VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

Bases para un uso racional de *Bacillus thuringiensis* para el control de las plagas del algodón

TESIS DOCTORAL

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CERTIFICAN: Que Dña. **ANNA ESTELA BOLTA**, Licenciada en Ciencias Biológicas, ha realizado bajo su dirección el trabajo de investigación recogido en esta memoria que lleva como título "Bases para un uso racional de *Bacillus thuringiensis* en las plagas del algodón" para optar al grado de Doctor por la Universitat de València.

Y para que así conste, en cumplimiento con la legislación vigente, expido el siguiente certificado en Burjassot, a 1 de septiembre de 2006.

Fdo: Dr. D. Juan Ferré Manzanero Fdo: Dr. D. Baltasar Escriche Soler

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CERTIFICAN: Que en la memoria presentada, como compendio de artículos, por Dña. ANNA ESTELA BOLTA, para optar al grado de Doctor en Ciencias Biológicas, en aquellas publicaciones en las que no figura como primer autor, su trabajo consistió en:

I. La preparación de las toxinas utilizadas para la realización de bioensayos, su posterior purificación mediante FPLC, la realización de los ensayos de unión ligando-receptor para determinar cuáles de las toxinas comparten sitios de unión en el intestino del insecto y en la posterior interpretación de los resultados presentados, en los artículos: Ibargutxi *et al.*, 2006, Appl. Environ. Microbiol. 72: 437-442, y Ruíz de Escudero *et al.*, 2006, Appl. Environ. Microbiol. 72: 4796-4804.

II. La realización de los bioensayos con *Bombyx mori, Manduca sexta,* y *Plutella xylostella* con la toxina Cry1Ia7, sus controles con Cry1Ab, y su posterior interpretación, en el artículo: Ruíz de Escudero *et al.*, 2006, Appl. Environ. Microbiol. 72: 4796-4804.

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A mis padres,
por su confianza y
apoyo incondicional

Agradecimientos

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ÍNDICE

APUNTE INICIAL

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El origen de la agricultura se encuentra en el Neolítico cuando la economía de las sociedades humanas evolucionó desde la recolección y la caza a la agricultura y la ganadería. Las primeras plantas cultivadas que se conocen fueron el trigo y la cebada. Sus orígenes se pierden en la prehistoria, pero al parecer se remontan a tres culturas que de forma independiente la practicaban: en Mesopotamia, en América Central (por las culturas precolombinas) y probablemente en el este asiático, produciéndose de este modo una transición más o menos gradual desde una economía basada en la caza-recolección a la agricultura. Se postula que una de las razones de la introducción de la agricultura pudo ser la aparición de un cambio climático hacia temperaturas más templadas. De esta manera, la agricultura permitió una mayor densidad poblacional que la economía basada en la caza y recolección, a la vez que una mejor disponibilidad de alimento, favoreciendo de este modo el asentamiento poblacional.

La evolución de la agricultura y posteriormente el almacenamiento del excedente de grano y semillas, produjo inevitablemente una adaptación de los insectos a esta nueva situación, convirtiéndose en especies plaga, más o menos específicas para cada tipo de cultivo, que ocasionan pérdidas importantes en éstos. Desde entonces, el hombre ha intentado paliar esta situación de diferentes formas y con diferentes estrategias. Entre ellas cabe destacar la utilización de diferentes productos químicos de amplio espectro, introducción de depredadores y parásitos específicos para determinadas especies plaga, baculovirus, hongos entomopatógenos, mediante el uso de las toxinas producidas por la bacteria *Bacillus thuringiensis* y, muy recientemente, con la

creación de plantas resistentes a estos insectos mediante la introducción de genes exógenos que les confieran propiedades insecticidas mediante la tecnología del ADN recombinante.

El abuso en la utilización de productos químicos de amplio espectro junto con una mala estrategia para prevenir la resistencia en campo ha generado la aparición de poblaciones de insectos resistentes a muchos de los tratamientos utilizados. El futuro de la agricultura a nivel mundial reside en el diseño no sólo de nuevas estrategias que sean efectivas para el control de estas especies fitopatógenas, sino en el estudio de los posibles mecanismos de resistencia que puedan aparecer para, de esta forma, poder evitar la aparición de insectos resistentes en campo y optimizar el rendimiento de la producción dentro del marco de una agricultura sostenible.

1. INTRODUCCIÓN

Lo que sabemos es una gota de agua;
lo que ignoramos es el océano
Isaac Newton

1. INTRODUCCIÓN

Bacillus thuringiensis es una bacteria aeróbica que ha sido clave para el control de muchas especies plaga de cultivos agrícolas tales como lo son *Helicoverpa armigera* y *Earias insulana* para el algodón. El potencial que tienen estas especies de desarrollar resistencia a productos fitosanitarios basados en *B. thuringiensis* es un tema preocupante que se pretende paliar mediante el diseño estrategias adecuadas y, más recientemente, con la introducción de las plantas transgénicas que expresan una o varias toxinas de *B. thuringiensis*. En la literatura podemos encontrar diferentes casos de aparición de resistencia a las proteínas de *B. thuringiensis* tras selección en laboratorio, pero en la mayoría de ellos esta resistencia se ha generado por alteración del lugar de unión de las toxinas a la diana del insecto, situada en el intestino medio de la larva. Por lo tanto, conocer estos mecanismos de resistencia es un paso crucial para poder diseñar estrategias adecuadas y evitar la aparición de resistencia en campo dentro de un marco de agricultura sostenible.

1.1. Generalidades de *B. thuringiensis*

B. thuringiensis es una bacteria aeróbica, Gram-positiva y formadora de endosporas que produce cristales paraesporales durante la fase estacionaria de su ciclo celular (Fig. 1A), estrechamente emparentada con *Bacillus cereus* y *Bacillus anthracis* (Daffonchio *et al.*, 1998; Logan y Berkeley, 1984). El tamaño del bacilo oscila entre 1,0-1,2 μm de ancho y 3-5 μm de largo (Fig. 1) (Iriarte y Caballero, 2001). Fue aislada por primera vez a principios del siglo XX (Ishiwata, 1901). Pero fue el científico alemán Berliner quien otorgó el término *Bacillus thuringiensis* en 1915 a un patógeno aislado de polillas de la harina procedente de la región alemana de Thuringia en el 1911 (Berliner, 1911, 1915).

B. thuringiensis se encuentra ampliamente distribuida alrededor del mundo en lugares tan diferentes como suelo, agua, interior de insectos, polvo de grano almacenado, excrementos y en el filoplano de las hojas (superficie de las hojas) (Martin y Travers, 1989; Schnepf *et al.* 1998). Su importancia radica

en la capacidad insecticida que le confieren mayoritariamente los cristales paraesporales que produce, y que han sido ampliamente utilizados como base de productos bioinsecticidas para el control de ciertas especies de insectos de los órdenes Coleoptera, Diptera y Lepidoptera (Schnepf *et al.*, 1998). También se han encontrado aislados con actividad en otros órdenes de insectos (Hymenoptera, Homoptera, Ortoptera y Mallofaga) y en nematodos, ácaros y protozoos (Feitelson, 1992).

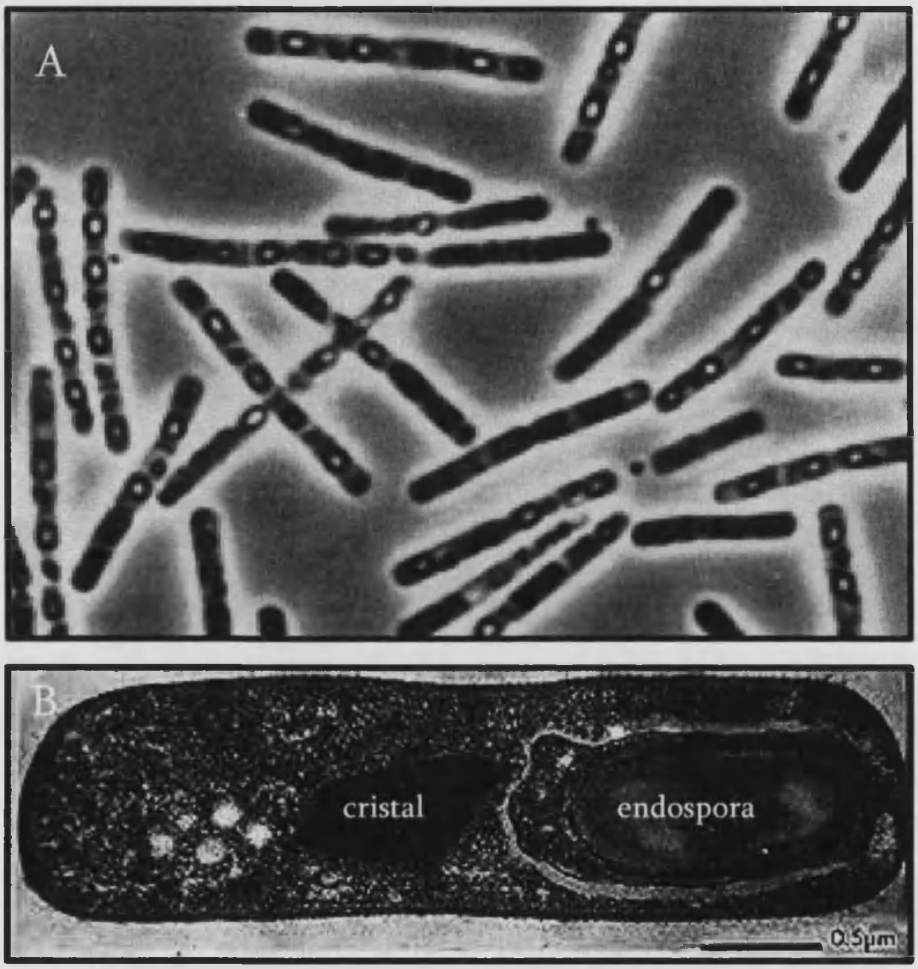

Fig 1. Morfología y ultraestructura de *B. thuringiensis*. (A) cultivo bacteriano; (B) micrografía electrónica de un bacilo.

