one embodiment an expression vector comprises a nucleic acid segment containing a *luc851* gene operably linked to a promoter which expresses the gene. Additionally, the coding region may also be operably linked to a transcription-terminating region, whereby the promoter drives the transcription of the coding region, and the transcription-terminating region halts transcription at some point 3' of the coding region.

10 As used herein, the term "operatively linked" means that a promoter is connected to an coding region in such a way that the transcription of that coding region is controlled and regulated by that promoter. Means for operatively linking a promoter to a coding region are well known in the art.

15 In a preferred embodiment, the recombinant expression of DNAs encoding the crystal proteins of the present invention is preferable in a *Bacillus* host cell. Preferred host cells include *B. thuringiensis*, *B. megaterium*, *B. subtilis*, and related bacilli, with *B. thuringiensis* host cells being highly preferred. Promoters that function in bacteria are well-known in the art. An exemplary and preferred promoter for the *Bacillus*-derived crystal proteins include any of the known crystal protein gene promoters, including the *luc851* gene promoter itself. Alternatively, mutagenized or recombinant promoters may be engineered by the hand of man and used to promote expression of the novel gene segments disclosed herein.

20 In an alternate embodiment, the recombinant expression of DNAs encoding the crystal proteins of the present invention is performed using a transformed Gram-negative bacterium such as an *E. coli* or *Pseudomonas* spp. host cell. Promoters which function in high-level expression of target polypeptides in *E. coli* and other Gram-negative host cells are also well-known in the art.

25 Where an expression vector of the present invention is to be used to transform a plant, a promoter is selected that has the ability to drive expression in plants. Promoters that function in plants are also well known in the art. Useful in expressing the polypeptide in plants are promoters that are inducible, viral, synthetic, constitutive as described (Poszkowski *et al.*, 1989; Odell *et al.*, 1985), and temporally regulated, spatially regulated, and spatio-temporally regulated (Chau *et al.*, 1989).

30 A promoter is also selected for its ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region. Structural genes can be driven by a variety of promoters in plant tissues. Promoters can be near-constitutive, such as the

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CaMV 35S promoter, or tissue-specific or developmentally specific promoters affecting dicots or monocots.

Where the promoter is a near-constitutive promoter such as CaMV 35S, increases in polypeptide expression are found in a variety of transformed plant tissues (e.g., callus, leaf, seed and root). Alternatively, the effects of transformation can be directed to specific plant tissues by using plant integrating vectors containing a tissue-specific promoter.

An exemplary tissue-specific promoter is the lectin promoter, which is specific for seed tissue. The Lectin protein in soybean seeds is encoded by a single gene (*Lel*) that is only expressed during seed maturation and accounts for about 2 to about 5% of total seed mRNA. The lectin gene and seed storage protein specific promoter have been fully characterized and used to direct seed specific expression in transgenic tobacco plants (Vodkin *et al.*, 1983; Lindstrom *et al.*, 1990.)

An expression vector containing a coding region that encodes a polypeptide of interest is engineered to be under control of the lectin promoter and that vector is introduced into plants using, for example, a protoplast transformation method (Dhir *et al.*, 1991a). The expression of the polypeptide is directed specifically to the seeds of the transgenic plant.

A transgenic plant of the present invention produced from a plant cell transformed with a tissue specific promoter can be crossed with a second transgenic plant developed from a plant cell transformed with a different tissue specific promoter to produce a hybrid transgenic plant that shows the effects of transformation in more than one specific tissue.

Exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989), corn light harvesting complex (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (Langridge *et al.*, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), CaMV 35S transcript (Odell *et al.*, 1985) and Potato patatin (Wenzler *et al.*, 1989). Preferred promoters are the cauliflower mosaic virus (CaMV 35S) promoter and the S-E9 small subunit RuBP carboxylase promoter.

The choice of which expression vector and ultimately to which promoter a polypeptide coding region is operatively linked depends directly on the functional properties desired, e.g., the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is

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capable of directing the expression of the polypeptide coding region to which it is operatively linked.

5 Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described (Rogers *et al.*, 1987). However, several other plant integrating vector systems are known to function in plants including pCaMVCN transfer control vector described (Fromm *et al.*, 1985). pCaMVCN (available from Pharmacia, Piscataway, NJ) includes the cauliflower mosaic virus CaMV 35S promoter.

10 In preferred embodiments, the vector used to express the polypeptide includes a selection marker that is effective in a plant cell, preferably a drug resistance selection marker. One preferred drug resistance marker is the gene whose expression results in kanamycin resistance; *i.e.*, the chimeric gene containing the nopaline synthase promoter, Tn5 neomycin phosphotransferase II (*aptII*) and nopaline synthase 3' non-translated region described (Rogers *et al.*, 1988).

15 RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

20 Means for preparing expression vectors are well known in the art. Expression (transformation vectors) used to transform plants and methods of making those vectors are described in U. S. Patent Nos. 4,971,908, 4,940,835, 4,769,061 and 4,757,011, the disclosures of which are specifically incorporated herein by reference in their entirety. Those vectors can be modified to include a coding sequence in accordance with the present invention.

25 A variety of methods have been developed to operatively insert a DNA sequence into a vector *via* complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA sequence to be inserted and to the vector DNA. The vector and DNA sequence are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

30 A coding region that encodes a polypeptide having the ability to confer insecticidal activity to a cell is preferably a t1C851 *B. thuringiensis* crystal protein-encoding gene. In preferred embodiments, such a polypeptide has the amino acid residue sequence of SEQ ID NO:8, or a functional equivalent thereof. In accordance with such embodiments, a coding region comprising the DNA sequence of SEQ ID NO:7 is also preferred.

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SEQ ID NO:8

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4.5 CHARACTERISTIC OF THE ϵ IC851 POLYPEPTIDE ISOLATED FROM EG4135

The present invention provides a novel polypeptide that defines a whole or a portion of a *B. thuringiensis* ϵ IC851 crystal protein.

In a preferred embodiment, the invention discloses and claims an isolated and purified ϵ IC851 protein. The ϵ IC851 protein isolated from EG4135 comprises a 632 amino acid sequence, and has a calculated molecular mass of approximately 69,527 Da. ϵ IC851 has a calculated isoelectric constant (pI) equal to 5.80. The amino acid composition of the ϵ IC851 protein is given in Table 2.

TABLE 2
AMINO ACID COMPOSITION OF ϵ IC851

Amino Acid	# Residues	% Total	Amino Acid	# Residues	% Total
Ala	45	7.1	Leu	29	4.6
Arg	13	2.1	Lys	51	8.1
Asn	40	6.3	Met	5	0.8
Asp	49	7.8	Phe	22	3.5
Cys	1	0.2	Pro	34	5.4
Gln	13	2.1	Ser	34	5.4
Glu	41	6.5	Thr	57	9.0
Gly	47	7.4	Trp	8	1.3
His	12	1.9	Tyr	25	3.9
Ile	62	9.8	Val	44	6.9
Acidic	(Asp + Glu)		90	14	
Basic	(Arg + Lys)		64	10	
Aromatic	(Phe + Trp + Tyr)		55	9	
Hydrophobic	(Aromatic + Ile + Leu + Met + Val)		195	31	

4.6 NOMENCLATURE OF THE NOVEL PROTEINS

The inventors have arbitrarily assigned the designation ϵ IC851 to the novel protein of the invention. Likewise, the arbitrary designation of ϵ IC851 has been assigned to the novel nucleic acid sequence which encodes this polypeptide. Formal assignment of gene and protein designations based on the revised nomenclature of crystal protein endotoxins will be assigned by a committee on the nomenclature of *B. thuringiensis*, formed to systematically classify *B. thuringiensis* crystal proteins. The inventors contemplate that the

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arbitrarily assigned designations of the present invention will be superseded by the official nomenclature assigned to these sequences, and that based on the lack of identity or substantial similarity to other known insecticidal protein isolated from *Bacillus thuringiensis*, the tC851 protein will be alone in a separate category and class of proteins.

5

4.7 TRANSFORMED HOST CELLS AND TRANSGENIC PLANTS

Methods and compositions for transforming a bacterium, a yeast cell, a plant cell, or an entire plant with one or more expression vectors comprising a crystal protein-encoding gene sequence are further aspects of this disclosure. A transgenic bacterium, yeast cell, plant cell or plant derived from such a transformation process or the progeny and seeds from such a transgenic plant are also further embodiments of the invention.

Means for transforming bacteria and yeast cells are well known in the art. Typically, means of transformation are similar to those well known means used to transform other bacteria or yeast such as *E. coli* or *Saccharomyces cerevisiae*. Methods for DNA transformation of plant cells include *Agrobacterium*-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, injection into immature embryos and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant strain may not necessarily be the most effective for another plant strain, but it is well known which methods are useful for a particular plant strain. Suitable methods for introducing transforming DNA into a cell consist of but are not limited to *Agrobacterium* infection, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of DNA coated particles, etc. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and van der Eb, 1973; Zatloukal *et al.*, 1992); (2) physical methods such as microinjection (Capecci, 1980), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985; U. S. Patent No. 5,384,253) and the gene gun (Johnston and Tang, 1994; Fynan *et al.*, 1993); (3) viral vectors (Clapp, 1993; Lu *et al.*, 1993; Eglitis and Anderson, 1988; Eglitis *et al.*, 1988); and (4) receptor-mediated mechanisms (Curiel *et al.*, 1991; 1992; Wagner *et al.*, 1992).

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4.7.1 MICROPROJECTILE BOMBARDMENT

5 A particularly advantageous method for delivering transforming DNA sequences into plant cells is microprojectile bombardment. In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

4.7.2 AGROBACTERIUM-MEDIATED TRANSFER

10 *Agrobacterium*-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described (Fraley *et al.*, 1985; Rogers *et al.*, 1987). Further, the integration of the Ti-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome as described (Spielmann *et al.*, 1986; Jorgensen *et al.*, 1987).

15 Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987), have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both 20 armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

25 It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, 30 exogenous genes that encode a polypeptide of interest. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

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4.7.3 GENE EXPRESSION IN PLANTS

5 To overcome limitations in foreign gene expression in plants, particular sequences and signals in RNAs that have the potential for having a specific effect on RNA stability have been identified. In certain embodiments of the invention, therefore, there is a desire to optimize expression of the disclosed nucleic acid segments *in planta*. One particular method of doing so, is by alteration of the bacterial gene to remove sequences or motifs which decrease expression in a transformed plant cell. The process of engineering a coding sequence for optimal expression *in planta* is often referred to as "plantizing" a DNA sequence.

10 Particularly problematic sequences are those which are A+T rich. Unfortunately, since *B. thuringiensis* has an A+T rich genome, native crystal protein gene sequences must often be modified for optimal expression in a plant. The sequence motif ATTTA (or AUUUA as it appears in RNA) has been implicated as a destabilizing sequence in mammalian cell mRNA (Shaw and Kamen, 1986). Many short lived mRNAs have A+T rich 3' untranslated regions, and these regions often have the ATTTA sequence, sometimes present in multiple copies or as multimers (e.g., ATTTATTTA...). Shaw and Kamen showed that the transfer of the 3' end of an unstable mRNA to a stable RNA (globin or VA1) decreased the stable RNA's half life dramatically. They further showed that a pentamer of ATTTA had a profound destabilizing effect on a stable message, and that this signal could exert its effect whether it was located at the 3' end or within the coding sequence. However, the number of ATTTA sequences and/or the sequence context in which they occur also appear to be important in determining whether they function as destabilizing sequences. Shaw and Kamen showed that a trimer of ATTTA had much less effect than a pentamer on mRNA stability and a dimer or a monomer had no effect on stability (Shaw and Kamen, 1987). Note that multimers of ATTTA such as a pentamer automatically create an A+T rich region. This was shown to be a cytoplasmic effect, not nuclear. In other unstable mRNAs, the ATTTA sequence may be present in only a single copy, but it is often contained in an A+T rich region. From the animal cell data collected to date, it appears that ATTTA at least in some contexts is important in stability, but it is not yet possible to predict which occurrences of ATTTA are destabilizing elements or whether any of these effects are likely to be seen in plants. Table 3 lists some of the more common AT rich sequences identified as problematic when present in a coding sequence for which high levels of expression are desired.

SEQUENCE - 5' to 3'

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The addition of a polyadenylate string to the 3' end is common to most eukaryotic mRNAs, both plant and animal. The currently accepted view of polyA addition is that the nascent transcript extends beyond the mature 3' terminus. Contained within this transcript are signals for polyadenylation and proper 3' end formation. This processing at the 3' end involves cleavage of the mRNA and addition of polyA to the mature 3' end. By searching for consensus sequences near the polyA tract in both plant and animal mRNAs, it has been possible to identify consensus sequences that apparently are involved in polyA addition and 3' end cleavage. The same consensus sequences seem to be important to both of these processes. These signals are typically a variation on the sequence AATAAA. In animal cells, some variants of this sequence that are functional have been identified; in plant cells there seems to be an extended range of functional sequences (Wickens and Stephenson, 1984; Dean *et al.*, 1986). Because all of these consensus sequences are variations on AATAAA, they all are A+T rich sequences.

TABLE 3
POLYADENYLATION SITES IN PLANT GENES

PA	AATAAA	Major consensus site
P1A	AATAAT	Major plant site
P2A	AACCAA	Minor plant site
P3A	ATATAA	"
P4A	AATCAA	"
P5A	ATACTA	"
P6A	ATAAAA	"
P7A	ATGAAA	"
P8A	AAGCAT	"
P9A	ATTAAAT	"
P10A	ATACAT	"
P11A	AAAATA	"
P12A	ATTAAA	Minor animal site
P13A	AATTAA	"
P14A	AATACA	"
P15A	CATAAA	"

The present invention provides a method for preparing synthetic plant genes which genes express their protein product at levels significantly higher than the wild-type

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genes which were commonly employed in plant transformation heretofore. In another aspect, the present invention also provides novel synthetic plant genes which encode non-plant proteins.

As described above, the expression of native *B. thuringiensis* genes in plants is often problematic. The nature of the coding sequences of *B. thuringiensis* genes distinguishes them from plant genes as well as many other heterologous genes expressed in plants. In particular, *B. thuringiensis* genes are very rich (~62%) in adenine (A) and thymine (T) while plant genes and most other bacterial genes which have been expressed in plants are on the order of 45-55% A+T.