1.2. Caracterización de *B. thuringiensis*

El estrecho parentesco de *B. thuringiensis* con bacterias productoras de toxinas activas contra organismos superiores como mamíferos ha sido en parte una de las razones que ha generado cierta controversia a nivel social

sobre el uso de *B. thuringiensis* como herramienta bioinsecticida. En base a criterios morfológicos y bioquímicos, como inicialmente se intentó establecer una clasificación en el género Bacillus, *B. thuringiensis* es prácticamente imposible de diferenciar de *B. cereus* (Schnepf *et al.*, 1998), guarda una estrecha relación con *B. anthracis,* y está muy emparentado con *Bacillus mycoides.* Dada la elevada similitud entre estas tres especies, algunos autores propusieron que deberían ser consideradas como una única especie (Helgason et al 2000; Mignot et al 2001). Recientemente, las diferencias encontradas a nivel molecular entre *B. thuringiensis, B. cereus* y *B. anthracis* apoyan la divergencia de *B. anthracis* respecto de *B. cereus* y *B. thuringiensis*, siendo estas dos últimas mucho más similares entre sí (Cherif *et al.*, 2003, Read *et al.*, 2003).

La clasificación de las subespecies de *B. thuringiensis* basada en la identificación serológica del antígeno flagelar o antígeno H, por su reproducibilidad, especificidad y fiabilidad, se introdujo a principios de los años 60 (de Banjac y Bonnefoi, 1962). Esta metodología permite separar las nuevas cepas aisladas en diferentes serotipos, en base a los factores antigénicos determinados por el flagelo bacteriano. De esta forma se han clasificado 82 serovares en base a 69 serotipos diferentes, pero incluso con esta técnica, existen cepas de *B. cereus* que presentan reacción cruzada al antígeno H de *B. thuringiensis* (Lecadet *et al.*, 1999).

Las evidencias encontradas tras la utilización de diversos métodos moleculares tales como hibridación del ácido desoxiribonucleico (ADN) cromosómico, análisis de la composición lipídica y de ácidos grasos, análisis de fragmentos de restricción amplificados (AFLP, siglas procedentes del inglés "Amplified Fragment Length Polymorphism") y análisis de restricción de ADN genómico, también apoyan la hipótesis de que *B. thuringiensis* y *B. cereus* son una única especie (Schnepf *et al.*, 1998). La propiedad que realmente caracteriza a *B. thuringiensis* y lo distingue de *B. cereus* es su capacidad de producir inclusiones cristalinas (Helgason *et al.*, 2000), aunque existen algunas cepas de *B. thuringiensis* que no producen dicho cristal llamadas cepas acristalóforas. La morfología del cristal paraesporal es variable, dependiendo de las proteínas que lo componen, encontrándose cristales con formas bipiramidales, rectangulares, cúbicas, esféricas, romboidales, triangulares y otros sin una estructura determinada o irregulares (Fig. 2). Además, algunas cepas de *B. cereus* son capaces de hibridar con sondas específicas para genes *cry1A* de *B. thuringiensis* (Schnepf *et al.*, 1998). En la

actualidad continúa la investigación para determinar las relaciones filogenéticas y taxonómicas entre estas especies del género Bacillus aplicando técnicas más avanzadas de biología molecular basadas en el ADN que permitan la separación tanto a nivel interespecífico como intraespecífico.

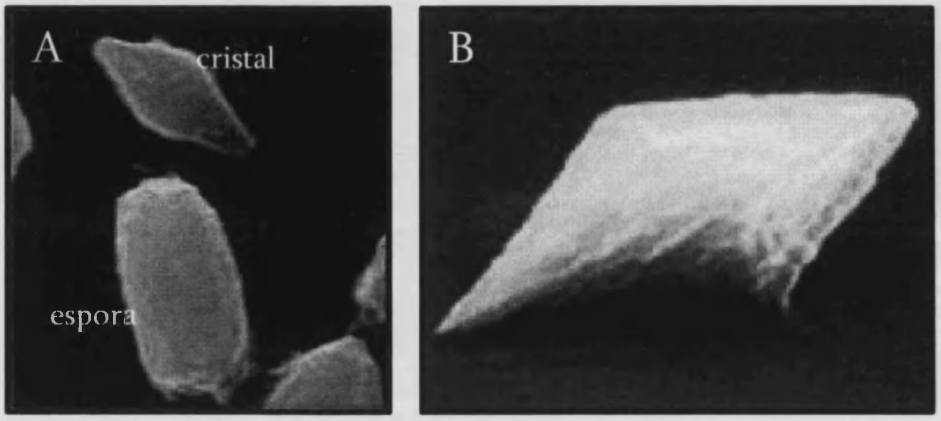

Fig 2. Micrografía electrónica de barrido de cristales y esporas de *B. thuringiensis*. A) cristales y esporas; B) detalle de un cristal bipiramidal.

1.3. Las toxinas de *B. thuringiensis*

B. thuringiensis produce varias formas de toxinas con diferentes propiedades tales como las β-exotoxinas, proteínas insecticidas producidas durante su fase vegetativa (toxinas VIP), δ-endotoxinas y factores tóxicos como enterotoxinas, exoenzimas y hemolisinas (Glare y O'Callaghan, 2000).

La *β*-exotoxina, conocida también como thuringiensina, es una molécula de tamaño pequeño y termoestable que se ha detectado en diferentes serovares de *B. thuringiensis* (Glare y O'Callaghan, 2000; Hernández *et al.*, 2001; Hernández *et al.*, 2003). Está compuesta de adenina, glucosa y ácido alárico, y parece que actúa durante el proceso de síntesis del ácido ribonucleico (ARN), inhibiendo a la ARN polimerasa por competencia con nucleótidos de adenosín trifosfato (ATP), dada su gran homología estructural con los nucleótidos de adenina. La presencia de *β*-exotoxina puede ser determinada con bioensayos con mosca común (*Musca domestica*) o mediante detección por cromatografía líquida de alta resolución (high-performance liquid chromatography o HPLC) (Campbell *et al.*, 1987; Hernández *et al.*, 2001). Tiene un amplio espectro de acción, incluyendo especies de insectos coleópteros, dípteros y lepidópteros, y presenta también

toxicidad frente a mamíferos a elevadas dosis (Meadows, 1993), por lo que algunos países tienen legislada y exigen su detección en las cepas nuevas de *B. thuringiensis* que se quieran registrar para uso comercial (McClintock *et al.* 1995).

Las proteínas insecticidas vegetativas (conocidas como proteínas Vip) se producen durante la fase de crecimiento vegetativo bacteriano y no forman parte del cristal. De entre ellas, la Vip3A tiene un amplio espectro de actividad incluyendo *Agrotis ipsilon, Spodoptera exigua, Spodoptera frugiperda* y *Helicoverpa zea* (Estruch *et al.*, 1996) y, aunque su mecanismo de acción todavía no está totalmente claro, se postula que es similar al de las proteínas Cry. La diana de unión de las proteínas Vip se encuentra en las células epiteliales del intestino medio del insecto, a las que se unen tras sufrir un procesamiento previo por la acción de determinadas proteasas intestinales. Este procesamiento las convierte en un fragmento activo que tras unirse a su diana da lugar a un fenómeno de lisis celular, provocando posteriormente la muerte del insecto (Yu *et al.*, 1997).

Las enterotoxinas siempre han sido halladas en cantidades muy reducidas en algunas cepas y formulados comerciales basados en *B. thuringiensis* (Damgaard, 1995; Damgaard *et al.*, 1996). Además, *B. thuringiensis* libera un gran número de exoenzimas tales como las quitinasas, proteasas y fosfolipasas que actúan como factores tóxicos y participan en la actividad tóxica global de esta bacteria (Glare y O'Callaghan, 2000). Algunas cepas de *B. thuringiensis* producen también hemolisinas con actividad lítica celular en los eritrocitos de vertebrados (Glare y O'Callaghan, 2000).

1.3.1. δ-endotoxinas

Las δ-endotoxinas son proteínas específicas contra insectos de los órdenes Coleoptera, Diptera, Lepidoptera, Hymenoptera, Homoptera, Ortoptera y Mallofaga (Schnepf *et al.*, 1998), y su diana de acción se localiza en el intestino medio de los insectos. Las δ-endotoxinas se expresan durante la fase de esporulación en el ciclo de vida de *B. thuringiensis.* Tienen la propiedad de formar inclusiones cristalinas dentro de la célula bacteriana y por eso se les atribuye el nombre de proteínas o toxinas Cry (de la palabra inglesa "crystal"). Estas formaciones cristalinas pueden contener una o varias proteínas diferentes lo que determinará la actividad insecticida final.

Algunas cepas de *B. thuringiensis* producen además otro tipo de endotoxinas, las endotoxinas citolíticas (Cyt), que pueden formar parte del cristal junto con las proteínas Cry (Glare y O'Callaghan, 2000), pero que no presentan homología de secuencia ni de estructura terciaria con las proteínas Cry. Estas toxinas muestran *in vitro* un amplio espectro de acción no específico, pero con actividad insecticida contra especies de dípteros *in vivo* (Knowles *et al.*, 1992).

1.3.1.1. Clasificación de las δ-endotoxinas

B. thuringiensis es esencial para el control de diferentes especies por la gran abundancia de δ-endotoxinas que produce con actividad específica contra insectos. Las toxinas de *B. thuringiensis* se clasificaron por primera vez atendiendo a su especificidad contra insectos. De esta forma las CryI eran específicas contra lepidópteros, las CryII eran activas contra lepidópteros y dípteros, las CryIII eran específicas contra contra coleópteros, y las CryIV tenían propiedades insecticidas contra dípteros (Höfte y Whiteley, 1989). Sin embargo, el sistema de clasificación se revisó posteriormente al aparecer nuevas toxinas, que por su especificidad podían ser ubicadas en más de uno de los grupos establecidos, o que compartían gran homología de secuencia con proteínas Cry previamente descritas pero con diferente especificidad contra insectos. El sistema actual de clasificación establece cuatro categorías cuyos rangos se asignan dependiendo del grado de identidad de secuencia aminoacídica (Crickmore *et al.*, 1998). El primer rango de la clasificación requiere al menos un 45% de identidad de secuencia entre las δ-endotoxinas (ej. Cry1); el segundo rango, una identidad de secuencia de al menos el 78% (ej. Cry1A); el tercero de ellos una identidad de secuencia de al menos el 95% (ej. Cry1Aa). En el cuarto rango (ej. Cry1Aa1), las secuencias tienen una identidad de secuencia superior al 95 %, y forman holotipos diferentes cuyas diferencias pueden ser debidas a mutaciones puntuales o a procesos de recombinación (Crickmore *et al.*, 1998). Estas diferencias dentro del cuarto nivel de clasificación pueden estar relacionadas con la fuente bacteriana utilizada para la obtención de la toxina y con pequeñas variaciones de secuencia producidas durante el proceso de síntesis.

De acuerdo con este sistema, hasta el momento, el primer nivel de clasificación lo constituyen un total de 49 familias de toxinas Cry, que forman un total de 145 holotipos de proteínas Cry, y dos grupos de proteínas Cyt formando un total de 9 holotipos, detallado en

http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/. (Fig. 3). Esto constituye un total de más de 300 proteínas Cry y Cyt diferentes, muchas de ellas utilizadas actualmente como insecticidas biológicos en programas de manejo integrado de plagas (programas IPM).

Según esta clasificación, el grupo de proteínas englobadas dentro de la familia Cry1, y concretamente el de Cry1A, es el más numeroso y mejor estudiado. Está constituido por proteínas de unos 130-140 kDa, que necesitan de un procesamiento proteolítico en el intestino del insecto diana, generando un tamaño entre 60 y 70 kDa, para desempeñar su función tóxica (Höfte y Whiteley, 1989). Las proteínas Cry1I (*CryV* según el sistema de clasificación anterior), con un tamaño de 70-81 kDa, son un caso especial dentro de la familia Cry1 ya que no forman parte del cristal paraesporal (Song *et al.*, 2003; Taylor *et al.*, 1992; Tounsi *et al.* 2003). Por este motivo no se han utilizado tradicionalmente como agente activo de productos insecticidas basados en *B. thuringiensis.* No obstante, diferentes trabajos demuestran la efectividad de esta familia de toxinas en la protección de cultivos mediante la transformación de éstos con toxinas del grupo Cry1I (Lagnaoui *et al.*, 2001; Liu *et al.*, 2004; Selvapandiyan *et al.*, 1998). Las proteínas Cry1I tienen un espectro de acción más amplio que la mayoría de las del tipo Cry1, e incluyen especies plaga importantes tanto de lepidópteros como de coleópteros (Tailor *et al.*, 1992). En otras familias menos representadas y estudiadas, el tamaño de la protoxina tiene un tamaño de 65-70 kDa (Cry2), y de 70 o 130 kDa (Cry3).

1.3.1.2. Características de los genes *cry.*

La mayoría de los genes *cry* de *B. thuringiensis* reside en plásmidos, aunque algunos también se encuentran ubicados en los cromosomas bacterianos (Schnepf *et al.*, 1998). Los genes *cry* se expresan mayoritariamente durante la etapa de esporulación bacteriana, aunque algunos de ellos lo hacen de forma independiente a ésta (Schnepf *et al.*, 1998). El producto de estos genes se acumula en el interior celular para dar lugar a la formación de inclusiones cristalinas que pueden llegar a constituir entre un 20 y 30% del peso seco total bacteriano (Schnepf *et al.*, 1998). La expresión de los genes *cry* dependientes de esporulación está controlada por determinados factores sigma. Estos factores sigma concretos promueven la unión de la ARN polimerasa a promotores específicos de esporulación ubicados aguas arriba de los genes que codifican para las δ-endotoxinas, activando de esta forma su transcripción (Baum y Malvar, 1995; Aronson, 2002).

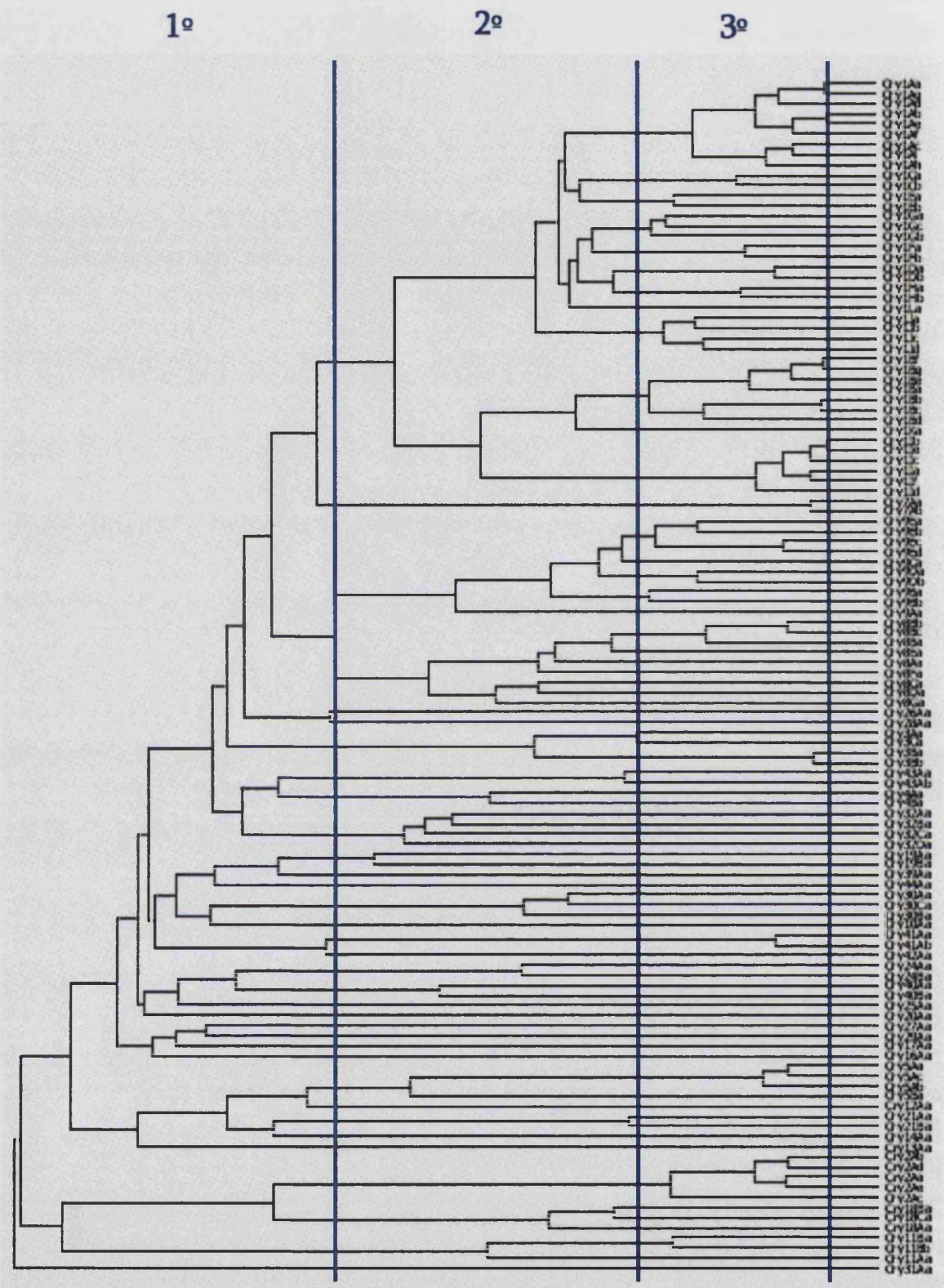

Fig. 3. Dendograma parcial de las familias de proteínas Cry de *B. thuringiensis*.

Existen otros casos donde la expresión de los genes *cry* es independiente de la esporulación, como en la toxina Cry3Aa y las del grupo Cry1I. Se ha demostrado que la expresión de *cry3Aa* se produce tanto durante la etapa de crecimiento vegetativo como, aunque en menor medida, durante la fase estacionaria bacteriana (Schnepf *et al.*, 1998). La expresión de los genes *cry1I* tampoco está determinada por la esporulación y su expresión se produce al principio de la fase estacionaria del crecimiento bacteriano (Kostichka *et al.*, 1996).