Due to the degeneracy of the genetic code and the limited number of codon choices for any amino acid, most of the "excess" A+T of the structural coding sequences of some *Bacillus* species are found in the third position of the codons. That is, genes of some *Bacillus* species have A or T as the third nucleotide in many codons. Thus A+T content in part can determine codon usage bias. In addition, it is clear that genes evolve for maximum function in the organism in which they evolve. This means that particular nucleotide sequences found in a gene from one organism, where they may play no role except to code for a particular stretch of amino acids, have the potential to be recognized as gene control elements in another organism (such as transcriptional promoters or terminators, polyA addition sites, intron splice sites, or specific mRNA degradation signals). It is perhaps surprising that such misread signals are not a more common feature of heterologous gene expression, but this can be explained in part by the relatively homogeneous A+T content (~50%) of many organisms. This A+T content plus the nature of the genetic code put clear constraints on the likelihood of occurrence of any particular oligonucleotide sequence. Thus, a gene from *E. coli* with a 50% A+T content is much less likely to contain any particular A+T rich segment than a gene from *B. thuringiensis*.

Typically, to obtain high-level expression of the δ -endotoxin genes in plants, existing structural coding sequence ("structural gene") which codes for the δ -endotoxin are modified by removal of ATTTA sequences and putative polyadenylation signals by site directed mutagenesis of the DNA comprising the structural gene. It is most preferred that substantially all the polyadenylation signals and ATTTA sequences are removed although enhanced expression levels are observed with only partial removal of either of the above identified sequences. Alternately if a synthetic gene is prepared which codes for the expression of the subject protein, codons are selected to avoid the ATTTA sequence and putative polyadenylation signals. For purposes of the present invention putative polyadenylation signals include, but are not necessarily limited to, AATAAA, AATAAT,

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AACCAA, ATATAA, AATCAA, ATACTA, ATAAAA, ATGAAA, AAGCAT, ATTAAT, ATACAT, AAAATA, ATTAAA, AATTAA, AATACA and CATAAA. In replacing the ATTTA sequences and polyadenylation signals, codons are preferably utilized which avoid the codons which are rarely found in plant genomes.

5 The selected DNA sequence is scanned to identify regions with greater than four consecutive adenine (A) or thymine (T) nucleotides. The A+T regions are scanned for potential plant polyadenylation signals. Although the absence of five or more consecutive A or T nucleotides eliminates most plant polyadenylation signals, if there are more than one of the minor polyadenylation signals identified within ten nucleotides of each other, then the
10 nucleotide sequence of this region is preferably altered to remove these signals while maintaining the original encoded amino acid sequence.

 The second step is to consider the about 15 to about 30 or so nucleotide residues surrounding the A+T rich region identified in step one. If the A+T content of the surrounding region is less than 80%, the region should be examined for polyadenylation
15 signals. Alteration of the region based on polyadenylation signals is dependent upon (1) the number of polyadenylation signals present and (2) presence of a major plant polyadenylation signal.

 The extended region is examined for the presence of plant polyadenylation signals. The polyadenylation signals are removed by site-directed mutagenesis of the DNA
20 sequence. The extended region is also examined for multiple copies of the ATTTA sequence which are also removed by mutagenesis.

 It is also preferred that regions comprising many consecutive A+T bases or G+C bases are disrupted since these regions are predicted to have a higher likelihood to form hairpin structure due to self-complementarity. Therefore, insertion of heterogeneous base
25 pairs would reduce the likelihood of self-complementary secondary structure formation which are known to inhibit transcription and/or translation in some organisms. In most cases, the adverse effects may be minimized by using sequences which do not contain more than five consecutive A+T or G+C.

30 **4.7.4 SYNTHETIC OLIGONUCLEOTIDES FOR MUTAGENESIS**

 When oligonucleotides are used in the mutagenesis, it is desirable to maintain the proper amino acid sequence and reading frame, without introducing common restriction sites such as *BglII*, *HindIII*, *SacI*, *KpnI*, *EcoRI*, *NcoI*, *PstI* and *SalI* into the modified gene. These
35 restriction sites are found in poly-linker insertion sites of many cloning vectors. Of course, the introduction of new polyadenylation signals, ATTTA sequences or consecutive stretches

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of more than five A+T or G+C, should also be avoided. The preferred size for the oligonucleotides is about 40 to about 50 bases, but fragments ranging from about 18 to about 100 bases have been utilized. In most cases, a minimum of about 5 to about 8 base pairs of homology to the template DNA on both ends of the synthesized fragment are maintained to insure proper hybridization of the primer to the template. The oligonucleotides should avoid sequences longer than five base pairs A+T or G+C. Codons used in the replacement of wild-type codons should preferably avoid the TA or CG doublet wherever possible. Codons are selected from a plant preferred codon table (such as Table 4 below) so as to avoid codons which are rarely found in plant genomes, and efforts should be made to select codons to preferably adjust the G+C content to about 50%.

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TABLE 4

PREFERRED CODON USAGE IN PLANTS

Amino Acid	Codon	Percent Usage in Plants
ARG	CGA	7
	CGC	11
	CGG	5
	CGU	25
	AGA	29
	AGG	23
	CUA	8
LEU	CUC	20
	CUG	10
	CUU	28
	UUA	5
	UUG	30
SER	UCA	14
	UCC	26
	UCG	3
	UCU	21
	AGC	21
	AGU	15
	ACA	21
THR	ACC	41
	ACG	7
	ACU	31
	CCA	45
	CCC	19
PRO	CCG	9
	CCU	26
	GCA	23
	GCC	32
	GCG	3
GLY	GCU	41
	GGA	32
	GGC	20
	GGG	11
	GGU	37
ILE	AUA	12
	AUC	45
	AUU	43
VAL	GUA	9
	GUC	20
	GUG	28

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TABLE 4 (CONTINUED)

Amino Acid	Codon	Percent Usage in Plants
LYS	GUU	43
	AAA	36
	AAG	64
ASN	AAC	72
	AAU	28
GLN	CAA	64
	CAG	36
HIS	CAC	65
	CAU	35
GLU	GAA	48
	GAG	52
ASP	GAC	48
	GAU	52
TYR	UAC	68
	UAU	32
CYS	UGC	78
	UGU	22
PHE	UUC	56
	UUU	44
MET	AUG	100
TRP	UGG	100

Regions with many consecutive A+T bases or G+C bases are predicted to have a higher likelihood to form hairpin structures due to self-complementarity. Disruption of these regions by the insertion of heterogeneous base pairs is preferred and should reduce the likelihood of the formation of self-complementary secondary structures such as hairpins which are known in some organisms to inhibit transcription (transcriptional terminators) and translation (attenuators).

Alternatively, a completely synthetic gene for a given amino acid sequence can be prepared, with regions of five or more consecutive A+T or G+C nucleotides being avoided. Codons are selected avoiding the TA and CG doublets in codons whenever possible. Codon usage can be normalized against a plant preferred codon usage table (such as Table 4) and the G+C content preferably adjusted to about 50%. The resulting sequence should be examined to ensure that there are minimal putative plant polyadenylation signals and ATTTA sequences. Restriction sites found in commonly used cloning vectors are also

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preferably avoided. However, placement of several unique restriction sites throughout the gene is useful for analysis of gene expression or construction of gene variants.

4.8 METHODS FOR PRODUCING INSECT-RESISTANT TRANSGENIC PLANTS

5 By transforming a suitable host cell, such as a plant cell, with a recombinant *ΔC857* gene sequence, the expression of the encoded crystal protein (*i.e.* a bacterial crystal protein or polypeptide having insecticidal activity against Coleopterans) can result in the formation of insect-resistant plants.

10 A transgenic plant of this invention thus has an increased amount of a coding region (*e.g.*, a gene) that encodes a polypeptide in accordance with SEQ ID NO:8. A preferred transgenic plant is an independent segregant and can transmit that gene and its activity to its progeny. A more preferred transgenic plant is homozygous for that gene, and transmits that gene to all of its offspring upon sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for, by way of example, increased insecticidal capacity against coleopteran insects, preferably in the field, under a range of environmental conditions.

20 Transgenic plants comprising one or more transgenes that encode a polypeptide in accordance with SEQ ID NO:8 will preferably exhibit a phenotype of improved or enhanced insect resistance to the target coleopteran insects as described herein. These plants will preferably provide transgenic seeds, which will be used to create lineages of transgenic plants (*i.e.* progeny or advanced generations of the original transgenic plant) that may be used to produce seed, or used as animal or human foodstuffs, or to produce fibers, oil, fruit, grains, or other commercially-important plant products or plant-derived components. In such instances, the progeny and seed obtained from any generation of the transformed plants will contain the selected and stably integrated transgene that encodes the δ -endotoxin of the present invention. The transgenic plants of the present invention may be crossed to produce hybrid or inbred lines with one or more plants that have desirable properties. In certain circumstances, it may also be desirable to create transgenic plants, seed, and progeny that contain one or more additional transgenes incorporated into their genome in addition to the transgene encoding the polypeptide of the invention. For example, the transgenic plants may contain a second gene encoding the same, or a different insect-resistance polypeptide, or alternatively, the plants may comprise one or more additional transgenes such as those conferring herbicide resistance, fungal resistance, bacterial resistance, stress, salt, or drought

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tolerance, improved stalk or root lodging, increased starch, grain, oil, carbohydrate, amino acid, protein production, and the like.

4.9 ISOLATING HOMOLOGOUS GENE AND GENE FRAGMENTS

The genes and δ -endotoxins according to the subject invention include not only the full length sequences disclosed herein but also fragments of these sequences, or fusion proteins, which retain the characteristic insecticidal activity of the sequences specifically exemplified herein.

It should be apparent to a person skill in this art that insecticidal δ -endotoxins can be identified and obtained through several means. The specific genes, or portions thereof, may be obtained from a culture depository, or constructed synthetically, for example, by use of a gene machine. Variations of these genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. Also, genes which code for active fragments may be obtained using a variety of other restriction enzymes. Proteases may be used to directly obtain active fragments of these δ -endotoxins.

Equivalent δ -endotoxins and/or genes encoding these equivalent δ -endotoxins can also be isolated from *Bacillus* strains and/or DNA libraries using the teachings provided herein. For example, antibodies to the δ -endotoxins disclosed and claimed herein can be used to identify and isolate other δ -endotoxins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the δ -endotoxins which are most constant and most distinct from other *B. thuringiensis* δ -endotoxins. These antibodies can then be used to specifically identify equivalent δ -endotoxins with the characteristic insecticidal activity by immunoprecipitation, enzyme linked immunoassay (ELISA), or Western blotting.

A further method for identifying the δ -endotoxins and genes of the subject invention is through the use of oligonucleotide probes. These probes are nucleotide sequences having a detectable label. As is well known in the art, if the probe molecule and nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and sample are essentially identical. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying formicidal δ -endotoxin genes of the subject invention.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be

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tolerated. Therefore, the probes of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, by methods currently known to an ordinarily skilled artisan, and perhaps by other methods which may become known in the future.

The potential variations in the probes listed is due, in part, to the redundancy of the genetic code. Because of the redundancy of the genetic code, *i.e.*, more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins. Therefore different nucleotide sequences can code for a particular amino acid. Thus, the amino acid sequences of the *B. thuringiensis* δ -endotoxins and peptides can be prepared by equivalent nucleotide sequences encoding the same amino acid sequence of the protein or peptide. Accordingly, the subject invention includes such equivalent nucleotide sequences. Also, inverse or complement sequences are an aspect of the subject invention and can be readily used by a person skilled in this art. In addition it has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser and Kazdy, 1984). Thus, the subject invention includes mutants of the amino acid sequence depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is substantially retained. Further, the invention also includes mutants of organisms hosting all or part of a δ -endotoxin encoding a gene of the invention. Such mutants can be made by techniques well known to persons skilled in the art. For example, UV irradiation can be used to prepare mutants of host organisms. Likewise, such mutants may include asporogenous host cells which also can be prepared by procedures well known in the art.

4.10 RECOMBINANT HOST CELLS

The nucleotide sequences of the subject invention may be introduced into a wide variety of microbial and eukaryotic hosts. As hosts for recombinant expression of tIC851 polypeptides, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and Gram-positive, include *Enterobacteriaceae*, such as *Escherichia*, *Erwinia*, *Shigella*, *Salmonella*, and *Proteus*; *Bacillaceae*; *Rhizobiaceae*, such as *Rhizobium*; *Spirillaceae*, such as *Photobacterium*, *Zymomonas*, *Serratia*, *Aeromonas*, *Vibrio*, *Desulfovibrio*, *Spirillum*; *Lactobacillaceae*; *Pseudomonadaceae*, such as *Pseudomonas* and *Acetobacter*; *Azotobacteraceae*, *Actinomycetales*, and *Nitrobacteraceae*. Among eukaryotes are fungi, such as *Phycomycetes*

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and *Ascomycetes*, which includes yeast, such as *Saccharomyces* and *Schizosaccharomyces*, and *Basidiomycetes* yeast, such as *Rhodotorula*, *Aureobasidium*, *Sporobolomyces*, and the like.

5 Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the genetic constructs of the present invention into the host cell, availability of expression systems, efficiency of expression, stability of the gene of interest in the host, and the presence of auxiliary genetic capabilities.

10 A large number of microorganisms known to inhabit the phylloplane (the surface of the plant leaves) and/or the rhizosphere (the soil surrounding plant roots) of a wide variety of important crops may also be desirable host cells for manipulation, propagation, storage, delivery and/or mutagenesis of the disclosed genetic constructs. These microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms, such as bacteria, e.g., genera *Bacillus* (including the species and subspecies *B. thuringiensis kurstaki* HD-1, *B. thuringiensis kurstaki* HD-73, *B. thuringiensis sotto*, *B. thuringiensis berliner*, 15 *B. thuringiensis thuringiensis*, *B. thuringiensis tolworthi*, *B. thuringiensis dendrolimus*, *B. thuringiensis alesti*, *B. thuringiensis gallertae*, *B. thuringiensis abisawai*, *B. thuringiensis subtoxicus*, *B. thuringiensis entomocidus*, *B. thuringiensis tenebrionis* and *B. thuringiensis san diego*); *Pseudomonas*, *Erwinia*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Streptomyces*, *Rhizobium*, *Rhodopseudomonas*, *Methylophilus*, *Agrobacterium*, *Acetobacter*, *Lactobacillus*, 20 *Artibacter*, *Azotobacter*, *Leuconostoc*, and *Alcaligenes*; fungi, particularly yeast, e.g., genera *Saccharomyces*, *Cryptococcus*, *Kluyveromyces*, *Sporobolomyces*, *Rhodotorula*, and *Aureobasidium*. Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Acetobacter xylinum*, *Agrobacterium tumefaciens*, *Rhodobacter sphaeroides*, *Xanthomonas campestris*, 25 *Rhizobium meliloti*, *Alcaligenes eutrophus*, and *Azotobacter vinlandii*; and phytosphere yeast species such as *Rhodotorula rubra*, *R. glutinis*, *R. marina*, *R. aurantiaca*, *Cryptococcus albidus*, *C. diffluens*, *C. laurentii*, *Saccharomyces rosei*, *S. pretoriensis*, *S. cerevisiae*, *Sporobolomyces roseus*, *S. odoratus*, *Kluyveromyces veronae*, and *Aureobasidium pullulans*.

30 Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing a selected genetic construct into the host, availability of expression systems, efficiency of expression, stability of the polynucleotide in the host, and the presence of auxiliary genetic capabilities. Other considerations include ease of formulation and handling, economics, storage stability, and the like.

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4.11 POLYNUCLEOTIDE SEQUENCES

DNA compositions encoding the insecticidally-active polypeptides of the present invention are particularly preferred for delivery to recipient plant cells, and ultimately in the production of insect-resistant transgenic plants. For example, DNA segments in the form of vectors and plasmids, or linear DNA fragments, in some instances containing only the DNA element to be expressed in the plant cell, and the like, may be employed.

4.12 METHODS FOR PREPARING MUTAGENIZED POLYNUCLEOTIDE SEQUENCES

In certain circumstances, it may be desirable to modify or alter one or more nucleotides in one or more of the polynucleotide sequences disclosed herein for the purpose of altering or changing the insecticidal activity or insecticidal specificity of the encoded polypeptide. In general, the means and methods for mutagenizing a DNA sequences are well-known to those of skill in the art. Modifications to such sequences may be made by random, or site-specific mutagenesis procedures. The polynucleotides may be modified by the addition, deletion, or substitution of one or more nucleotides from the sequence encoding the insecticidally-active polypeptide.

Mutagenesis may be performed in accordance with any of the techniques known in the art such as and not limited to synthesizing an oligonucleotide having one or more mutations within the sequence of a particular region. In particular, site-specific mutagenesis is a technique useful in the preparation of mutants, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to about 75 nucleotides or more in length is preferred, with about 10 to about 25 or more residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those

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skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

5 The preparation of sequence variants of the selected δ -endotoxin-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of DNA sequences may be obtained. For example, recombinant vectors encoding the desired sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

10 As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" also is intended to refer to
15 a process that involves the template-dependent extension of a primer molecule. The term template-dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (Watson, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA
20 vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent No. 4,237,224.

A number of template dependent processes are available to amplify the target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR™) which is described in detail in U. S. Patent Nos.
25 4,683,195, 4,683,202 and 4,800,159. Briefly, in PCR™, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase (e.g., *Taq* polymerase). If the target sequence is present in a sample, the primers will bind to the target and the polymerase will cause the primers to be extended
30 along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction products and the process is repeated. Preferably a reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction
35 methodologies are well known in the art.

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Another method for amplification is the ligase chain reaction (referred to as LCR), disclosed in Eur. Pat. Appl. Publ. No. 320,308. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U. S. Patent No. 4,883,750, incorporated herein by reference in its entirety, describes an alternative method of amplification similar to LCR for binding probe pairs to a target sequence.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]triphosphates in one strand of a restriction site (Walker *et al.*, 1992, incorporated herein by reference in its entirety), may also be useful in the amplification of nucleic acids in the present invention.

4.13 POST-TRANSCRIPTIONAL EVENTS AFFECTING EXPRESSION OF TRANSGENES IN PLANTS

In many instances, the level of transcription of a particular transgene in a given host cell is not always indicative of the amount of protein being produced in the transformed host cell. This is often due to post-transcriptional processes, such as splicing, polyadenylation, appropriate translation initiation, and RNA stability, that affect the ability of a transcript to produce protein. Such factors may also affect the stability and amount of mRNA produced from the given transgene. As such, it is often desirable to alter the post-translational events through particular molecular biology techniques. The inventors contemplate that in certain instances it may be desirable to alter the transcription and/or expression of the polypeptide-encoding nucleic acid constructs of the present invention to increase, decrease, or otherwise regulate or control these constructs in particular host cells and/or transgenic plants.

4.13.1 EFFICIENT INITIATION OF PROTEIN TRANSLATION

The 5'-untranslated leader (5'-UTL) sequence of eukaryotic mRNA plays a major role in translational efficiency. Many early chimeric transgenes using a viral promoter used an arbitrary length of viral sequence after the transcription initiation site and fused this to the AUG of the coding region. More recently studies have shown that the 5'-UTL sequence and the sequences directly surrounding the AUG can have a large effect in

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translational efficiency in host cells and particularly certain plant species and that this effect can be different depending on the particular cells or tissues in which the message is expressed.

5 In most eukaryotic mRNAs, the point of translational initiation occurs at the
AUG codon closest to the 5' cap of the transcript. Comparison of plant mRNA sequences
and site directed mutagenesis experiments have demonstrated the existence of a consensus
sequence surrounding the initiation codon in plants, 5'-UAAACAAUGGCU-3' (SEQ ID
NO:4) (Joshi, 1987; Lutcke *et al.*, 1987). However, consensus sequences will be apparent
amongst individual plant species. For example, a compilation of sequences surrounding the
10 initiation codon from 85 maize genes yields a consensus of 5'-(C/G)AUGGCG-3' (Luehrsen
et al., 1994). In tobacco protoplasts, transgenes encoding β -glucuronidase (GUS) and
bacterial chitinase showed a 4-fold and an 8-fold increase in expression, respectively, when
the native sequences of these genes were changed to encode 5'-ACCAAUGG-3' (Gallie *et al.*,
1987b; Jones *et al.*, 1988). Interestingly, *B. thuringiensis* has chosen to utilize an alternative
15 initiation codon for the native gene encoding tIC851. The inventors find, as described below,
that this codon, although not generally known to encode for other than leucine, is believed to
code for methionine in the first position of the tIC851 polypeptide toxin as judged by N-
terminal amino acid sequence analysis of the purified toxin. Therefore, for efficiency
in planta, it is intended that the more frequently utilized ATG initiation codon will be used
20 instead.

When producing chimeric transgenes (*i.e.* transgenes comprising DNA segments
from different sources operably linked together), often the 5'-UTL of plant viruses are used.
The alfalfa mosaic virus (AMV) coat protein and brome mosaic virus (BMV) coat protein
5'-UTLs have been shown to enhance mRNA translation 8-fold in electroporated tobacco
25 protoplasts (Gallie *et al.*, 1987a; 1987b). A 67-nucleotide derivative (Ω) of the 5'-UTL of
tobacco mosaic virus RNA (TMV) fused to the chloramphenicol acetyltransferase (CAT)
gene and GUS gene has been shown to enhance translation of reporter genes *in vitro* (Gallie
et al., 1987a; 1987b; Sleat *et al.*, 1987; Sleat *et al.*, 1988). Electroporation of tobacco
mesophyll protoplasts with transcripts containing the TMV leader fused to reporter genes
30 CAT, GUS, and LUC produced a 33-, 21-, and 36-fold level of enhancement, respectively
(Gallie *et al.*, 1987a; 1987b; Gallie *et al.*, 1991). Also in tobacco, an 83-nt 5'-UTL of potato
virus X RNA was shown to enhance expression of the neomycin phosphotransferase II
(*NptII*) 4-fold (Poogin and Skryabin, 1992).

The effect of a 5'-UTL may be different depending on the plant, particularly
35 between dicots and monocots. The TMV 5'-UTL has been shown to be more effective in

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tobacco protoplasts (Gallie *et al.*, 1989) than in maize protoplasts (Gallie and Young, 1994). Also, the 5'-UTLs from TMV-Ω (Gallie *et al.*, 1988), AMV-coat (Gehrke *et al.*, 1983; Jobling and Gehrke, 1987), TMV-coat (Goelet *et al.*, 1982), and BMV-coat (French *et al.*, 1986) worked poorly in maize and inhibited expression of a luciferase gene in maize relative to its native leader (Koziel *et al.*, 1996). However, the 5'-UTLs from the cauliflower mosaic virus (CaMV) 35S transcript and the maize genes glutelin (Boronat *et al.*, 1986), PEP-carboxylase (Hadapath and Grula, 1989) and ribulose biphosphate carboxylase showed a considerable increase in expression of the luciferase gene in maize relative to its native leader (Koziel *et al.*, 1996).

These 5'-UTLs had different effects in tobacco. In contrast to maize, the TMV Ω 5'-UTL and the AMV coat protein 5'-UTL enhanced expression in tobacco, whereas the glutelin, maize PEP-carboxylase and maize ribulose-1,5-bisphosphate carboxylase 5'-UTLs did not show enhancement relative to the native luciferase 5'-UTL (Koziel *et al.*, 1996). Only the CaMV 35S 5'-UTL enhanced luciferase expression in both maize and tobacco (Koziel *et al.*, 1996). Furthermore, the TMV and BMV coat protein 5'-UTLs were inhibitory in both maize and tobacco protoplasts (Koziel *et al.*, 1996).

4.13.2 USE OF INTRONS TO INCREASE EXPRESSION

Including one or more introns in the transcribed portion of a gene has been found to increase heterologous gene expression in a variety of plant systems (Callis *et al.*, 1987; Maas *et al.*, 1991; Mascarenhas *et al.*, 1990; McElroy *et al.*, 1990; Vasil *et al.*, 1989), although not all introns produce a stimulatory effect and the degree of stimulation varies. The enhancing effect of introns appears to be more apparent in monocots than in dicots. Tanaka *et al.*, (1990) has shown that use of the catalase intron 1 isolated from castor beans increases gene expression in rice. Likewise, the first intron of the alcohol dehydrogenase 1 (*Adh1*) has been shown to increase expression of a genomic clone of *Adh1* comprising the endogenous promoter in transformed maize cells (Callis *et al.*, 1987; Dennis *et al.*, 1984). Other introns that are also able to increase expression of transgenes which contain them include the introns 2 and 6 of *Adh1* (Luehrsen and Walbot, 1991), the catalase intron (Tanaka *et al.*, 1990), intron 1 of the maize bronze 1 gene (Callis *et al.*, 1987), the maize sucrose synthase intron 1 (Vasil *et al.*, 1989), intron 3 of the rice actin gene (Luehrsen and Walbot, 1991), rice actin intron 1 (McElroy *et al.*, 1990), and the maize ubiquitin exon 1 (Christensen *et al.*, 1992).

Generally, to achieve optimal expression, the selected intron(s) should be present in the 5' transcriptional unit in the correct orientation with respect to the splice junction

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sequences (Callis *et al.*, 1987; Maas *et al.*, 1991; Mascarenhas *et al.*, 1990; Oard *et al.*, 1989; Tanaka *et al.*, 1990; Vasil *et al.*, 1989). Intron 9 of *Adhl* has been shown to increase expression of a heterologous gene when placed 3' (or downstream of) the gene of interest (Callis *et al.*, 1987).

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4.13.3 USE OF SYNTHETIC GENES TO INCREASE EXPRESSION OF HETEROLOGOUS GENES IN PLANTS

When introducing a prokaryotic gene into a eukaryotic host, or when expressing a eukaryotic gene in a non-native host, the sequence of the gene must often be altered or modified to allow efficient translation of the transcript(s) derived from the gene. Significant experience in using synthetic genes to increase expression of a desired protein has been achieved in the expression of *Bacillus thuringiensis* in plants. Native *B. thuringiensis* genes are often expressed only at low levels in dicots and sometimes not at all in many species of monocots (Koziel *et al.*, 1996). Codon usage in the native genes is considerably different from that found in typical plant genes, which have a higher G+C content. Strategies to increase expression of these genes in plants generally alter the overall G+C content of the genes. For example, synthetic *B. thuringiensis* crystal-protein encoding genes have resulted in significant improvements in expression of these endotoxins in various crops including cotton (Perlak *et al.*, 1990; Wilson *et al.*, 1992), tomato (Perlak *et al.*, 1991), potato (Perlak *et al.*, 1993), rice (Cheng *et al.*, 1998), and maize (Koziel *et al.*, 1993).

In a similar fashion the inventors contemplate that the genetic constructs of the present invention, because they contain one or more genes of bacterial origin, may in certain circumstances be altered to increase the expression of these prokaryotic-derived genes in particular eukaryotic host cells and/or transgenic plants which comprise such constructs. Using molecular biology techniques which are well-known to those of skill in the art, one may alter the coding or non coding sequences of the particular δ C851-encoding gene sequences to optimize or facilitate its expression in transformed plant cells at levels suitable for preventing or reducing insect infestation or attack in such transgenic plants.

4.13.4 USE OF PROMOTERS IN EXPRESSION VECTORS

The expression of a gene which exists in double-stranded DNA form involves transcription of messenger RNA (mRNA) from the coding strand of the DNA by an RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. Transcription of DNA into mRNA is regulated by a region of DNA referred to as the "promoter". The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using one of

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the DNA strands as a template to make a corresponding strand of RNA. The particular promoter selected should be capable of causing sufficient expression of the coding sequence to result in the production of an effective insecticidal amount of the *B. thuringiensis* protein.

A promoter is selected for its ability to direct the transformed plant cell's or transgenic plant's transcriptional activity to the coding region, to ensure sufficient expression of the enzyme coding sequence to result in the production of insecticidal amounts of the *B. thuringiensis* protein. Structural genes can be driven by a variety of promoters in plant tissues. Promoters can be near-constitutive (*i.e.* they drive transcription of the transgene in all tissue), such as the CaMV35S promoter, or tissue-specific or developmentally specific promoters affecting dicots or monocots. Where the promoter is a near-constitutive promoter such as CaMV35S or FMV35S, increases in polypeptide expression are found in a variety of transformed plant tissues and most plant organs (*e.g.*, callus, leaf, seed and root). Enhanced or duplicate versions of the CaMV35S and FMV35S promoters are particularly useful in the practice of this invention (Kay *et al.*, 1987; Rogers, U. S. Patent 5,378,619).