1.3.1.3. Estructura de las δ-endotoxinas

Hasta la fecha se ha determinado la estructura tridimensional de diversas δ-endotoxinas de *B. thuringiensis,* activadas con quimotripsina, papaína o tripsina, pertenecientes a las familias Cry1 (Grochulski *et al.*, 1995), Cry2 (Morse *et al.* 2001), Cry3 (Galitsky *et al.*, 2001; Li *et al.*, 1991) y Cry4 (Boonserm *et al.*, 2005) mediante cristalografía de rayos-X (Fig. 4). La estructura terciaria de las toxinas Cry es muy similar aunque no compartan gran homología de secuencia (Schnepf *et al.*, 1998; de Maagd *et al.*, 2001), lo cual sugiere que esta estructura debe jugar un papel fundamental en su similitud funcional a nivel biológico (Whalon y Wingerd, 2003). La estructura de las toxinas Cry consiste en tres regiones estructurales diferentes. El Dominio I, situado en el extremo amino-terminal, está formado por un entramado de 5 a 7 hélices α antiparalelas (Boonserm *et al.*, 2005; de Maagd *et al.*, 2001; Grochulski *et al.*, 1995; Li *et al.*, 1991; Schnepf *et al.*, 1998). Las hélices α del Dominio I son necesarias para su inserción en la membrana, agregación, formación de poros y regulación de los canales. El Dominio II está formado por tres hojas β simétricamente plegadas y dispuestas en un pliege tipo prisma β, que juegan un papel importante en el reconocimiento y posterior unión al receptor. El Dominio III consiste en una estructura tipo sándwich formada por dos hojas β antiparalelas involucradas en el reconocimiento y unión al receptor, así como en la formación de poros y especificidad de canales (Grochulski *et al.*, 1995; Li *et al.*, 1991). Los tres dominios forman una "L" desde una perspectiva apical, donde el dominio III se introduce el dominio II, y el dominio I cuelga por fuera de ambos. (de Maagd *et al.*, 2001; Schnepf *et al.*, 1998) (Fig. 4).

1.3.1.4. Formación del cristal

La capacidad de formar cristal de las δ-endotoxinas reside, en parte, en la estructura secundaria de la toxina, en los puentes disulfuro que existen entre las cadenas de la toxina y, en algunos casos, en la presencia de otras proteínas (Schnepf *et al.*, 1998). La composición proteica del cristal determina su estructura final. Los cristales formados por toxinas Cry1 tienen una forma bipiramidal, los de las Cry2 son cuboidales, los de las Cry3A forman un rectángulo plano, los Cry3B tienen una forma irregular, los Cry4A y Cry4B son esféricos, y los Cry11 tienen una morfología romboidal. La formación espontánea de los cristales se postula que se debe a los enlaces disulfuro en la zona carboxi-terminal de la mayoría de las protoxinas Cry1, Cry4, Cry5 y Cry7 (Schnepft *et al.*, 1998). Las toxinas que no poseen la región carboxi-terminal, tales como Cry3A, utilizan los puentes salinos para la formación del cristal (Li *et al.*, 1991). Otras toxinas como Cry2A y Cyt1A se postula que requieren la ayuda de otras proteínas para la formación del cristal (Baum y Malvar, 1995). Las toxinas del grupo Cry1I no forman cristales (Tounsi *et al.*, 2003).

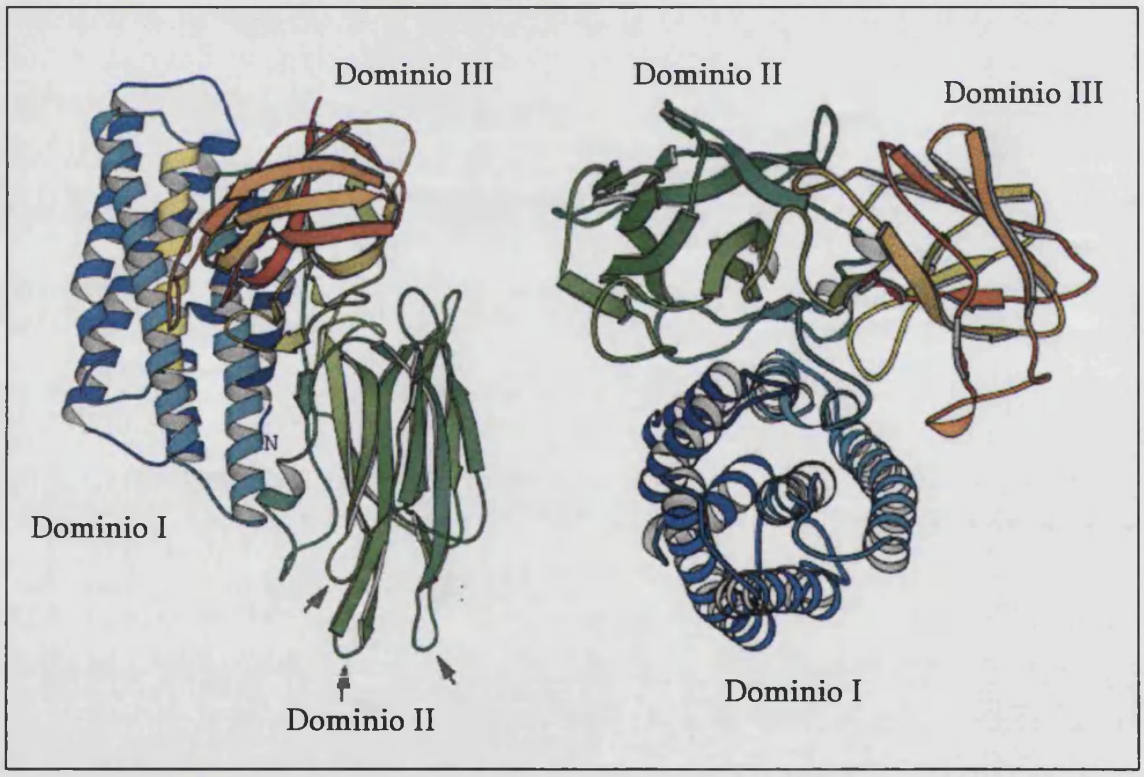

Fig 4. Estructura tridimensional de la proteína Cry3Aa de *B. thuringiensis:* vista frontal (izquierda) y apical (derecha). Imágenes tomadas de http://www.bioc.cam.ac.uk/~dje1.

1.3.1.5. Mecanismo de acción de las toxinas Cry

a) GENERALIDADES

La especificidad de las toxinas Cry viene determinada por todos y cada uno de los pasos que forman su mecanismo de acción global. Tras la ingesta de los cristales, éstos se solubilizan en el interior del intestino, y son procesados para convertirse en toxinas activas, gracias a la acción de determinadas proteasas intestinales. Tras la activación proteolítica, las toxinas necesitan atravesar la membrana peritrófica para acceder a las células epiteliales intestinales, donde se unirán a determinadas moléculas, a las que llamamos receptores, en la membrana de las células del borde en cepillo intestinales. La unión de las toxinas Cry a sus receptores se produce de manera reversible inicialmente, pero posteriormente la toxina se inserta en la membrana de forma irreversible contribuyendo a la formación de poros en ella. Estos poros generados a través de la membrana dan lugar a un desequilibrio osmótico celular que conlleva a una lisis y, posteriormente, a la muerte celular (Schnepf *et al.*, 1998).

b) RECEPTORES CANDIDATOS

Aminopeptidasas N

Estas enzimas son metaloproteasas que tienen como función el corte de ciertos aminoácidos de la región amino-terminal de las proteínas y requieren Zn^{2+} para ejercer su función (Hua *et al.*, 1998). Las aminopeptidasas N de insectos producen el corte preferentemente de los aminoácidos alanina, leucina y metionina. La unión de las aminopeptidasas N a las membranas se produce generalmente a través del glicosilfosfatidilinositol (GPI), como apoyan experimentalmente tratamientos realizados con fosfolipasa C específica para fosfatidilinositol (Knight *et al.*, 1995; Lee *et al.*, 1996). La participación de estas moléculas como receptores de diferentes toxinas Cry se ha demostrado por diferentes técnicas en *Epiphyas postvittana* (Simpson y Newcomb, 2000), *Bombyx mori* (Yaoi *et al.*, 1997; Jenkins y Dean, 2001), *H. armigera* (Rajagopal *et al.*, 2003; Ingle *et al.*, 2001; Liao *et al.*, 2005), *Heliothis virescens* (Luo *et al.*, 1997; Banks *et al.*, 2001; Oltean *et al.*, 1999; Gill *et al.*, 1995), *Limantria dispar* (Valaitis *et al.*, 1995; Lee *et al.*, 1996), *Manduca sexta* (Knight *et al.*, 1994; Lee *et al.*, 1996; Denolf *et al.*, 1997; Masson *et al.* 1995;

Sangadala *et al.*, 1994), *Ostrinia nubilalis* (Hua *et al.*, 2001), *Plutella xylostella* (Denolf *et al.*, 1997) y *Trichoplusia ni* (Lorente *et al.*, 1997).

Cadherinas

Son glicoproteínas transmembrana que participan en la adhesión celular con la ayuda de iones calcio extracelulares. Se ha determinado experimentalmente la participación de las cadherinas como receptores de diferentes toxinas Cry mediante cromatografía de afinidad de intercambio iónico con vesículas preparadas a partir del ribete en cepillo intestinal (BBMV, siglas procedentes del inglés "Brush Border Membrane Vesicles") y a partir de preparaciones crudas de membrana de *B. mori* (Ihara *et al.*, 1998; Jenkins y Dean, 2001; Nagamatsu *et al.*, 1998), *H. armigera* (Wang *et al.*, 2005), *H. virescens* (Gahan *et al.*, 2001; Jurat-Fuentes *et al.*, 2004), *M. sexta* (Keeton y Bulla, 1997; Vadlamudi *et al.*, 1993), *O. nubilalis* (Hua *et al.*, 2001), y *Pectinophora gossypiella* (Morin *et al.*, 2003). Además, en la cepa YHD2 de *H. virescens* se determinó la existencia de ligamiento entre la resistencia desarrollada a la toxina Cry1Ac por esta cepa y el gen *BtR-4*, que codifica para una cadherina, mediante experimentos de búsqueda de ligamiento (Gahan *et al.*, 2001). La cepa resistente contenía una cadherina truncada tras la inserción de un retrotransposón en su secuencia, lo que daba lugar a un alelo truncado que afectaba la expresión correcta del gen. En otro trabajo, Morin *et al.* (2003) también demostraron ligamiento entre la resistencia a Cry1Ac y tres alelos mutantes diferentes de un gen que codifica para una cadherina en tres cepas resistentes de *P. gossypiella*, otra plaga importante del cultivo del algodón.