Those skilled in the art will recognize that there are a number of promoters which are active in plant cells, and have been described in the literature. Such promoters may be obtained from plants or plant viruses and include, but are not limited to, the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *A. tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the light-inducible promoter from the small subunit of ribulose 1,5-bisphosphate carboxylase (rRUBISCO, a very abundant plant polypeptide), the rice *Act1* promoter and the Figwort Mosaic Virus (FMV) 35S promoter. All of these promoters have been used to create various types of DNA constructs which have been expressed in plants (see e.g., McElroy *et al.*, 1990, U. S. Patent 5,463,175).

25 In addition, it may also be preferred to bring about expression of the *B. thuringiensis* δ -endotoxin in specific tissues of the plant by using plant integrating vectors containing a tissue-specific promoter. Specific target tissues may include the leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity. Therefore, promoter function should be optimized by selecting a
30 promoter with the desired tissue expression capabilities and approximate promoter strength and selecting a transformant which produces the desired insecticidal activity in the target tissues. This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants since there is variation between transformants containing the same heterologous gene due to the site of gene insertion within
35 the plant genome (commonly referred to as "position effect"). In addition to promoters

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which are known to cause transcription (constitutive or tissue-specific) of DNA in plant cells, other promoters may be identified for use in the current invention by screening a plant cDNA library for genes which are selectively or preferably expressed in the target tissues and then determine the promoter regions.

5 An exemplary tissue-specific promoter is the lectin promoter, which is specific for seed tissue. The lectin protein in soybean seeds is encoded by a single gene (*Lel*) that is only expressed during seed maturation and accounts for about 2 to about 5% of total seed mRNA. The lectin gene and seed-specific promoter have been fully characterized and used to direct seed specific expression in transgenic tobacco plants (Vodkin *et al.*, 1983; 10 Lindstrom *et al.*, 1990). An expression vector containing a coding region that encodes a polypeptide of interest can be engineered to be under control of the lectin promoter and that vector may be introduced into plants using, for example, a protoplast transformation method (Dhir *et al.*, 1991). The expression of the polypeptide would then be directed specifically to the seeds of the transgenic plant.

15 A transgenic plant of the present invention produced from a plant cell transformed with a tissue specific promoter can be crossed with a second transgenic plant developed from a plant cell transformed with a different tissue specific promoter to produce a hybrid transgenic plant that shows the effects of transformation in more than one specific tissue.

20 Other exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989), corn light harvesting complex (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (McBride and Summerfelt, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 25 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), CaMV 35s transcript (Odell *et al.*, 1985) and Potato patatin (Wenzler *et al.*, 1989). Preferred promoters are the cauliflower mosaic virus (CaMV 35S) promoter and the S-E9 small subunit RuBP carboxylase promoter.

30 The promoters used in the DNA constructs of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV35S promoter may be ligated to the portion of the *ssRUBISCO* gene that represses the expression of *ssRUBISCO* in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. For purposes of this description, the phrase "CaMV35S" promoter thus includes variations of CaMV35S 35 promoter, *e.g.*, promoters derived by means of ligation with operator regions, random or

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controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay *et al.* (1987). Chloroplast or plastid specific promoters are known in the art (Daniell *et al.*, US Pat. No. 5,693,507; herein incorporated by reference),
5 for example promoters obtainable from chloroplast genes, such as the *psbA* gene from spinach or pea, the *rbcL* and *atpB* promoter region from maize, and rRNA promoters. Any chloroplast or plastid operable promoter is within the scope of the present invention.

The RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected
10 to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. As shown below, a plant gene leader sequence
15 which is useful in the present invention is the petunia heat shock protein 70 (*hsp70*) leader (Winter *et al.*, 1988).

An exemplary embodiment of the invention involves the plastid targeting or plastid localization of the *B. thuringiensis* amino acid sequence. Plastid targeting sequences have been isolated from numerous nuclear encoded plant genes and have been shown to
20 direct importation of cytoplasmically synthesized proteins into plastids (reviewed in Keegstra and Olsen, 1989). A variety of plastid targeting sequences, well known in the art, including but not limited to ADPGPP, EPSP synthase, or ssRUBISCO, may be utilized in practicing this invention. In alternative embodiments preferred, plastidic targeting sequences (peptide and nucleic acid) for monocotyledonous crops may consist of a genomic coding fragment
25 containing an intron sequence as well as a duplicated proteolytic cleavage site in the encoded plastidic targeting sequences.

Tables 5 - 7 list promoters which are illustrative of those known in the art, but which are not meant to be limiting.

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TABLE 5
PLANT PROMOTERS

Promoter	Reference
Viral	
Figwort Mosaic Virus (FMV)	U. S. Patent No. 5,378,619
Cauliflower Mosaic Virus (CaMV)	U. S. Patent No. 5,530,196
	U. S. Patent No. 5,097,025
	U. S. Patent No. 5,110,732
Plant	
Elongation Factor	U. S. Patent No. 5,177,011
Tomato Polygalacturonase	U. S. Patent No. 5,442,052
Arabidopsis Histone H4	U. S. Patent No. 5,491,288
Phaseolin	U. S. Patent No. 5,504,200
Group 2	U. S. Patent No. 5,608,144
Ubiquitin	U. S. Patent No. 5,614,399
P119	U. S. Patent No. 5,633,440
α -amylase	U. S. Patent No. 5,712,112
Viral enhancer/Plant promoter	
CaMV 35S enhancer/mannopine synthase promoter	U. S. Patent No. 5,106,739

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TABLE 6
TISSUE SPECIFIC PLANT PROMOTERS

Tissue Specific Promoter	Tissue(s)	Reference
Blec	epidermis	U. S. Patent No. 5,646,333
malate synthase	seeds; seedlings	U. S. Patent No. 5,689,040
isocitrate lyase	seeds; seedlings	U. S. Patent No. 5,689,040
patatin	tuber	U. S. Patent No. 5,436,393
ZRP2	root	U. S. Patent No. 5,633,363
ZRP2(2.0)	root	U. S. Patent No. 5,633,363
ZRP2(1.0)	root	U. S. Patent No. 5,633,363
RB7	root	U. S. Patent No. 5,459,252
	root	U. S. Patent No. 5,401,836
	fruit	U. S. Patent No. 4,943,674
	meristem	U. S. Patent No. 5,589,583
	guard cell	U. S. Patent No. 5,538,879
	stamen	U. S. Patent No. 5,589,610
SodA1	pollen; middle layer; stomium of anthers	Van Camp <i>et al.</i> , 1996
SodA2	vascular bundles; stomata; axillary buds; pericycle; stomium; pollen flowers; root tips	Van Camp <i>et al.</i> , 1996
CHS15		Faktor <i>et al.</i> , 1996
Paam-1	phloem tissue; cortex; root tips	Vander <i>et al.</i> , 1996
ACT11	elongating tissues and organs; pollen; ovules	Huang <i>et al.</i> , 1997
zmGBS	pollen; endosperm	Russell and Fromm, 1997
zm227	endosperm	Russell and Fromm, 1997
oaAGP	endosperm	Russell and Fromm, 1997
oaGT1	endosperm	Russell and Fromm, 1997
RoIC	phloem tissue; bundle sheath; vascular parenchyma	Graham <i>et al.</i> , 1997
Sh	phloem tissue	Graham <i>et al.</i> , 1997
CMd	endosperm	Grosset <i>et al.</i> , 1997
Bum1	pollen	Treacy <i>et al.</i> , 1997
rice tungro bacilliform virus	phloem	Yin <i>et al.</i> , 1997a; 1997b
S2-RNase	pollen	Pickar <i>et al.</i> , 1998
LaB4	seeds	Baumlein <i>et al.</i> , 1991
gf-2.8	seeds; seedlings	Berna and Bernier, 1997

The ability to express genes in a tissue specific manner in plants has led to the production of male and female sterile plants. Generally, the production of male sterile plants

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involves the use of anther-specific promoters operably linked to heterologous genes that disrupt pollen formation (U. S. Patent Nos. 5,689,051; 5,689,049; 5,659,124). U. S. Patent No. 5,633,441 discloses a method of producing plants with female genetic sterility. The method comprises the use of style-cell, stigma-cell, or style- and stigma-cell specific promoters that express polypeptides that, when produced in the cells of the plant, kills or significantly disturbs the metabolism, functioning or development of the cells.

TABLE 7
INDUCIBLE PLANT PROMOTERS

Promoter	Reference
heat shock promoter	U. S. Patent No. 5,447,858
Em	U. S. Patent No. 5,139,954
Adh1	Kyozoka <i>et al.</i> , 1991
HMG2	U. S. Patent No. 5,689,056
cinnamyl alcohol dehydrogenase	U. S. Patent No. 5,633,439
asparagine synthase	U. S. Patent No. 5,595,896
GST-II-27	U. S. Patent No. 5,589,614

4.13.5 CHLOROPLAST SEQUESTERING AND TARGETING

Another approach for increasing expression of A+T rich genes in plants has been demonstrated in tobacco chloroplast transformation. High levels of expression of an unmodified *Bacillus thuringiensis* crystal protein-encoding genes in tobacco has been reported by McBride *et al.*, (1995).

Additionally, methods of targeting proteins to the chloroplast have been developed. This technique, utilizing the pea chloroplast transit peptide, has been used to target the enzymes of the polyhydroxybutyrate synthesis pathway to the chloroplast (Nawrath *et al.*, 1994). Also, this technique negated the necessity of modification of the coding region other than to add an appropriate targeting sequence.

U. S. Patent 5,576,198 discloses compositions and methods useful for genetic engineering of plant cells to provide a method of controlling the timing or tissue pattern of expression of foreign DNA sequences inserted into the plant plastid genome. Constructs include those for nuclear transformation which provide for expression of a viral single subunit RNA polymerase in plant tissues, and targeting of the expressed polymerase protein into plant cell plastids. Also included are plastid expression constructs comprising a viral gene promoter region which is specific to the RNA polymerase expressed from the nuclear

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expression constructs described above and a heterologous gene of interest to be expressed in the transformed plastid cells.

4.13.6 EFFECTS OF 3' REGIONS ON TRANSGENE EXPRESSION

The 3'-end regions of transgenes have been found to have a large effect on transgene expression in plants (Ingelbrecht *et al.*, 1989). In this study, different 3' ends were operably linked to the neomycin phosphotransferase II (*NptII*) reporter gene and expressed in transgenic tobacco. The different 3' ends used were obtained from the octopine synthase gene, the 2S seed protein from *Arabidopsis*, the small subunit of *rbcS* from *Arabidopsis*, extension from carrot, and chalcone synthase from *Antirrhinum*. In stable tobacco transformants, there was about a 60-fold difference between the best-expressing construct (small subunit *rbcS* 3' end) and the lowest expressing construct (chalcone synthase 3' end).

4.14 ANTIBODY COMPOSITIONS AND METHODS OF MAKING

In particular embodiments, the inventors contemplate the use of antibodies, either monoclonal or polyclonal which bind to one or more of the polypeptides disclosed herein. Means for preparing and characterizing antibodies are well known in the art (See, e.g., Harlow and Lane, 1988). The methods for generating monoclonal antibodies (mAbs) generally begin along the same lines as those for preparing polyclonal antibodies. mAbs may be readily prepared through use of well-known techniques, such as those exemplified in U. S. Patent 4,196,265. Antibody use is well known in the art and can be used for purification, immunoprecipitation, ELISA and western blot for resolving the presence of molecules having identifiable epitopes. Those skilled in the art would not encounter undue experimentation in using antibodies and such methods to isolate, identify, and characterize genes and proteins expressed from such genes as contemplated herein. Immuno-based detection methods for use in conjunction with Western blotting include enzymatically-, radiolabel-, or fluorescently-tagged secondary antibodies against the toxin moiety are considered to be of particular use in this regard.

4.15 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes may be made in the structure of the peptides of the present invention and DNA sequences which encode them and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even

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an improved, second-generation molecule. In particular embodiments of the invention, mutated crystal proteins are contemplated to be useful for increasing the insecticidal activity of the protein, and consequently increasing the insecticidal activity and/or expression of the recombinant transgene in a plant cell. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the codons given in Table 8.

TABLE 8

Amino Acids	Codon Abbreviations ¹		Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Asparagine	Asn	N	AAC	AAU				
Aspartic acid	Asp	D	GAC	GAU				
Cysteine	Cys	C	UGC	UGU				
Glutamic acid	Glu	E	GAA	GAG				
Glutamine	Gln	Q	CAA	CAG				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Lysine	Lys	K	AAA	AAG				
Methionine	Met	M	AUG	UUG*				
Phenylalanine	Phe	F	UUC	UUU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				
Valine	Val	V	GUA	GUC	GUG	GUU		

* the codon UUG is also utilized as an initiation codon as a part of the tlc151 coding sequence
1- three letter code and corresponding single letter code abbreviations

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be

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made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

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5 It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

10 As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

5.0 EXAMPLES

15 The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 - *BACILLUS THURINGIENSIS* STRAINS WITH SEQUENCES RELATED TO *CryET70*

25 We previously identified a *B. thuringiensis* strain expressing a protein which we designated CryET70. The CryET70 protein had effective coleopteran specific bioactivity when provided in bioassay feeding studies to western corn rootworm larvae, but not against southern corn rootworm larvae. We were interested in identifying additional *B. thuringiensis* strains which contained DNA encoding CryET70 and closely related genes. Colony blot hybridization experiments were completed as indicated below, using a probe prepared from *cryET70* DNA. Wild-type *B. thuringiensis* strains were patched onto LB plates and incubated at 30°C for four hours. A Nytran® Maximum-Strength Plus (Schleicher and Schuell, Keene, NH) circular (82 mm) membrane filter was then placed on the plates and the plates and filters were incubated at 25°C overnight. The filters, which contained an exact replica of the

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patches, were then placed on fresh LB plates, and the filters and the original plates were incubated at 30°C for 4 hr to allow for growth of the colonies. To release the DNA from the *B. thuringiensis* cells onto the nitrocellulose filter, the filters were placed, colony-side up, on Whatman 3 MM Chromatography paper (Whatman International LTD., Maidstone, England) soaked with 0.5 N NaOH, 1.5 M NaCl for 15 min. The filters were then neutralized by placing the filters, colony-side up, on Whatman paper soaked with 1 M NH₄-acetate, 0.02 M NaOH for 10 min. The filters were then rinsed in 3X SSC, 0.1% SDS, air dried, and baked for one hr at 80°C in a vacuum oven to prepare them for hybridization.