Otros receptores

Otras moléculas candidatas relacionadas con la unión de las toxinas Cry incluyen glicoconjugados en *L. dispar* (Valaitis *et al.*, 2001) y *Caenorhabditis elegans* (Griffitts *et al.*, 2005), y la fosfatasa alcalina en *M. sexta* (Syagala *et al.*, 1994), *H. virescens* (Jurat-Fuentes *et al.*, 2004) y *Aedes aegypti* (Fernández *et al.* 2006).

c) DETALLES DEL PROCESO

En lepidópteros, posteriormente a la ingestión de los cristales, éstos se disuelven en el tracto intestinal del insecto, generalmente bajo condiciones reductoras y de pH alcalino, liberando la protoxina. Estas condiciones son

necesarias para que se produzca la rotura de los puentes disulfuro existentes entre las cadenas que forman parte de la protoxina, dando lugar a la solubilización de los cristales (Du *et al.*, 1994). Las variaciones en este proceso se hacen patentes entre diferentes insectos y vienen determinadas por las condiciones intestinales intrínsecas de cada especie, influyendo directamente en el grado de toxicidad (Aronson *et al.*, 1991; Du *et al.*, 1994; Monnerat *et al.*, 1999). El pH del intestino de la mayoría de coleópteros no es tan básico como el de los lepidópteros, rondando en la mayoría de ellos entre 4.5 y 6.0 (Koller *et al.*, 1992). Estas diferencias halladas entre lepidópteros y coleópteros también se reflejan en la composición enzimática intestinal, siendo las serín-proteasas la principal proteasa intestinal en lepidópteros, y las cisteín- y aspártico-proteasas más importantes en coleópteros (de Maagd *et al.*, 2001). A pesar de estas diferencias, existen toxinas con actividad dual contra especies de lepidópteros y coleópteros, como Cry3A y Cry1B (Bradley *et al.*, 1995), ésta última además con actividad adicional contra especies de dípteros (Zhong *et al.*, 2000). Por tanto, en el modelo general propuesto para lepidópteros, después de la solubilización de las protoxinas, éstas son parcialmente digeridas por la acción de proteasas intestinales del tipo tripsina y quimotripsina para pasar a ser activas para ejercer su función (Schnepf *et al.* 1998). Estas enzimas producen un corte de unos 500 aminoácidos en el extremo carboxi-terminal y de 28 aminoácidos en el extremo amino-terminal en las toxinas Cry1A (Knowles, 1994), cuyo producto es un fragmento activo de unos 60-70 kDa.

Las proteasas intestinales pueden a veces cortar dentro del fragmento activo de la toxina, produciendo variaciones en su toxicidad, e incluso pueden provocar su inactivación. A modo de ejemplo, la digestión del dominio I de la toxina Cry1Ab con proteasas intestinales procedentes de *M. sexta* produce la liberación de un fragmento activo; en cambio, la digestión adicional dentro del dominio II, produce dos fragmentos con toxicidad reducida (Miranda *et al.*, 2001). En el caso de Cry9Ca, el tratamiento con tripsina *in vitro* produce un fragmento intermedio activo, pero su digestión prolongada con esta enzima da lugar a un producto inactivo de 55 kDa (Lambert *et al.*, 1996).

Tras la activación proteolítica, la toxina atraviesa la membrana peritrófica para unirse a las células epiteliales de borde en cepillo intestinales, específicamente a través de determinadas moléculas que denominamos "receptores" (Whalon y Wingerd, 2003). La membrana peritrófica protege al insecto del ataque de bacterias y otros parásitos, forma parte del sistema

digestivo del insecto y es capaz de unir las toxinas de *B. thuringiensis,* aunque esta unión no siempre va ligada a toxicidad sino que más bien le confiere protección. Se han propuesto varias moléculas para realizar la función de receptor de las proteínas Cry de *B. thuringiensis,* pero sobre las que más pruebas se tienen son las aminopeptidasas-N y cadherinas.

Existen varios modelos para explicar el mecanismo por el que las proteínas Cry, una vez unidas a sus receptores en la membrana celular, se agregan para formar una estructura tipo poro, provocando de esta forma un desequilibrio iónico celular. Por analogía estructural de las toxinas Cry con otras toxinas como la colicina A, exotoxina A y la toxina diftérica, se supone que el dominio I tiene una estructura más afín para participar en la inserción en la membrana y en la posterior formación de poros en ella (Parker y Pattus, 1993). Las hélices α exteriores del Dominio I de las toxinas Cry, originalmente posicionadas hacia el interior de la molécula (Fig. 5A), son las que se postula orientan su parte hidrofóbica mirando hacia el entorno lipídico exterior durante la inserción en la membrana, por tanto es necesario que se produzca un cambio conformacional para esta reubicación (Knowles, 1994; Schwartz *et al.*, 1997). En el modelo de navaja (Fig. 5B), se propone que la hélices α5 y α6 del Dominio I forman parte del poro tras sufrir un giro desde su ubicación original, de forma que quedan dispuestas mirando hacia afuera, y se insertan en la membrana, y que las otras hélices α del Dominio I permanecen inmóviles (Knowles, 1994). En el modelo del paraguas (Fig. 5C) se propone que son las hélices α4 y α5 las que se introducen en la membrana mientras las otras hélices α se disponen de manera paralela a ella, asemejando la estructura que le da nombre al modelo (Gazit *et al.*, 1998). La inserción del Dominio I en la membrana celular se ha relacionado con la unión irreversible de las toxinas Cry (Li *et al.*, 2001), y a su vez con la toxicidad (Chen *et al.* 1995). Los Dominios II y III están relacionados con la especificidad y la unión al receptor como se ha demostrado mediante experimentos de mutagénesis dirigida (de Maagd *et al.*, 1999; de Maagd et al 1996; Jenkins y Dean, 2000; Smith y Ellar, 1994).

Algunos trabajos proponen que las moléculas relacionadas con la unión de las toxinas Cry se ubican en regiones ricas en lípidos insolubles, llamadas "balsas lipídicas" o "lipids rafts", caracterizadas por contener una proporción abundante de colesterol, esfingolípidos y proteínas ancladas a membrana mediante GPI (Brown y Rose, 1992). El primer trabajo donde se demostró la ubicación diferencial de determinadas aminopeptidasas-N y otras moléculas

de mayor peso molecular de *M. sexta* y *H. virescens* en estas regiones lo llevaron a cabo Zhuang *et al.* (2002). En este trabajo las balsas lipídicas se relacionaron con la unión de las toxinas Cry1A y en la posterior formación de poros.

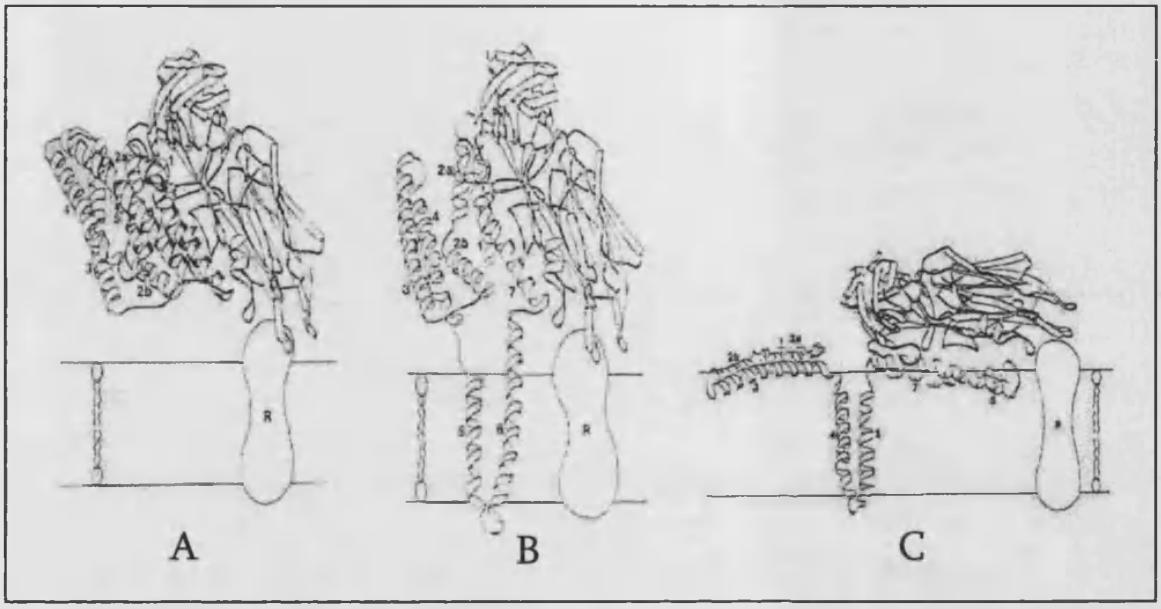

Fig. 5. Modelos de inserción en la membrana propuesto para las toxinas Cry de *B. thuringiensis.* Tras la unión de la toxina Cry a sus receptores de membrana (A), en el modelo de navaja (B) se produce la inserción de las hélices α5 y α6 en la membrana y las otras hélices quedan en el exterior formando parte de la estructura proteica. En el modelo en paraguas (C), las hélices α4 y α5 atraviesan la membrana como una horquilla y las otras hélices quedan extendidas en la supercie de la membrana. Figuras tomadas de Knowles, 1994.