10 Oligonucleotide primers were designed based on the cryET70 sequence (SEQ ID NO:1):
AM34: 5'-GACATGATTTTACTTTTAGAGC-3' (SEQ ID NO:3)
AM43: 5'-CATCACTTTCCCATAGC-3' (SEQ ID NO:4)

15 A PCR™ with primers AM 34 and AM 43 was used to amplify a cryET70 fragment from pEG1648 DNA. This PCR™ product was labeled with [α -³²P]dATP using the Prime-a-Gene® kit (Promega Corporation, Madison, WI) to generate a cryET70-specific probe. Hybridizations were performed overnight with the hybridization temperature at 63°C. Filters were washed in 1X SSC, 0.1% SDS at 63°C. Hybridizing colonies were detected by autoradiography using Kodak X-OMAT AR X-ray film. The results indicated that several *B.*
20 *thuringiensis* strains in our collection contained DNA sequences which hybridized to cryET70 sequences under specified conditions. The strains identified by colony blot hybridization are listed in Table 9.

5.2 EXAMPLE 2 – PRODUCTION OF ANTIBODY TO CryET70

25 CryET70 specific polyclonal antibody was prepared so that proteins containing CryET70-related epitopes could be identified using immunological methods. Recombinant *B. thuringiensis* strain EG11839 containing plasmid pEG1648 expressing CryET70 was grown in C2 medium for four days at 25°C. The resulting spores and crystals were washed in 2.5X volume H₂O and resuspended at 1/20 the original volume in 0.005% Triton X-100®.
30 The spore-crystal suspension was then loaded on a sucrose step gradient consisting of 79%, 72% and 55% sucrose. The gradient was spun overnight in a Beckman SW28 at 18,000 RPM. CryET70 crystals banded between the 79% and the 72% sucrose layers. CryET70 crystals were washed several times in H₂O and resuspended in 0.005% Triton X-100®. The purified crystals were then solubilized in 50 mM sodium carbonate (pH 10), 5 mM DTT, and
35 any contaminating vegetative cells or spores were removed by centrifugation. The

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supernatant was neutralized with boric acid to pH 8.4, and the solubilized crystals were sent to Rockland Laboratories (Gilbertsville, PA) for antibody production in rabbits according to standard procedures. The rabbits received two intradermal injections on days zero and seven with 50% CryET70 protein in sterile phosphate buffered saline, 50% complete Freund's adjuvant. Two additional boosts were given subcutaneously on days 14 and 28 before a test bleed on day 38. Two hundred fifty μ g of CryET70 were used per rabbit for the initial injection, and 125 μ g of CryET70 were used per rabbit for the subsequent boosts. On day 56 the rabbits were boosted again, as before, prior to a production bleed on day 71. The final boost was with 160 μ g CryET70 on day 80, followed by a termination bleed on day 90.

5.3 EXAMPLE 3 - SOUTHERN AND WESTERN BLOT ANALYSES

Strains identified in Example 1 as containing sequences related to cryET70 were examined further by Southern and Western blot analyses.

Total DNA was prepared from the strains by the following procedure. Vegetative cells were resuspended in a lysis buffer containing 50 mM glucose, 25mM Tris-HCl (pH 8.0), 10 mM EDTA, and 4 mg/ml lysozyme. The suspension was incubated at 37°C for one hr. Following incubation, SDS was added to 1%. The suspension was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2). DNA was precipitated from the aqueous phase by the addition of one-tenth volume 3 M sodium acetate, and two volumes of 100% ethanol. The precipitated DNA was collected with a glass rod, washed with 70% ethanol, and resuspended in dH₂O.

Total DNA was digested with EcoRI and separated on a 0.8% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM Na₂EDTA, pH 8). The DNA was blotted onto an Immobilon-NC nitrocellulose filter (Millipore Corp., Bedford, MA) according to the method of Southern (1975). DNA was fixed to the filter by baking at 80°C in a vacuum oven.

The blot was then hybridized with the cryET70 probe described in Example 1. The filters were exposed to the labeled probe diluted in 3X SSC, 0.1% SDS, 10X Denhardt's reagent (0.2% bovine serum albumin (BSA), 0.2% polyvinylpyrrolidone, 0.2% Ficoll®), 0.2 mg/ml heparin and incubated overnight at 60°C. Following the incubation, the filters were washed in three changes of 3X SSC, 0.1% SDS at 60°C. The filters were blotted dry and exposed to Kodak X-OMAT AR X-ray film (Eastman Kodak Company, Rochester, NY) overnight at -70°C with an intensifying screen (Fisher Biotech, Pittsburgh, PA). Strains containing hybridizing DNA fragments are listed in Table 9.

For the Western blot analysis, *B. thuringiensis* strains were grown in C2 medium (Donovan *et al.*, 1988) at 25°C for four days until sporulation and cell lysis had occurred.

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5 The resulting spores and crystals were harvested by centrifugation, washed in approximately 2.5 times the original volume with H₂O, and resuspended in 0.005% Triton X-100® at one-tenth the original volume. Proteins from 10-fold concentrated cultures of the strains were run on a 10% SDS-polyacrylamide gel (Owl Separation Systems, Woburn, MA). Twenty µl of culture was added to 10 µl of 3x Laemmli buffer and heated at 100°C for five minutes. Fifteen µl were loaded per lane. Following electrophoresis, the gel was blotted to nitrocellulose following standard Western blotting procedures (Towbin *et al.*, 1979). The filter was blocked with TBSN (10 mM Tris, pH 7.8, 0.9% NaCl, 0.1% globulin-free BSA, 0.03% NaN₃) + 2% BSA. The filter was then washed with TBSN twice and then incubated with anti-CryET70 rabbit antiserum diluted 1/1,000 in TBSN. The filter was then washed in TBSN and incubated with alkaline phosphatase conjugated sheep anti-rabbit IgG (1/1,000 dilution in TBSN). After washing in TBSN, proteins antigenically related to CryET70 were detected with ImmunoPure® NBT/BCIP Substrate Kit (Pierce, Rockford, IL). *B. thuringiensis* strains producing proteins antigenically related to CryET70 as judged by Western blot analysis are indicated in Table 9.

5.4 EXAMPLE 4 - BIOASSAY EVALUATION OF *B. THURINGIENSIS* STRAINS

20 Insect bioassays were used to characterize *B. thuringiensis* strains having activity directed against western corn rootworm larvae. *B. thuringiensis* strains were grown in C2 medium (Donovan *et al.*, 1988) at 25°C for four days at which time sporulation and lysis had occurred. The resulting spores and crystals were harvested by centrifugation, washed in approximately 2.5 times the original volume with water, and resuspended in 0.005% Triton X-100® at one-tenth the original culture volume. The spore-crystal suspensions were used directly in bioassay.

25 Insecticidal activity against WCRW larvae was determined via a surface contamination assay on an artificial diet (20 g agar, 50 g wheat germ, 39 g sucrose, 32 g casein, 14 g fiber, 9 g Wesson salts mix, 1 g methyl paraben, 0.5 g sorbic acid, 0.06 g cholesterol, 9 g Vacterzan's vitamin mix, 0.5 ml linseed oil, 2.5 ml phosphoric/propionic acid per liter) in a plastic feeding cup (175 mm² surface). All bioassays were performed using 128-well trays containing approximately 1 ml of diet per well with perforated mylar sheet covers (C-D International Inc., Pitman, NJ). Thirty-two larvae (one per well) were tested per bioassay screen at 50 µl of a spore-crystal suspension per well of diet. The results of the bioassay screen are shown in Table 9.

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TABLE 9
SUMMARY OF SOUTHERN, WESTERN, AND BIOASSAY ANALYSES

Strains	Southern blot	Western blot	% Control WCRW
EG2929	+	+	26
EG3218	+/-	-	30
EG3221	+/-	-	63
EG3303	+/-	-	15
EG3304	+/-	-	0
EG3707	+	-	45
EG3803		-	0
EG3953	+		100
EG3966	+	-	7
EG4113	-	-	40
EG4135	+	+	45
EG4150	-	-	64
EG4268	-	+	46
EG4375		-	100
EG4447	+/-		0
EG4448	+	-	100
EG4503	+/-	-	56
EG4541	+/-	-	72
EG4580	+	+	33
EG4640	-	-	95
EG4737	-	-	72
EG4741	+	-	73
EG5233	-	-	52
EG5366	+	-	69
EG5370	-	-	16
EG5422		-	8

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5.5 EXAMPLE 5 - ANALYSIS OF WILD-TYPE *B. THURINGIENSIS* STRAINS

The CryET70 peptide sequence has previously been shown to share significant amino acid sequence identity with Cry22Aa. Based on the known nucleotide and amino acid

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sequences of CryET70 and Cry22Aa, thermal amplification primers were designed for sequences similar or identical to those of the CryET70 and Cry22Aa coding sequences.

Table 10.

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Thermal Amplification Oligonucleotide Sequence Alignment in *cry22Aa* and *cryET70*

Oligo ^a	Sequence (5'-3') & Corresponding SEQ ID NO	Corresponding Position of Oligo in:	
		<i>cry22Aa</i> (SEQ ID NO:9)	<i>cryET70</i> (SEQ ID NO:1)
2270-1	GCATTCATAGAGGATCAAT SEQ ID NO:4	262-281	350-369
2270-2	ATGATCCTCTATGAAATGC SEQ ID NO:11	281-262	369-350
2270-3	GTTTCCCAAATGGATATCC SEQ ID NO:12	428-446	516-534
2270-4	GGATATCCATTGGGAAAC SEQ ID NO:13	446-428	534-516
2270-5	ATCTAATAACCTACATCAGA SEQ ID NO:14	726-745	814-833
2270-6	TCTGATGTAGGTTATTAGAT SEQ ID NO:15	745-726	833-814
2270-7	TATGGGGAAGTGTGAAAA SEQ ID NO:16	973-992	1061-1080
2270-8	TTTCATCACTTTCCCATTA SEQ ID NO:5	992-973	1080-1061
2270-9	ATGTTGAATTAGAAATAG SEQ ID NO:17	1280-1297	1368-1385
2270-10	CTATTCTAATTCAACAT SEQ ID NO:18	1297-1280	1385-1368
2270-11	AAGTCCTTGTTCTAGGAGAA SEQ ID NO:19	1481-1500	1569-1588
2270-12	TTCTCTAGAACAAAGGACTT SEQ ID NO:20	1500-1481	1588-1569
2270-13	TATGTATTCTATGATTCTAG SEQ ID NO:21	1840-1859	1928-1947
2270-14	CTAGAATCATAGAATACATA SEQ ID NO:22	1859-1840	1947-1928

a: odd numbered oligonucleotides represent sequences identical to the indicated position for each gene (SEQ ID NO), and even numbered oligonucleotides represent sequences complementary to the indicated position for each gene (SEQ ID NO).

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Even numbered oligonucleotides were paired with odd numbered oligonucleotides in various combinations in thermal amplification reactions in order to confirm the expected size of fragments from amplification of sequences from both *cryET70* and *cry22Aa*. DNA obtained from strains EG4135 and EG4268 was also used in separate thermal reactions with all primer pairs. While all pairs produced amplification fragments from both *cryET70* and *cry22Aa*, the only oligonucleotide primer pair which produced a

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product from DNA of strains EG4135 and EG4268 was the 2270-1 and 2270-8 primer pair (SEQ ID NO:4 & SEQ ID NO:5 respectively).

Amplification reactions were performed using 'Taq-Beads' (Pharmacia Biotech), a Stratagene Robocycler™, and the following cycling regimen: 94 C for 30 seconds, 45 C for 45 seconds, and 72 C for 1 minute for 30 cycles. Thermocycling was preceded by a 5 minute incubation at 94 C, followed by a 5 minute incubation at 72 C. The amplification products produced from strains EG4135 and EG4268 were cloned as blunt-end fragments into the SmaI site of pBluescript KSII(+) and sequenced. The sequences of the DNA inserts indicated the presence of an open reading frame (ORF) which displayed approximately 65% sequence identity to the corresponding region from either CryET70 or Cry22Aa.

5.6 EXAMPLE 6 — SEQUENCE ANALYSIS OF THE FULL-LENGTH GENE

Genomic DNA libraries from strains EG4135 and EG4268 were constructed in the Lambda Zap® II vector (Stratagene; La Jolla, CA) and used to isolate recombinant clones containing the entire ORF identified in Example 5. The ORF encodes a protein of 632 amino acids, designated tIC851. The nucleotide sequence encompassing the tIC851 gene (SEQ ID NO:7) is shown below:

AAATATTTT AAAGGGGGAT ACGTAATTGG AATTCTAAAT CTATCATCGA AAAAGGGGTA 60
CAAGAGAATC AATATATTGA TATTCGTAAC ATATGTAGCA TTAATGGTTC TGCTAAATTT 120
GATCCTAATA CTAACATTAC AACCTTAACA GAAGCTATCA ATTCTCAAGC AGGAGCGATT 180
GCTGGAAAAA CTGCCCTAGA TATGAGACGT GATTTTACTC TCGTAGCAGA TATATACCTA 240
GGGTCATAAA GTAGTGGAGC TGATGGTATT GCTATAGCGT TTCATAGAGG ATCAATTGGT 300
TTTATCGGTA CCATGGGTGG AGGCTTAGGG ATTCTAGGAG CACCAACCGG GATAGGATTT 360
GAAATAGATA CGTATTGGAA AGCAACTTCA GATGAAACAG GCGATTTCATT TGGACATGGT 420
CAATGAATG GAGCACATGC GGGATTTGTA AGTACAAATC GAAATGCAAG CTATTTAACA 480
GCCTTAGCTC CTATGCAAAA AATACCTGCA CCTAATAATA AATGGCGGGT TCTAACTATC 540
AATTGGGATG CCGTAACAA CAACTAACA GCACGGCTTC AAGAGAAAAG TAATGATGCT 600
TCTACTAGCA CTCCTAGTCC AAGATATCAA ACATGGGAAC TATTAAATCC TCGCTTTGAT 660
TTAAATCAGA AATATACTTT TATTATCGGC TCAGCTACAG GGGCTGCTAA TAACAAGCAT 720
CAGATTGGAG TTACTTTGTT TGAAGCATAC TTTACAAAAC CAACTATAGA GGCAAATCCT 780
GTTGATATTG AACTAGGCAC AGCGTTTGAT CCATTAAACC ATGAGCCAAT TGGACTCAA 840
GCAACAGATG AAGTAGATGG AGATATAACA AAGGACATTA CGGTAGAATT TAATGACATA 900
GATACCTCCA AACCAGGTGC ATACCGTGTG ACATATAAAG TAGTAAATAG TTATGGAGAA 960
AGTGATGAGA AAACAATAGA AGTCGTAGTA TACACGAAAC CAACTATAAC TGCACATGAT 1020
ATTACGATTA AGAAAGACTT AGCATTTGAT CCATTAAACT ATGAACCAAT TGGACTCAA 1080
GCAACCGATC CAATTGATGG AGATATAACA GATAAAATCG CTGTAAATTT TAATAATGTC 1140

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GATACCTCTA AACCGGGTAA ATACCATGTA ACATATAAAG TGATAAATAG TTATGAAAAA 1200
ATTGATGAAA AAACAATAGA GGTCACAGTA TATACGAAC CATCTATAGT GGCACATGAT 1260
GTTGAGATTA AAAAAGATAC GGCATTGAT CCGTTAACT ATGAACCAAT TGGGCTCAA 1320
GCAACCGATC CAATTGATGG AGATATAACA GATAAAATTA CGGTAGAATC TAATGATGTT 1380
GATACCTCTA AACCAGGTGC ATATAGTGTG AAATATAAAG TAGTAAATAA TTATGAAGAA 1440
AGTGACGAAA AAACAATTGC CATTACAGTA CCTGTTATAG ATGATGGGTG GGAGAATGGC 1500
GATCCGACAG GATGGAAATT CTTCTCTGGT GAAACCAITA CTCTAGAAGA TGATGAAGAG 1560
CATGCTCTTA ATGGTAAATG GGTATTTTAT GCTGATAAAC ATGTAGCAAT ATACAAACAA 1620
GTAGAGTTGA AGAATAATAT CCTTATCAA ATTACAGTAT ATGTTAAACC AGAAGATGAA 1680
GGAACGTGG CACACCATAT TGTAAAGTA TCTTTCAAT CTGATTCTGC TGGTCCAGAA 1740
AGTGAGAAG TTATAAATGA AAGATTAAAT GATGCAGAAC AGATACAAA AGGATACAGA 1800
AAGTAACAA GTATTCCATT TACACCAACA ACCATTGTTT CCAACAAAA ACCAGTGATA 1860
ATTGTTGAAA ACTTTTACC AGGATGGATA GGTGGAGTTA GAATAATTGT AGAGCCTACA 1920
AAGTAAAGAT TATAAAGTAG CTTTAATAA ATATATTTAA AAAAT 1965

The tIC851 ORF initiation codon is TTG beginning at nucleotide 28 of the sequence shown above. The deduced amino acid sequence (SEQ ID NO. 8) of the tIC851 protein is shown below, as translated from the ORF described above:

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MNSEKIIKRG VQESQYIDIR NICSINGSAK FDPWTHITL TRAINSQAGA IAGKTALDMR 60
RDFTLVADIY LGSKSSGADG IALAFERGSI GFIGTNGGGL GILGAFNGIG FEIDTYWKAT 120
SDFTGDSFGH GQMSGANAGF VSTRKASLYL TALAFNQKIP APNKKNEVLT INWDARKNKL 180
TARLQEKEND ASTSTPSRY QTWELNPAF DLMQKTYFII GSATGAANWK RQIGVTLFEA 240
YFTEPTIEAN FVDIELGTAF DPLNHEPIGL KATDEVGDI TKDITVEFND IDTSKPGAYR 300
VYTKVNSYG ESDEKTIENV VYTKPTITAE DITAKDLAF DPLNHEPIGL KATDPIDGDI 360
TDKIAVKFEM VDTSKPGKYH VYTKVNSYE KIDEKTIENV VYTKPSIVAH DVEIKKDTAF 420
DPLNHEPIGL KATDPIDGDI TDKITVESND VDTSKPGAYS VKYKVNRYE ESDEKTIENV 480
VPVIDDGHEN GQFTGWEFFS QETITLQDE EHALNGKNTF YADKEVALYK QVELKNWIPY 540
QITVYVKPED EGTVAHHIVK VSPKDSAGP ESEEVLEKRL IDAQIQNGY RKLTSIPPTP 600
TTIVPKKIPV IIVENFLPGW IGGVRIIEVP TK 632

The predicted molecular weight for this protein is 69,398 Daltons.

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The amino acid sequences of tIC851, CryET70, and Cry22Aa were aligned as shown below using the CLUSTAL alignment program (PC/GENE®). The tIC851 protein shares approximately 56% amino acid sequence identity with CryET70 and approximately 57% amino acid sequence identity with Cry22Aa. According to current *Bacillus thuringiensis* crystal protein nomenclature rules, the tIC851 protein should be assigned to a new secondary class of Cry proteins.

SEQUENCE - CRY851

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For the three way alignment, the K-tuple value was set at 1, the gap penalty value was set at 5, the window size was set at 10, the filtering level was set at 2.5, the open gap cost was set at 10, and the unit gap cost was set at 10. An "*" indicates that a position in the alignment is perfectly conserved, and a "." indicates that a position is well conserved.

5				
	Cry22Aa	MIKQNLNKYDEITVQAASDYIDIRPIFQTEGSAITFMSQTNITTLTQAINS	50	
	ET70	MIKDSISNGYDEITVQA-SDYIDIRSIFQTEGSAITFMSQTNITTLTQAINS	49	
	tIC851	MN---SKSIIEKGVQE-NQYIDIENICSINGSAKFDPTNITTLTQAINS	46	
		*. . . * .*** . .***** * .****.*.*****.*.***		
10				
	Cry22Aa	QAGAIAGKTALDMRHDFTFRADIFLGTKENGADGIAIAFHRGSGIGFVGTK	100	
	ET70	QAGAIAGKTALDMRHDFTFRADIFLGTKENGADGIAIAFHRGSGIGFVGK	99	
	tIC851	QAGAIAGKTALDMRHDFTLVADIYLGSKSSGADGIAIAFHRGSGIGFIQTN	96	
		*****.***.***.***.***.*****.***.***		
15				
	Cry22Aa	GGGLGILGAPKSGIGFELDTYANAPQDEQDSFGHGAMGSGFSPFPNGYPK	150	
	ET70	GGGLGILGALGSGIGFELDTYANAPQDEQDSFGHGAMGSLFPFGFPNGYPK	149	
	tIC851	GGGLGILGAPNGIGFELDTYWEKATSDFTGDSFGHGQNG-----AR	137	
		*****.*****.***.***.***.*****.***.***		
20				
	Cry22Aa	AGFVSTDKNSRWLSALAQMQRIAAPNGRWRLERINDARMKELTANLQUL	200	
	ET70	AGFVSTDKNRGWSALAQMQRIAAPNGRWRLERINDARMKELTANLEDL	199	
	tIC851	AGFVSTERNASYLTALAPMQKIPAPNKKRVLITINDARMKELTARLQER-	186	
		*****.*.***.***.***.***.***.***.***.***.***		
25				
	Cry22Aa	TFPDITVGEKPTPTPTATNRLVNPAPFELQKYTFVIGSATGASNNLEQIG	250	
	ET70	TFPDSTVLVXPRTPEYARWELNPAPFELQKYTFVIGSATGASNNLEQIG	249	
	tIC851	--KSDASTSTPSPRYQTWELNPAPFELQKYTFVIGSATGAANNKEQIG	234	
		** . * * *****.*.*****.*****.*.***		
30				
	Cry22Aa	IIEFDAYFTKPTIEANRVVVPVGATFNPKTYPGINLRATDEKIDGILTSEI	300	
	ET70	IIEFDAYFTKPTIEANRVVVPVGATFNPKTYPGINLRATDEKIDGILTSEI	299	
	tIC851	VTLFRAYFTKPTIEANRVVDIELGTAFDPLNHEPIGLKATDEVDGDITKDI	284	
		. . *.*****.***.***.***.***.***.***.***.***		
35				
	Cry22Aa	IVKANVNTSKTGVYVYTYVENSYGESDEKTIETVTFSEPTIIASDVBI	350	
	ET70	IVTDMNVNTSKBGVYVYTYVENSYGESDEKTIETVTFSEPTIIASDVBI	349	
	tIC851	TVFMDIDTSKPGAYRVYTKVENSYGESDEKTIETVYVYTKPTITAEHITI	334	
		.*.***.***.***.***.***.***.***.***.***.***.***		
40				
	Cry22Aa	EKGESFNPFLTDSRVGLSAQDSLGNDITQSVKVKSSNVDTSKPGGEYEVVFE	400	
	ET70	EKGESFNPFLTDSRVGLSAQDSLGNDITSKVVKSSNVDTSKPGGEYDVVFE	399	
	tIC851	KKDLAFDPL-----NYE----	346	
		.*.***.***.***.***.***.***.***.***.***.***		
45				
	Cry22Aa	VTDSPGGKAEEKDKVTVLGQPSIEANDVELEIDDSLDPLTDAKVLRAKD	450	
	ET70	VTDNFGGKAEEKIKVTVLGQPSIEANDVELEIGDLFNPFLTDSQVGLRAKD	449	
	tIC851	-----PIGLKATD	354	
		.		
50				
	Cry22Aa	SLGNDITKDIKVKFNNVDTSNGSKYEVIFFVTDSPGKKAEEKSIEVLVLGE	500	
	ET70	SLGNDITNDVKVKSSNVDTSKPGGEYEVVFEVTDSPGKKAEEKSIEVLVLGE	499	
	tIC851	PIDGDITDKLAVKFNVDTSKPGKYHVYTKVINSYKIDDKTIETVTVTK	404	
		. . . ***.***.***.***.***.***.***.***.***.***		

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5	Cry22Aa	PSIRANDVEVNRGRTTPEPLTDSRVGLRAKDSLGNDDTKOVKIKSSNVDT8	550
	ET70	PSIRANNVEIEKDERFDPLTDSRVGLRAKDSLKGDITMDVKVSSNVDT8	549
	tIC851	PSIVARDVEIKKDTAFDPLNYPEIGLKATDPIDGDIITDKITVESNDVDT8	454
*** *...			
10	Cry22Aa	KPGYEYVVFVTDTRFGKYVEKTIGVIVPVIDDEWEDGNVGNKPYAGQDI	600
	ET70	KPGYEYVVFVTDTRFGKYVKLIIVVIVPVIDDEWEDGNVGNKPYAGQDI	599
	tIC851	KPGAYSVKYKVVNMYEESDEKTLAVTVFVIDDGWERQDPTGNKFFSGETI	504
***... ..* *			
15	Cry22Aa	KLLKDPDKAYKGYVFDSEHVAISKTIPLTDLQININYEITVYAKA8-	649
	ET70	TLLKDPKAYKGYVFDSEHAAISKTIPTVTLQVGGHYEITVYVKA8-	648
	tIC851	TLEDDEEHALNGKNVFDYADKHAIVYKQV---ELKQNIIPYQITVYKPEDE	551
* ..* ..*			
20	Cry22Aa	---GDHRLKVTYKKDPAGPPEPFVFNRLISTGTLVENDYRELKGT-FEVT	695
	ET70	---GDHRLKVTYKKDPPEPPEPFVFNRLISTGTLVENDYRELKGT-FEVT	694
	tIC851	GTVAHHIVKVSFXSDSAGPSEKEVINERLIDAEQIQKGYRKLTSIPFTPT	601
..* ..*... ..*... ..*			
25	Cry22Aa	EL--NKAPLIIVENFGAGYIGGIRIV--KIS	722
	ET70	EL--NQAPLIIVENFGAGYIGGIRIV--KIS	721
	tIC851	TIVPNKKFVIIVENFLPGWIGGVRIIVEPTK	632
.. ..*... ..*... ..			

5.7 EXAMPLE 7 - EXPRESSION OF THE tIC851 PROTEIN IN *B. THURINGIENSIS* AND BIOASSAY EVALUATION

The coding region for tIC851 was cloned into the *B. thuringiensis* shuttle vector pEG597 (Baum *et al.*, 1990) together with about 0.6 kb of flanking native DNA both up and down stream of the ORF, giving rise to the recombinant plasmids pIC17501 and pIC17502. These plasmids contain a gene which confers chloramphenicol resistance on a *B. thuringiensis* host cell. Plasmid pMON56207, containing the cryET70 coding sequence, confers erythromycin resistance to a *B. thuringiensis* host. These plasmids were introduced into the Cry- *B. thuringiensis* strain EG10650 by electroporation. Recombinants harboring the correct plasmids were selected for growth on starch agar medium supplemented with the appropriate antibiotic.

Recombinants were grown in C2 medium for 72-96 hours, at which time the cultures were sporulated and the cells lysed. Plasmids pIC17501 and pIC17502, differing only with respect to the orientation of the tIC851 gene insert, directed the production of a protein with an apparent molecular mass of approximately 75 kDa, as judged by SDS polyacrylamide gel electrophoresis. EG10650 recombinants harboring the cloning vector

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PEG597 did not produce a crystal protein. Plasmid pMON56207 directed the production of CryET70, with an apparent molecular mass of approximately 80 kDa.

tIC851 was tested against boll weevil larvae and western corn rootworm (WCRW) larvae in an insect feeding bioassay and shown not to have activity against WCRW, but surprisingly good activity against boll weevil. Based on the similarity of tIC851 to CryET70 and Cry22Aa, these two proteins were also tested against boll weevil. Dose-response studies on the susceptibility of the boll weevil to these *B. thuringiensis* toxins was performed by diet incorporation (Stone *et al.* 1991). A series of 3 to 8 concentrations prepared by serial dilution was used in each instance. First instar larvae were manually infested onto the diet. Mortality and weight measurements were recorded 10 days after infestation. Larvae that were dead or were still at the neonate stage were considered dead in tabulating larval responses to the individual proteins. Concentration-mortality regressions were estimated assuming the probit model (SAS software 1995). Weight records were used to calculate effective concentrations using the non-linear regression model (SAS 1995).