Esta estructura tetramérica de la toxina es más estable que su análoga monomérica (Rausell *et al.*, 2004) y se ha observado en experimentos *in vitro* utilizando membranas sintéticas (Vie *et al.*, 2001). Posteriormente a la actuación de la cadherina, la aminopeptidasa-N es la encargada de dirigir al complejo pre-poro hacia las balsas lipídicas donde se produce la inserción en la membrana que da lugar a la formación del poro (Bravo *et al.*, 2004). Tras la formación de poros en la membrana celular del insecto se produce un desequilibrio iónico que da lugar a una concentración de iones en el interior celular y, consecuentemente, produce la lisis de las células afectadas. La formación de poros causada por las toxinas Cry tiene como consecuencia un aumento de la permeabilidad celular a iones, destruyéndose de esta forma la diferencia de potencial inicial a través de la membrana (Wolfersberger, 1992),

y provocando un aumento del pH citoplasmático lo que afecta al metabolismo celular. A determinadas concentraciones, las toxinas Cry pueden producir daños en el epitelio intestinal que no pueden ser regenerados por la maquinaria celular, dando lugar a la muerte final del insecto. A concentraciones menores de toxina, los daños causados pueden afectar al desarrollo normal de la larva e inhibir su crecimiento.

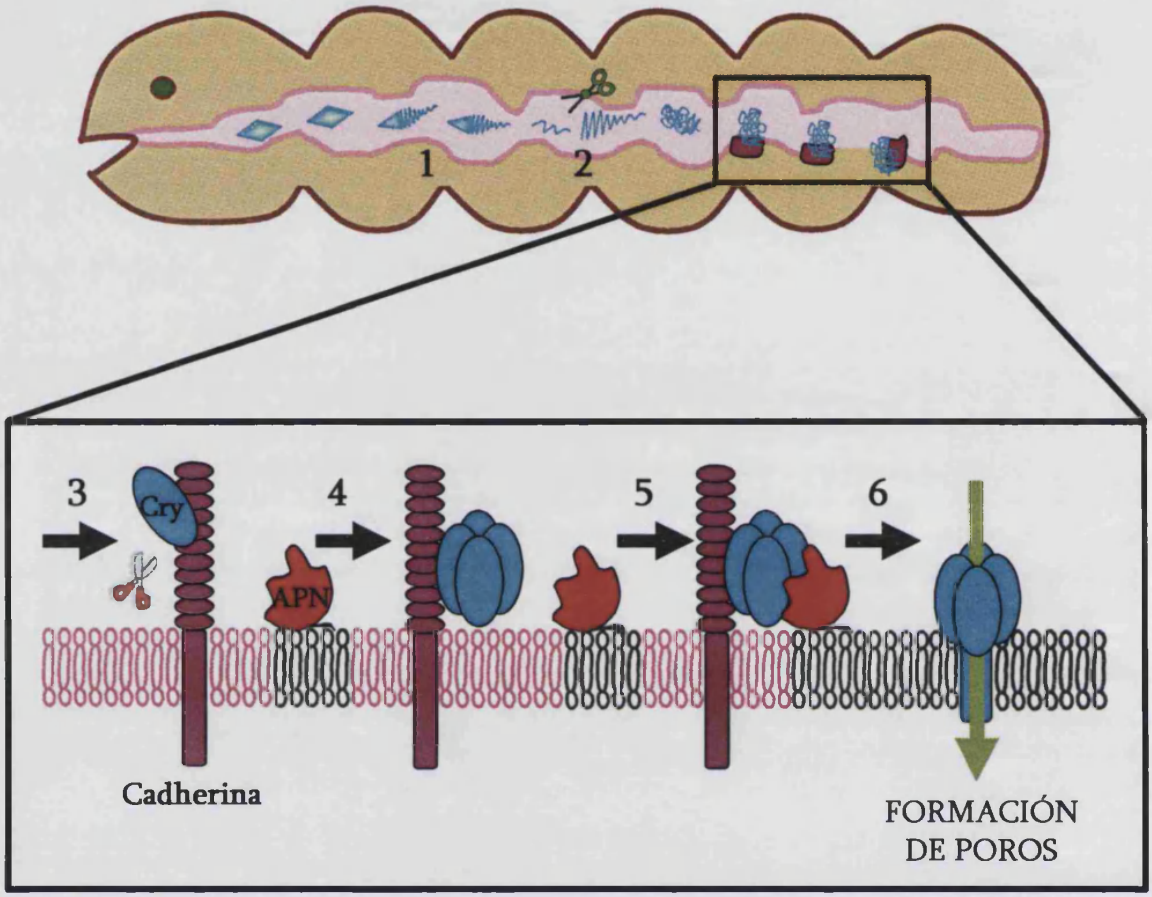

Fig 6. Modelo del mecanismo de acción de las toxinas Cry de *B. thuringiensis*. (1) solubilización del cristal; (2) activación proteolítica de la protoxina; (3) unión de la toxina Cry (monómero) a la cadherina de membrana y procesamiento de la hélice α1; (4) formación de la estructura pre-poro oligomérica; (5) unión del oligómero a la aminopeptidasa N y movilización hacia las balsas lipídicas; (6) estructura del poro.

En un trabajo publicado muy recientemente, Broderick *et al.* (2006) proponen además la participación de parte de las bacterias entéricas ubícuas del intestino del insecto en la toxicidad final de las proteínas Cry de *B. thuringiensis* en *Lymantria dispar*. Éste es el primer trabajo donde se describe la existencia de un sinergismo entre especies del género Enterobacter,

presentes de forma natural en la flora intestinal bacteriana del insecto, y las proteínas Cry, ya que contribuyen a la septicemia asociada tras la ingesta de toxinas que dan lugar a la muerte del insecto diana.

1.4. Aplicaciones bioinsecticidas de *B. thuringiensis*

1.4.1. Bioinsecticidas convencionales

Los formulados comerciales basados en *B. thuringiensis* pueden presentarse en diferentes formatos: como polvos con agentes humectantes, granulados, emulsiones líquidas o suspensiones acuosas, dependiendo del insecto diana y de las características del cultivo (Fig. 7). Están formados generalmente por una mezcla de esporas y cristales, procedentes de cepas o aislados de *B. thuringiensis* de la naturaleza (productos de primera generación), o tras sufrir una modificación mediante técnicas de ingeniería genética para modificar su contenido génico e incluir gen/es de interés (productos de segunda generación). Para el control de especies plagas de cultivos agrícolas, como el algodón, se requiere la aplicación de productos tipo spray ya sea bien por vía aérea o terrestre para intentar cubrir la totalidad de la planta. Tales productos pueden estar compuestos totalmente por una única toxina o por una combinación de diversas toxinas de *B. thuringiensis.* A modo de ejemplo, los productos tipo MVP están basados en una sola toxina, mientras que Dipel® y Xentari® son activos contra varias especies de insectos lepidópteros, en parte, por contener una combinación múltiple de toxinas.

Fig. 7. Ejemplo de diferentes productos fitosanitarios comerciales basados en toxinas de *B. thuringiensis.*

El uso adecuado de estos productos viene limitada por factores tales como el deterioro del producto insecticida tras su aplicación, y el estadio del insecto diana. Las toxinas de *B. thuringiensis* son sensibles a la luz ultravioleta, se lavan fácilmente por el efecto de la lluvia y se degradan a los pocos días tras su aplicación (Aronson *et al.*, 1986), requiriendo en muchas ocasiones la realización de varias aplicaciones espaciadas durante el periodo de infestación en campo. Además, las aplicaciones en spray no cubren la totalidad de la planta, pudiendo quedar partes de ella susceptibles de ser atacadas por la larva. La diana de estos formulados son mayoritariamente los insectos neonatos y los de los primeros estadios larvarios por necesitar una menor cantidad de producto para ser erradicados, dificultándose la tarea cuando los insectos alcanzan los últimos estadios larvarios (Liao *et al.*, 2002). El problema se agrava cuando se trata de insectos con hábitos endófitos como el gusano rosado (*P. gossypiella),* ya que cuando la larva se introduce dentro de la planta, el producto insecticida no puede actuar sobre ella (Novillo *et al.*, 1999). La poca persistencia de estos productos se ha paliado con la introducción de los genes *cry* en otras bacterias como *Pseudomonas fluorescens* por transformación (Schnepf *et al.*, 1998).

El primer producto comercial a base de *B. thuringiensis,* Sporeine®, fue utilizado en Francia para erradicar una plaga de la harina en 1938 (Beegle y Yamamoto, 1992; Shelton *et al.*, 2002). Pero no fue hasta la década de los años 1950 cuando se empezaron a producir diferentes productos comerciales a base de *B. thuringiensis,* cuyas pruebas de campo para su comercialización se intensificaron una década posterior. El descubrimiento de la cepa HD-1 de la subespecie *kurstaki* (Dulmage, 1970) fue toda una revolución por la gran actividad insecticida contra diferentes especies de insectos al producir varias toxinas de *B. thuringiensis.* Por esta razón, HD-1 ha sido incluida en muchos de los productos fitosanitarios comerciales y se ha utilizado como cepa de referencia para cálculos de actividad relativa de otros productos de nueva generación.

1.4.2. Plantas transgénicas

Las plantas transgénicas que expresan toxinas de *B. thuringiensis,* o plantas Bt, se desarrollaron para paliar la poca persistencia de las toxinas Cry en campo y para tener una producción continua de toxina en la planta, así como para prolongar su eficacia durante todos los estadios de la especie diana. Esta continuada expresión también ha repercutido en una disminución

importante de las aplicaciones de insecticidas químicos sintéticos, reduciéndose de esta forma la mortalidad de las especies auxiliares beneficiosas del cultivo genéticamente modificado (cultivo GM) (Velkov *et al.*, 2005). Por ejemplo, en EEUU, el número medio de aplicaciones insecticidas disminuyó de 4.6, entre 1992-1995, a 0.8, durante el período 1999-2001, gracias en gran medida a la introducción del algodón Bt (Romeis *et al.*, 2006). Otras ventajas de los cultivos Bt son el incremento del rendimiento (Figs. 8 y 9) y reducción de costes para la protección del cultivo, selectividad y especificidad contra especies plaga diana, conservación de la biodiversidad, y demostrada inocuidad contra el medio ambiente y la salud humana (Nester *et al.*, 2002). Entre los problemas asociados a las plantas transgénicas cabe destacar los posibles efectos sobre organismos beneficiosos no diana, que incluyen a agentes naturales de control biológico de la plaga, tanto parásitos como depredadores (Mohan y Manjunath, 2005). Por tanto, es importante incluir estudios sobre el efecto de las toxinas de *B. thuringiensis* en estas especies que forman parte de la fauna auxiliar del cultivo Bt, para asegurarnos su inocuidad, antes de su comercialización (Romeis *et al.*, 2006).

Las toxinas Cry de *B. thuringiensis* están presenten en cultivos tan diversos como algodón, arroz y maíz. La última revisión a nivel mundial indica que los cultivos Bt representan el 18% del total de los cultivos GM, aproximadamente 16.2 millones de hectáreas plantadas en el 2005 (James, 2005). De entre éstos, el algodón Bt es el segundo cultivo Bt más extendido, después del maíz, ocupando una superficie de 9.8 millones de hectáreas a nivel mundial en el 2005 (11 % del total de cultivos GM) (James, 2005). La superficie total de algodón Bt se espera que continúe con tendencia al alza en los próximos años, basándonos tanto en el crecimiento continuado durante estos últimos años en cinco de los principales países productores (China, India, Argentina, Brasil y Sudáfrica), como por la introducción de estos cultivos en otros países por primera vez (James, 2005).

La situación de los cultivos Bt en España, aunque presenta cierta reticencia a los productos transgénicos, es la mejor dentro la Unión Europea. Solamente está permitida la comercialización del maíz Bt que representa el 11 % del total de maíz cultivado a nivel estatal (James, 2005). En el resto de Europa, al igual que en España, tampoco se ha aprobado todavía ningún evento de algodón Bt, aunque hay una gran demanda por parte del sector.

Fig. 8. Imagen de un campo de pruebas de algodón de la zona de Sevilla donde se aprecian los daños causados por las plagas. La parte de la derecha corresponde a una variedad de algodón Bt y la de la izquierda es algodón convencional. Fotografía cedida por Monsanto-España.

Fig. 9. Detalle de la protección contra el ataque de insectos que confiere una variedad de algodón Bt (izquierda) frente a una variedad convencional (derecha). Imagen cedida por Monsanto-España.

1.4.2.1. Variedades de algodón Bt

La comercialización del algodón que expresa la toxina Cry1Ac de *B. thuringiensis* (algodón de primera generación) se inició en 1996 en Australia y Estados Unidos para el control de determinadas especies de lepidópteros (Krattiger, 1997; Perlak *et al.,* 2001). La variedad Bollgard® (En Estados Unidos) se desarrolló principalmente para el control de *H. virescens,* mientras que la variedad Ingard® (en Australia) tenía como finalidad erradicar las plagas *H. armigera* y *Helicoverpa punctigera,* ambos casos mediante la expresión de Cry1Ac en el cultivo Bt. Otra plaga importante a nivel mundial, que es controlada por el algodón Bt es *P. gossypiella.* El problema de estas variedades transformadas radica en la variación de la expresión de la toxina en la planta a lo largo del ciclo. Se ha demostrado que durante la floración y el periodo de maduración de las cápsulas los niveles de toxina en planta son menores que en los periodos anteriores (Fitt *et al,* 1994). Por tanto, la expresión de Cry1Ac en el algodón Bt disminuye a lo largo del crecimiento de la planta. Además, tanto en el algodón como en otros cultivos Bt (maíz, arroz y tabaco) también se ha observado una producción variable de toxina Cry en los diferentes tejidos de la planta (Sharma *et al.*, 2005).

Para proporcionar una mayor protección al cultivo del algodón se han desarrollado otras variedades comerciales de algodón Bt en las que se incluyen combinaciones de varias toxinas Cry en la misma planta (variedades de algodón Bt de segunda generación). En el evento Bollgard II® (Monsanto) se combinan las toxinas Cry1Ac y Cry2Ab de *B. thuringiensis* simultáneamente en la misma planta. Esta combinación confiere una mayor protección contra el ataque de insectos plaga (Stewart *et al.*, 2001) al expresar dos toxinas en el mismo cultivo ya que amplía el espectro de acción tóxico. Además ayudará a evitar la aparición de resistencia gracias a combinar dos toxinas que no comparten los mismos sitios de unión en el intestino del insecto (Alcántara *et al.*, 2004; English *et al.*, 1994). En septiembre del 2002 se aprobó la comercialización del Bollgard II® en Australia y Estados Unidos (James, 2002). Otra variedad comercial desarrollada en China por la CAAS (Chinese Academy of Agricultural Sciences) es el algodón Bt transformado para la expresión de un gen de fusión *cry1Ab/cry1Ac* híbrido, que indudablemente ejercerá una importante presión de selección a las poblaciones de lepidóperos en este ecosistema. Syngenta y Dow AgroSciences, empresas importantes del sector, anunciaron en el 2002 el futuro lanzamiento de dos nuevos eventos transformantes de algodón Bt

(James, 2002). La combinación de Cry1Ac y Cry1F en el algodón Bt por Dow AgroSciences tenía como finalidad ampliar el espectro de acción a otras especies de lepidópteros. Pero para conferir una buena protección al cultivo, las toxinas expresadas no deben de compartir los mismos sitios de unión en el intestino de la larva (Ferré y Van Rie, 2002) y, dichos requisitos han demostrado no cumplirse recientemente en algunas de las especies más importantes del algodón (González-Cabrera *et al.*, 2003; Hernández y Ferré, 2005; Herrero *et al.*, 2001a). Por otra parte, Syngenta desarrolló el algodón Bt que expresa una toxina diferente de *B. thuringiensis,* una toxina Vip (James, 2002), nunca expresada en este tipo de cultivo anteriormente.

1.5. La resistencia

Cuando *B. thuringiensis* se utilizó por primera vez para el control biológico de plagas se pensaba que los insectos no serían capaces de desarrollar resistencia a este agente. El uso de productos a base de *B. thuringiensis* tales como Dipel® ocasionó la aparición de resistencia en poblaciones de laboratorio de *Plodia interpunctella* (McGaughey, 1985). Otras muchas especies se han seleccionado posteriormente con diferentes toxinas de *B. thuringiensis* en condiciones de laboratorio para poder investigar el mecanismo de resistencia generado en ellas. Entre estas especies se encuentran por ejemplo *H. armigera, H. virescens, O. nubilalis, P. gossypiella y T. ni* (Akhurst *et al.*, 2003; Estada y Ferré, 1994; Gould *et al.*, 1992; Siqueira *et al.*, 2004; Tabashnik *et al.*, 2000).

1.5.1. Mecanismos de resistencia

La adquisición de la resistencia en los insectos puede ocasionarse por uno o varios cambios producidos en cualquiera de los diversos pasos que forman el mecanismo de acción de las toxinas Cry de *B. thuringiensis* (Fig. 6) (Ferré y Van Rie, 2002). De entre todos estos pasos, el mecanismo de resistencia mejor caracterizado, el que se ha observado experimentalmente como base de resistencia cruzada y que generalmente confiere niveles elevados de resistencia, es la alteración del sitio de unión en la especie diana (Ferré y Van Rie, 2002). Las alteraciones en la unión pueden incluir pérdidas de afinidad por el receptor, pérdida total de la unión al receptor o una disminución de los sitios de unión en el intestino medio del insecto. Estas variaciones en la unión de las toxinas Cry son el mecanismo más específico de resistencia y/o resistencia cruzada en insectos. Por esta razón, es importante

determinar cuáles son las moléculas responsables de esta unión como clave para poder desarrollar marcadores para su uso en programas de seguimiento de la resistencia en campo para poder evitarla.

En otros casos, la resistencia se ha relacionado con un aumento o disminución del procesado proteolítico de las toxinas Cry en el intestino de los insectos (Forcada *et al.*, 1996; Oppert *et al.*, 1994), o a la habilidad de los insectos de reparar las alteraciones ocasionadas por las toxinas en el epitelio intestinal (Martínez-Ramírez *et al.*, 1999).

En algunas poblaciones de insectos también se ha observado la capacidad de desarrollar diversos mecanismos de resistencia tras la exposición a altas concentraciones de toxinas Cry. En una línea resistente de *Plodia interpunctella* se observó, por ejemplo, tanto una reducción del procesado proteolítico de la protoxina como una disminución de la afinidad por los sitios de unión, así como una reducción del número de receptores (Herrero *et al.*, 2001b; Oppert *et al.*, 1997). Estos diferentes mecanismos de resistencia pueden deberse por ejemplo a cambios en varias moléculas que pueden participar o influir en cualquiera de los pasos del mecanismo de acción de las toxinas Cry en el insecto.

1.5.2. Genética de la resistencia

Para determinar el grado de adaptación de las especies diana en un cultivo Bt es importante conocer las bases genéticas que puedan ocasionar la aparición de la resistencia para poder prevenirla. En algunas especies de insectos existe una gran variabilidad genética tanto a nivel intraespecífico como interespecífico, y aunque no disponemos de mucha información a este nivel, se conocen más estudios de variabilidad dentro de una misma población (González-Cabrera *et al.*, 2001; Ferré y Van Rie, 2002). Por tanto, uno de los pasos claves para el diseño de estrategias apropiadas para evitar la aparición de resistencia en zonas donde se utiliza *B. thuringiensis* como insecticida biológico es estimar la frecuencia inicial de los alelos que confieran dicha resistencia. En un estudio con una población de campo de una de las plagas más importantes del algodón, *H. virescens,* se estimó que la frecuencia de los alelos de resistencia era de 1.5×10^{-3} (Gould *et al.*, 1997), valor dentro del rango esperado en poblaciones naturales.