Surprisingly, Cry22Aa was also found to have significant toxicity to boll weevil larvae comparable to that of CryET70, as indicated in Table 11. This is the first report that Cry22Aa and CryET70 have activity against this target insect pest.

Table 11. Cotton boll weevil Bioassay

Protein	LC ₅₀ (µg/well)	EC ₅₀ (µg/well)
CryET70	3.12 (1.95-5.00)	1.92 ± 0.37
Cry22Aa	0.72 (0.022-1.70)	0.36 ± 0.18

The toxin encoded by the tIC851 gene has interesting similarities as well as differences when compared with the toxins encoded by the CryET70 and Cry22Aa genes. Both CryET70 and Cry22Aa have within their primary sequence four repeating regions of approximately 80 amino acids each, aligned in a head-to-tail fashion. The sequence of tIC851 shows that the tIC851 protein has only three of the four 'repeat domains' found in CryET70 and Cry22Aa. This accounts for most of the approximately 90 amino acids by which the tIC851 coding sequence is shorter than that of either CryET70 or Cry22Aa. Despite this difference in structure, tIC851 has significant activity on boll weevil larvae. The novel modular structure of these three Bt toxins should be of value in semi-rational engineering of variants, which could have increased potency or spectrum of activity.

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5.8 EXAMPLE 8 -- TRANSGENIC PLANTS EXPRESSING tIC851

One or more transgenes, each containing a structural coding sequence of the present invention can be inserted into the genome of a plant by any suitable method such as those detailed herein. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella (1983), Bevan (1983), Klee (1985) and Eur. Pat. Appl. Publ. No. EP0120516. In addition to plant transformation vectors derived from the Ti or root-inducing (RI) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using viruses or pollen (Fromm *et al.*, 1986; Armstrong *et al.*, 1990; Fromm *et al.*, 1990). For efficient expression of the polynucleotides disclosed herein in transgenic plants, the selected sequence region encoding the insecticidal polypeptide must have a suitable sequence composition (Diehn *et al.*, 1996).

Expression of the tIC851 protein from within a plant expression vector is then confirmed in plant protoplasts by electroporation of the vector into protoplasts followed by protein blot and ELISA analysis. This vector can be introduced into the genomic DNA of plant embryos such as cotton by particle gun bombardment followed by paromomycin selection to obtain cotton plants expressing the *cry* gene essentially as described in U. S. Patent No. 5,424,412. For example, the plant transformation and expression vector can be introduced via co-bombardment with a hygromycin resistance conferring plasmid into transformation susceptible cotton tissue, followed by hygromycin selection, and regeneration. Transgenic cotton lines expressing the tIC851 protein can then identified by ELISA analysis. Progeny seed from these events can then subsequently be tested for protection from susceptible insect feeding.

The *B. thuringiensis* polypeptides described herein are primarily localized to the cytoplasm of the plant cell, and this cytoplasmic localization results in plants that are insecticidally effective. However, in certain embodiments, it may be advantageous to direct the *B. thuringiensis* polypeptide to other compartments of the plant cell. Localizing *B. thuringiensis* proteins in compartments other than the cytoplasm may result in less exposure of the *B. thuringiensis* proteins to cytoplasmic proteases leading to greater accumulation of the protein yielding enhanced insecticidal activity.

Utilizing SSU CTP sequences to localize crystal proteins to the chloroplast might also be advantageous. Localization of the *B. thuringiensis* crystal proteins to the chloroplast could protect these from proteases found in the cytoplasm. This could stabilize the proteins

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and lead to higher levels of accumulation of active toxin. *cry* genes containing the CTP may be used in combination with the SSU promoter or with other promoters such as CaMV35S.

5 In addition to t1C851 expression in plants as described herein, it is specifically intended that Cry22Aa and CryET70 be used alone or in combination with each other or in combinations along with t1C851 in plants to protect plants from boll weevil infestation and in particular combinations to prevent the onset of resistance of boll weevils to any of the proteins when used alone.

10 All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and
15 physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims. Accordingly, the exclusive rights sought to be patented are as described in the claims below.

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6.0 REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

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- U. S. Patent 4,196,265, issued Apr. 1, 1980.
- U. S. Patent 4,237,224, issued Dec. 2, 1980.
- U. S. Patent 4,554,101, issued Nov. 19, 1985.
- U. S. Patent 4,683,195, issued Jul. 28, 1987.
- U. S. Patent 4,683,202, issued Jul. 28, 1987.
- 30 U. S. Patent 4,757,011, issued Jul. 12, 1988.
- U. S. Patent 4,766,203, issued Aug. 23, 1988.
- U. S. Patent 4,769,061, issued Sep. 6, 1988.
- U. S. Patent 4,771,131, issued Sep. 13, 1988.
- U. S. Patent 4,797,279, issued Jan. 10, 1989.

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- U. S. Patent 4,800,159, issued Jan. 24, 1989.
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ABSTRACT OF THE INVENTION

A novel gene encoding a Coleopteran inhibitory *Bacillus thuringiensis* insecticidal crystal protein is disclosed. The protein, tIC851, is insecticidally active and provides plant protection from at least cotton boll weevil, *Anthonomus grandis*,
5 when applied to plants in an insecticidally effective composition.

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What is claimed is:

1. An isolated and purified protein comprising the amino acid sequence as set forth in SEQ ID NO:8.
- 5 2. An isolated an purified polynucleotide sequence encoding the polypeptide of SEQ ID NO:8, comprising SEQ ID NO:7.
3. A plant transformed to contain a polynucleotide sequence encoding the protein of SEQ ID NO:8.
- 10 4. A method for protecting a plant from cotton boll weevil infestation comprising providing to a boll weevil in its diet a plant transformed to express a protein toxic to said weevil wherein said protein is expressed in sufficient amounts to control boll weevil infestation and wherein said protein is selected from the group consisting of
15 Cry22Aa, ET70, and tIC851.
5. A method for protecting a plant from cotton boll weevil infestation comprising providing to a boll weevil in its diet a plant transformed to express one or more
20 proteins toxic to said weevil wherein said proteins are expressed in sufficient amounts alone or in combination to control boll weevil infestation and wherein said protein are selected from the group consisting of Cry22Aa, ET70, and tIC851.

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SEQUENCE LISTING

<110> Barbara G. Isaac
Elysia K. Joyce
Anne-Marie Light Mettus
Farhad Moshiri
Sakuntala Sivasupramaniam

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and Methods of Use

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Asn Val Asp Thr Ser Lys Pro Gly Glu Tyr Asp Val Val Phe Glu Val 385 390 395 400		
Thr Asp Asn Phe Gly Gly Lys Ala Glu Lys Glu Ile Lys Val Thr Val 405 410 415		
Leu Gly Gln Pro Ser Ile Glu Ala Asn Asp Val Glu Leu Glu Ile Gly 420 425 430		
Asp Leu Phe Asn Pro Leu Thr Asp Ser Gln Val Gly Leu Arg Ala Lys 435 440 445		

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Asp	Ser	Leu	Gly	Lys	Asp	Ile	Thr	Asn	Asp	Val	Lys	Val	Lys	Ser	Ser
450					455					460					
Asn	Val	Asp	Thr	Ser	Lys	Pro	Gly	Glu	Tyr	Glu	Val	Val	Phe	Glu	Val
465					470					475					480
Thr	Asp	Arg	Phe	Gly	Lys	Lys	Ala	Glu	Lys	Ser	Ile	Lys	Val	Leu	Val
				485					490					495	
Leu	Gly	Glu	Pro	Ser	Ile	Glu	Ala	Asn	Asn	Val	Glu	Ile	Glu	Lys	Asp
			500					505					510		
Glu	Arg	Phe	Asp	Pro	Leu	Thr	Asp	Ser	Arg	Val	Gly	Leu	Arg	Ala	Lys
	515						520					525			
Asp	Ser	Leu	Gly	Lys	Asp	Ile	Thr	Asn	Asp	Val	Lys	Val	Lys	Ser	Ser
530					535						540				
Asn	Val	Asp	Thr	Ser	Lys	Pro	Gly	Glu	Tyr	Glu	Val	Val	Phe	Glu	Val
545					550					555					560
Thr	Asp	Arg	Phe	Gly	Lys	Tyr	Val	Lys	Lys	Leu	Ile	Val	Val	Ile	Val
				565					570					575	
Pro	Val	Ile	Asp	Asp	Glu	Tyr	Glu	Asp	Gly	Asn	Val	Asn	Gly	Tyr	Lys
			580					585					590		
Phe	Tyr	Ala	Gly	Gln	Asp	Ile	Thr	Leu	Leu	Lys	Asp	Pro	Glu	Lys	Ala
	595					600						605			
Tyr	Lys	Gly	Glu	Tyr	Val	Phe	Tyr	Asp	Ser	Arg	His	Ala	Ala	Ile	Ser
	610					615					620				
Lys	Thr	Ile	Pro	Val	Thr	Asp	Leu	Gln	Val	Gly	Gly	Asn	Tyr	Glu	Ile
625					630					635					640
Thr	Val	Tyr	Val	Lys	Ala	Glu	Ser	Gly	Asp	His	His	Leu	Lys	Val	Thr
				645					650					655	
Tyr	Lys	Lys	Asp	Pro	Lys	Gly	Pro	Glu	Glu	Pro	Pro	Val	Phe	Asn	Arg
			660					665					670		
Leu	Ile	Ser	Thr	Gly	Lys	Leu	Val	Glu	Lys	Asp	Tyr	Arg	Glu	Leu	Lys
		675					680					685			
Gly	Thr	Phe	Arg	Val	Thr	Glu	Leu	Asn	Gln	Ala	Pro	Leu	Ile	Ile	Val
	690					695						700			
Glu	Asn	Phe	Gly	Ala	Gly	Tyr	Ile	Gly	Gly	Ile	Arg	Ile	Val	Lys	Ile
705				710						715					720
Ser															
721															

SEQ ID NO:3 (see example 1; ET70 specific primer used in colony blot hybridization searching for strains containing ET70 hybridizable similar sequence)AM43

catcacttttc cccatagc
18

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SEQ ID NO:4 (see example 1; ET70 specific primer used in colony blot hybridization searching for strains containing ET70 hybridizable similar sequence)AM34

gacatgattt tacttttaga gc
22

SEQ ID NO:5 (see example 6)

2270-1 5' - GCATTTTCATAGAGGATCAATTGG - 3' SEQ ID NO: 5

SEQ ID NO:6 (see example 6)

2270-8 5' - TTTTCATCACTTTCCCCATA - 3' SEQ ID NO: 6

SEQ ID NO:7 (tIC851)

AAATATTTT AAAGGGGGAT ACCTAATTG AATCTAAT CTATCATCG AAAAGGGGTA 60
CAGAGAAATC AATATATTGA TATTGTTAC ATATGTAGCA TTAATGTTTC TGCTAAATTT 120
GATCCTAATA CTACATTAC AACCTTACA GAAGCTATCA ATTCTCAGC AGGAGCGATT 180
GCTGGAAAAA CTGCCCTAGA TATGAGACTT GATTTTACTC TCGTAGCAGA TATATACCTA 240
GGGTCTAAAA GTAGTGGAGC TGATGGTATT GCTATAGCCT TCCATAGAGG ATCAATTGGT 300
TTTATCGGTA CCATGGGTGG AGCCTTAGGG ATTCTAGGAG CACCAAACCG GATAGGATTT 360
GAAATAGATA CGTATTGAA AGCAACTTCA GATGAAACAG GCGATTCAIT TGGACATGGT 420
CAATGGAATG GAGCAGATGC GCGATTGTA ATACAAATC GAAATGCAG CTATTTAACA 480
GCCTTAGCTC CTATGCAAAA AATACCTGCA CTAATAATA AATGCCGGGT TCTAACTATC 540
AATTGGGATG GCGCTACAA CAACTTACA GCACGCTTC AAGAGAAAG TAATGATGCT 600
TCTACTAGCA CTCCTAGTCC AAGATATCA ACATGGGAC TATTAAATCC TCGTTTGT 660
TTAATCAGA AATACTTTT TATTATGCG TCGCTACAG GGGCTGCTAA TAACAGCAT 720
CAGATTGGAG TTACTTTGTT TGAAGCATAC TTACAAAAC CAACTATAGA GGCAAATCCT 780
GTTGATATTG AACTAGGCAC AGCGTTTGT CCAATAAACC ATGAGCCAA TGGACTCAA 840
GCAACGATG AAGTAGATGG AGATATAACA AAGGACATTA CGGTAGAAAT TAATGACATA 900
GATACCTCCA AACCGGTGC ATACCGTGT ACATATAAG TAGTAATAG TTATGAGAA 960
AGTGATGAGA AAACAATAGA AGTCCTAGTA TACACAAAC CAACTATAAC TGCAATGAT 1020
ATTAGGATTA AGAAGACTT AGCATTGAT CCAATAAACC ATGAGCCAA TGGACTCAA 1080
GCAACCGATC CAATTGATGG AGATATAACA GATAAAATG CTGTAAAT TAAATATGTC 1140
GATACCTCTA AACCGGTGA ATACCTGTA ACATATAAG TGATAAATAG TTATGAAAA 1200
ATTGATGAAA AAACAATAGA GGTCACTGTA TATACGAAAC CATCTATAGT GGCACATGAT 1260
GTTGAGATTA AAAAGATAC GGCATTGAT CCGTAACCT ATGAGCCAA TGGGCTCAA 1320
GCAACCGATC CAATTGATGG AGATATAACA GATAAAATTA CGGTAGATC TAATGATGTT 1380
GATACCTCTA AACCGGTGC ATATGTTGT AATATAAG TAGTAATTA TGTGAGAA 1440
AGTGACGAAA AAACAATTGC CGTTACAGTA CCTGTTATAG ATGATGGGTG GAGAGATGTC 1500
GATCGACAG GATGGAAAT CTCTCTGCT GAAACCATTA CTCTAGAGA TGATGAGAG 1560
CATGCTCTTA ATGTTAAATG GGTATTTTAT GCTGATAAAC ATGTAGCAAT ATACAAACA 1620

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GTAGAGTTGA AGAATAATAT CCTTATCAA ATTACAGTAT ATGTTAAACC AGAAGATGAA 1680
GGAAGTGTGG CACACCATAT TGTAAAGTA TCTTTCAAT CTGATTCTGC TGGTCCAGAA 1740
AGTGANGAAG TTATAAATGA AAGATTAAAT GATGCGAAGC AGATACAAA AGGATACAGA 1800
AAGTTAACAA GTATTCCATT TACACCAACA ACCATTGTTT CCAACAAAA ACCAGTGATA 1860
ATTGTTGAAA ACTTTTACC AGGATGGATA GGTGGAGTGA GAATAATTGT AGAGCCTACA 1920
AAGTAAGAAT TATAACTAG CTTTAAATAA ATATATTTAA AAAAT 1965

SEQ ID NO:8 (tICSS1 protein)

MRKSIIRNG VQKQYIDIR MICSINGSAK FDMTHITTL TEAINBOAGA IAGKTALDNE 60
RDFTLVADIY LSKSSGAGG IAIAPRHSI GFIGNGGGL GILGAPNGIG FRIDTTWAT 120
SDFTGDSFGM GQKGAHAGF VSTRMBASYL TALAPNOKIP APNKKNEVLT INWDARNKIL 180
TARLQKKEHD ASTSTPSRY QTWELLHPAF DLNDKYTFII GSATGAANWK EQIGVTLFPA 240
YFTKPTIEAN FVDIKLQYAF DPLAKPIQL KATDEVGGDI TKDITVEFED IDTSKPGAYR 300
VTYKVMBSYG ESEKTIIEVV VTYKPTTIAH DITIKKILAF DPLMYEPIGL KATDPIDGDI 360
TDKIAVIFNM VDTSKPGKYH VTYKVINSYE KIDKTIIEVT VTYKPSIVAH DVEIKKDTAF 420
DPLMYEPIGL KATDPIDGDI TDKITVESHD VDTSKPGAYS VKYKVMBSYE ESEKTIIVT 480
VFVIDDQWEN GDFTGNKFFS GSTITLEDDE EHALNGENVF YADKEVAIYK QVELKNIPY 540
QITVYVKPED EGTVAHIVK VSPKDSAGP ESEKIVNERL IDAEQIQKGY RKLTSIPFTF 600
TTIVFHKIPV IIVENFLPGW IGGVRIIVKP TK 632

Cry22: SEQ ID NO:9 (coding sequence)

atgaangaac aaatctaaa taatatgat gaataactg tacaagcagc aagcgattat 60
atcgacattc gtcgattttt taaacaant ggcctgcta catttaattc taataccat 120
attcaactt taacacagc tatanatagt caagcagag caattgcag aaagactgct 180
ctagatataa gacatgactt taotttttag gcagatattt ttcttggaac taanagttaac 240
ggagcagagc gtattgcaat cgcatttcat agggatcaa ttgggtttgt tggacaaaa 300
ggcggaggac ttggaatatt aggtgaacot aaagggatag ggtttgaatt agacacatat 360
gogaatgaac ctggggacga agtaggcgat tgcgtttggc atggggcaat gaagagatca 420
ttccctagtt tcccaaatgy atatoccont gctggctttg taagtactga taanaatagt 480
agatggttat cagctotagc tccatgcag cgaatcgtc ctccaacgg gcttgagga 540
cgtctggaga ttctgtggga tgcctgtant aaagagtaa ctgcaaatct tcaggattta 600
acttttaant acataactgt tggagagag ccactactc caagaactgc aacttgaggy 660
ttagttaant ctgcatttga acttgatcag aagtataact ttgttattgy ttccggcagc 720
ggtgcatota ataacctaca tccgattggy attatagaat ttgatgcata ctttactaaa 780
ccgacaatag aagcgataa tgaatatgtc ccagtggag caacatttaa tccaaaaaca 840
tatccaggaa taatatttaag agcaacagat gngatagatg gggatttgac atcgagatt 900
attgtgaag caaacatgt taatacgtc aaacgggtg tgtattatgt gactattat 960
gtagaganta gttatgggga aagtgatga aaacaatcy aagtaactgt gttttcaaac 1020
cctacaatta ttgcagtgga tgttgaaatt gaanaagggg aatcttttaa cccactaact 1080
gattcaagag taggtcttcc tgcacaggat tcnttaggca atgatattac ccaaatgta 1140
aaggtaaaat cagtaantgt ggtacttca aagcagggg aatatgaagt tgtatttga 1200
gtgacagata gctttggtgg aaagcagaa aaagatttca aggtacagt tttaggacag 1260
ccaagtatag aagcgaatan tgttgaaata gaatatagtg attcatttga tccattaaac 1320
gatgcaaaag taggtctccg tgcnaaggt tcattaggta atgatattac gaagacata 1380
aaagtaagt tcaataacgt agatacttca aattcaggaa agtatgaagt tatatttga 1440
gtgacggacc gttttggaaa aaagcagaa aaagtattg aagtccttgt tctaggagaa 1500
ccaagcattg agcaaatga tgttgaggtt aataaggtg aaagcttga accattaaac 1560
gattcaagag ttggcctccg tgcnaagac tcattaggta atgatattac gaagatgtg 1620
aaataaaaat caagtaantgt ggtacttca aaaccagtg aatatgaagt tgtatttga 1680
gtgacagatc gttttggtaa atatgtaga aaacaattg gagttatagt gccagtaatt 1740
gatgatgaat gggagatgy aatgtgaat ggttggaaat tctatgctgy gcaagatatt 1800
aaactgttga aggatcctga taagcctat aaagcgatt atgtattcta tgattctaga 1860

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cacgttgcta tttctaaac aattccacta acggatttgc aaataaatat aaactatgaa 1920
attacagtgt atgotaaagc agaaagcggc gatcatcact taanaagtgac gtataagaaa 1980
gaccocggcag gtccagaaga gccgcaggtt ttcaatagac tgattagcac aggcacattg 2040
gtagaanaag attatagaga attaaaaggg acgttcggcg taacagaatt aaacaaagca 2100
ccattgataa tcgtagagaa ttttggagct ggatatatag gtgganttag anttgtgaaa 2160
atatcgtaat aa 2172

Cry22: SEQ ID NO:10 (amino acid sequence)

1 MKSQMLNKYDEITVQASDYIDIRPIFYNGSATFNSNTNITLTQAINR 50
51 QAGALAGKTALDMRHDFTPRADIFLGTKENGADGIAIAFERGSSIGPVGYK 100
101 GGGGLGILGAFEGIGFELDTYANAFEDFVGDSPGHGAMKGSFFSFPMGYPH 150
151 AGFVSTDKSKNLSALAQMQRIAAPNGENRRLEIRNDARNKELTANLQDL 200
201 TFMNITVGEKPRTPRTATNRLVHPAFELDQKYTFVIGSATGASNNLHQIG 250
251 IIEFDAYFTKPTIEANFVVPVGAIFMFKTYPGINLRATDSIDGDLTSKI 300
301 IVKANFVSTSKTGVIYVITYYVENSYGESDEKTEIVTVFENPTILASDVEI 350
351 EKESFNPLTDSRVGLSAQDSLGNDITQNVKVKSSNVDTSKPGYEYEVVFE 400
401 VTDSFGGKAERKDFKTVLQQPSIRANFVLEIDDSLDPLTDAKVGLRAD 450
451 SLGNDITKDIKVFENVDTSNGRYEVIFEVTDKPKKAERKIEVLVLGR 500
501 PSIRANDVFNKGEITFEPLTDSRVGLRAKDSLGNDITKDVKIKSSNVDTK 550
551 KPGYEYEVVFEVIDRFGKYVEKTIQVIVFVIDDEWEDGKVGKWFYAGQDI 600
601 KLLKDFDKAYKQDVVYFDSRNVAKSTIPLTDLQINTNYEITVYAKAESG 650
651 DBELKVTKIDPAGFPEEPFVFRLLISTGTLVEKDYRELKGTFFVTELNKA 700
701 FLIIVENFGAGYIGGIRIVKIS 722

SEQ ID NO:11
ATTGATCCTCT ATGAAATGC 19

SEQ ID NO:12
GTTTCCCAAT GGATATCC 18

SEQ ID NO:13
GGATATCCATT TGGGAAC 18

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SEQ ID NO:14	
ATCTAATAACC TACATCAGA	19
SEQ ID NO:15	
TCTGATGTAG GTTATTAGAT	20
SEQ ID NO:16	
TATGGGGAAA GTGATGAAAA	20
SEQ ID NO:17	
ATGTTGAATT AGAATAG	18
SEQ ID NO:18	
CTATTCTAA TTCAACAT	18
SEQ ID NO:19	
AAGTCCTGT TCTAGGAGAA	20
SEQ ID NO:20	
TTCTCCTAGA ACAAGGACTT	20
SEQ ID NO:21	
TATGTATTCT ATGATTCTAG	20
SEQ ID NO:22	
CTAGAATCAT AGAATACATA	20

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2do. EXAMEN DE FORMA PATENTE DE INVENCION

Folio 348

Radicación no 01-38451
Concepto Técnico no. 497
Clasificación Internacional de Patentes : A01N61/00

Practicado el examen de acuerdo a lo establecido en el Art. 38 de la Decisión 486 se determinó que esta solicitud de Patente de Invención:

- | | |
|--|---|
| SI ☒ NO ☐ | Contiene un resumen de la invención (Art.26-e). |
| SI ☒ NO ☐ | Contiene el título o nombre de la invención (Art.27-d). |
| SI ☒ NO ☐ | Contiene la descripción de la invención (Art.28-b). |
| SI ☒ NO ☐ | NO ES APLICABLE ☐ Contiene los dibujos necesarios para comprender la invención (Art.28-d) |
| SI ☒ NO ☐ | Contiene una o más reivindicaciones. (Art.28-c). |
| SI ☐ NO ☐ | NO ES APLICABLE ☒ Han sido desarrollados los productos y/o procedimientos a partir de recursos genéticos o de sus productos derivados de los que cualquiera de los Países Miembros es país de origen. (Art. 28- h). |
| SI ☐ NO ☐ | NO ES APLICABLE ☒ Los productos y/o procedimientos han sido obtenidos o desarrollados a partir de los conocimientos tradicionales de las comunidades indígenas, afroamericanas, o locales de los países miembros, de acuerdo a lo establecido en la Decisión 391 y sus modificaciones y reglamentaciones vigentes.(Art.28-i). |
| SI ☒ NO ☐ | La invención se refiere a un producto relativo a un material biológico. (Art.28-J). |
| SI ☒ NO ☐ | La prioridad coincide con la materia reivindicada. |
| SI ☒ NO ☐ | CUMPLE LOS REQUISITOS TÉCNICOS |

OBSERVACIONES Y OTROS: SI ☒ NO ☐ ver hoja(s) anexa(s)

EXAMINADOR TÉCNICO
202044

Bogotá D. C. Marzo 4 de 2003.

Carrera 13 No. 27-00 Piso 5 y 10
Conmutador: 334 12 21-2-3-4
Fax: 281 31 25 e-mail: info@sic.gov.co
Santa Fe de Bogotá D.C. - Colombia

~~Folio 349~~

Concepto técnico no. 497
OBSERVACIONES

- ▣ Anexa el documento mediante el cual reivindica prioridad # 60/204,387 Follos 244-347.
- ▣ Anexa el documento de cesión de derechos del inventor al solicitante. Follos 230-239.
- ▣ Anexa el documento de poder y el documento que acredita la representación legal de la sociedad solicitante. Follos 240-243

 **EXAMINADOR TECNICO**
202044

Bogotá, Marzo 4 de 2003.

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Folio 350

2020
Bogotá DC

Doctor(a)
EMILIO FERRERO
Apoderado(a)

REFERENCIA:	Radicación No.	01-38451
	Trámite	002
	Evento	001
	Actuación	416
	Oficio No.	492
	Folios	001

Teniendo en cuenta que la solicitud de privilegio de patente que se tramita bajo el expediente indicado en la referencia, reúne los requisitos de forma establecidos en los artículos 26 y 27 de la Decisión 486 de la Comisión de la Comunidad Andina, y en las demás disposiciones vigentes sobre la materia, se envía el extracto de la solicitud a la Oficina de Comunicaciones para efecto de su publicación en la Gaceta de Propiedad Industrial, conforme lo establece el artículo 40 de la misma Decisión.

Cúmplase

Dado en Bogotá DC, a los

25 MAR. 2003

ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

202044

DIVISION DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 01-038451

**EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION
FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 530, DE
FECHA 31 DE JULIO DE 2003, EL TERMINO HABIL SEÑALADO POR LA LEY
EXPIRA EL 28 DE OCTUBRE DE 2003.**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI

NO **X**

Bogotá D.C., Febrero de 2004

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RADICACIÓN: 1-38451

EDICTO No. 3214

LA SECRETARÍA GENERAL AD-HOC
HACE SABER:

Que esta Superintendencia profirió Resolución No. 2463 de 12-FEB-2004. Que el contenido de la parte resolutive es como sigue: El Superintendente de Industria y Comercio.

RESUELVE

ARTICULO PRIMERO: Declarar abandonada la solicitud de privilegio de patente de invención contenida en el expediente de la referencia.

ARTICULO SEGUNDO: Notificar personalmente el contenido de la presente resolución al doctor Emilio Ferrero, o a quien haga sus veces, quien actúa como apoderado de Monsanto Technology Llc., entregándole copia de la misma, advirtiéndole que contra ella procede el recurso de reposición Interpuesto ante el Superintendente de Industria y Comercio, al momento de la notificación o dentro de los 5 días hábiles siguientes a ella.

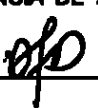
NOTIFÍQUESE Y CUMPLASE

Dada en Bogotá, D.C., a los 12 FEB. 2004

EL SUPERINTENDENTE DE INDUSTRIA Y COMERCIO,


JAIME AUGUSTO ESCOBAR

Para notificar a los interesados, se fija el presente EDICTO en lugar visible al público en la SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO, por el término de (10) diez días hábiles contados a partir de las 8:30 a.m. de hoy.

Secretaría General Ad-Hoc 23-MAR-2004  Este edicto se desflja hoy 05-ABR-2004 a las cinco (5:00 p.m.) 
Secretaría General Ad-Hoc
EDICTO No. 3214