La identificación de los genes que codifican proteínas que participan en la resistencia es otro punto clave para evitar o prevenir la resistencia a las toxinas de *B. thuringiensis.* En *P. gossypiella* se han detectado mutaciones en un gen homólogo a la cadherina que han sido asociadas a la aparición de resistencia a Cry1Ac y al algodón Bt (Morin *et al.*, 2003; Morin *et al.*, 2004). En otro estudio con las cepas CP73 y YHD2 de *H. virescens* se ha determinado recientemente, mediante análisis de ligamiento utilizando marcadores moleculares del tipo AFLP y alozimas, que la resistencia a las toxinas Cry1Ac y Cry2Aa no se debe a alteraciones dentro de un mismo grupo de ligamiento (Gahan *et al.*, 2005). Por tanto, conocer las características de las poblaciones a este nivel nos permitiría diseñar marcadores moleculares con el fin de detectar estos alelos y determinar la dinámica de la resistencia antes de que se extienda al resto de la población.

Tradicionalmente, la resistencia a las toxinas de *B. thuringiensis* se ha asociado a un carácter autosómico recesivo o parcialmente recesivo, aunque en algunas especies se ha comprobado un tipo de transmisión hereditario dominante o parcialmente dominante, o influenciado por el sexo (Ferré y Van Rie, 2002). Las estrategias diseñadas para evitar la aparición de resistencia en campo a los cultivos Bt parten de la premisa de una herencia recesiva o parcialmente recesiva. De esta forma la creación de refugios, zonas colindantes al cultivo Bt que no expresan las toxinas de *B. thuringiensis,* servirá para que los posibles individuos resistentes seleccionados en el campo Bt se crucen con los sensibles de la zona refugio (no Bt) y, por tanto, se diluya la resistencia y no se transmita a las generaciones futuras. El conocimiento del modo de herencia en cado caso podrá ayudar al diseño de estrategias futuras que se ajusten a cada situación con el fin de evitar la aparición de resistencia en campo (Ferré y Van Rie, 2002).

1.5.3. Estabilidad de la resistencia

En muchos estudios con líneas resistentes de insectos, cuando se elimina la presión de selección, los insectos tienden a perder la resistencia adquirida al cabo de varias generaciones sin estar en contacto con el agente de selección (Ferré y Van Rie, 2002). Generalmente la adquisición de la resistencia va en detrimento de la eficacia biológica de los insectos (Bird y Akhurst, 2004; Bird y Akhurst, 2005; Carriére *et al.*, 2001a; Carriére *et al.*, 2001b), y se postula que ésta puede ser una de las causas por las que la resistencia se pierde al cabo de varias generaciones sin selección. Tras cesar la

presión de selección, se produce una disminución de la frecuencia de alelos de resistencia en la población y por tanto, una menor tolerancia al agente de selección. No obstante, en algunas poblaciones estudiadas se reestablecen los niveles de resistencia al volver a reanudarse la selección tras varias generaciones sin exposición a las toxinas de *B. thuringiensis* (Tabashnik *et al.*, 1994a).

1.5.4. Resistencia cruzada

La selección de insectos con formulados comerciales a base de esporas y cristales de *B. thuringiensis* generalmente puede ocasionar resistencia a diversas toxinas ya que algunas cepas de *B. thuringiensis* expresan varias toxinas Cry (Tabashnik *et al.*, 1994b; Tabashnik *et al.*, 1996). Además, la resistencia adquirida tras la exposición de los insectos a una única toxina de *B. thuringiensis* puede ocasionar la aparición de resistencia adicional frente a otras toxinas no utilizadas como fuente de selección. Por tanto, esta resistencia múltiple o cruzada también se puede observar tras la selección con una sola toxina de *B. thuringiensis*, siempre que ambas toxinas compartan los mismos sitios de unión en el intestino del insecto. A modo de ejemplo, al exponer a *H. armigera* a la toxina Cry1Ac durante varias generaciones, se observó la aparición de resistencia cruzada a Cry1Ab (Akhurst *et al.*, 2003). *H. virescens* y *P. gossypiella* también desarrollaron resistencia cruzada a las toxinas Cry1Aa, Cry1Ab y Cry1F, tras haber sido seleccionadas únicamente con Cry1Ac (Lee *et al.*, 1995; Tabashnik *et al.*, 2000). Basándonos en estos y otros casos de resistencia, generalmente la aparición de resistencia cruzada se suele producir entre toxinas de grupos o clases similares.

1.5.5. Estrategias para prevenir la resistencia

La aparición de resistencia en los insectos ha impulsado el desarrollo de diferentes estrategias para el seguimiento de las poblaciones con el fin de detectar individuos resistentes mediante marcadores moleculares o por bioensayo. De esta forma se puede realizar una detección temprana de los alelos de resistencia antes de que ésta se extienda a toda la especie.

La rotación de cultivos, alternando cultivos Bt y cultivos no-Bt, con tratamientos químicos también se ha utilizado para evitar la aparición de individuos resistentes en el cultivo, aunque esta estrategia sólo sería eficaz en

el caso de que la resistencia no sea estable y desaparezca tras eliminar la presión de selección (Mohan y Manjunath, 2005).

Otra estrategia consiste en la expresión de elevadas dosis de toxinas de *B. thuringiensis* en la planta Bt combinada con la creación de refugios. Estos refugios pueden ser tanto del mismo cultivo como de otros tipos de cultivos donde la especie diana se puede alimentar y aparear. De esta forma, los individuos resistentes que hayan sobrevivido de la zona Bt, se aparearían con los insectos sensibles de los refugios y la resistencia se diluiría, siempre considerando que la resistencia fuera un carácter recesivo en la población. Una mejora a esta estrategia es la combinación dos o más toxinas en la misma planta, que no compartan sitios de unión, para conferir una mejor protección al cultivo al ejercer una presión de selección mayor (Ferré y Van Rie, 2002).

Actualmente, en nuestro país no está permitida la comercialización del algodón Bt, con lo que para el control de las especies plagas de este cultivo se utilizan sólo insecticidas químicos de amplio espectro (Avilla *et al.,* 2005). *H. armigera* y *E. insulana,* las especies más importantes del cultivo algodonero en España, son especies polífagas que pueden alimentarse de otros cultivos de los alrededores del algodón y que podrían servir como refugios naturales en caso de que en un futuro se aprobara a la utilización del algodón Bt. Además, se ha demostrado que *H. armigera* tiene una gran capacidad de vuelo, propiedad que le permitiría acceder a zonas relativamente alejadas al cultivo de origen (Farrow, 1984; Feng *et al.*, 2004; Feng *et al.*, 2005; Gregg *et al.*, 1993; Pedgley, 1985). La zona española donde se planta mayoritariamente el algodón en España es en los alrededores del valle del Guadalquivir por lo que los campos y/o invernaderos de la zona o de las provincias colindantes, como Sevilla y Huelva, podrían servir como refugios para hacer frente a los insectos resistentes que pudieran aparecer en caso de permitirse la comercialización de variedades de algodón Bt en la zona en un futuro.

1.6. Insectos objeto de estudio

1.6.1. *H. armigera*

H. armigera (Lepidoptera: Noctuidae) (Hübner) (Fig. 10) es una de las plagas de insectos más importantes en muchos países productores de algodón como Australia, China, España, India, Indonesia, Pakistán y algunas regiones africanas. Es una especie polífaga que ataca a más de 60 cultivos y 67

huéspedes silvestres vegetales entre los que cabe destacar al algodón, fresa, garbanzo, girasol, guisante, maíz, soja, sorgo, tabaco y tomate, entre otros (Trivedi *et al.*, 2005). Pero las mayores pérdidas económicas que ocasiona *H. armigera* en la agricultura es tras atacar los botones florales y cápsulas del algodón (*Gossypium hirsutum)* causando daños en el cultivo y provocando una disminución en el rendimiento global de la producción.

En zonas subtropicales y de clima templado, esta especie pasa el periodo invernal en diapausa, necesitando temperaturas superiores a 17ºC para salir de ella. Presenta una elevada fecundidad, llegando a depositar entre 500 y 3000 huevos por hembra (Trivedi *et al.*, 2005). La mayor actividad de esta especie se produce durante la noche, con desplazamientos variables dependiendo de la zona y condiciones climáticas (Feng *et al.*, 2004; Feng *et al.*, 2005; Trivedi *et al.*, 2005).

Fig. 10. Diferentes estadios de desarrollo del ciclo de vida de *H. armigera.* (A) huevos; (B) larva de último estadio; (C) pupa; (D) adulto.

1.6.2. *E. insulana*

Las especies del género Earias están muy distribuidas a lo largo del mundo, y algunas de éstas, como *E. insulana* (Boisduval) (Fig. 11), son plagas importantes del cultivo del algodón en África, la región mediterránea, en el este de la India, China y sudeste asiático (Reed *et al.*, 1994). *E. insulana* es una especie polífaga (Abul-Nasr *et al.*, 1973), pero su importancia económica radica en las pérdidas que ocasiona, en el cultivo del algodón en países como España, Egipto e Israel (Durán *et al.*, 2000; Hamed Amin *et al.*, 2001; Horowitz, 1997). Aunque se trata de una plaga secundaria comparada con *H. armigera*, puede llegar a tener una importancia considerable en el cultivo algodonero.

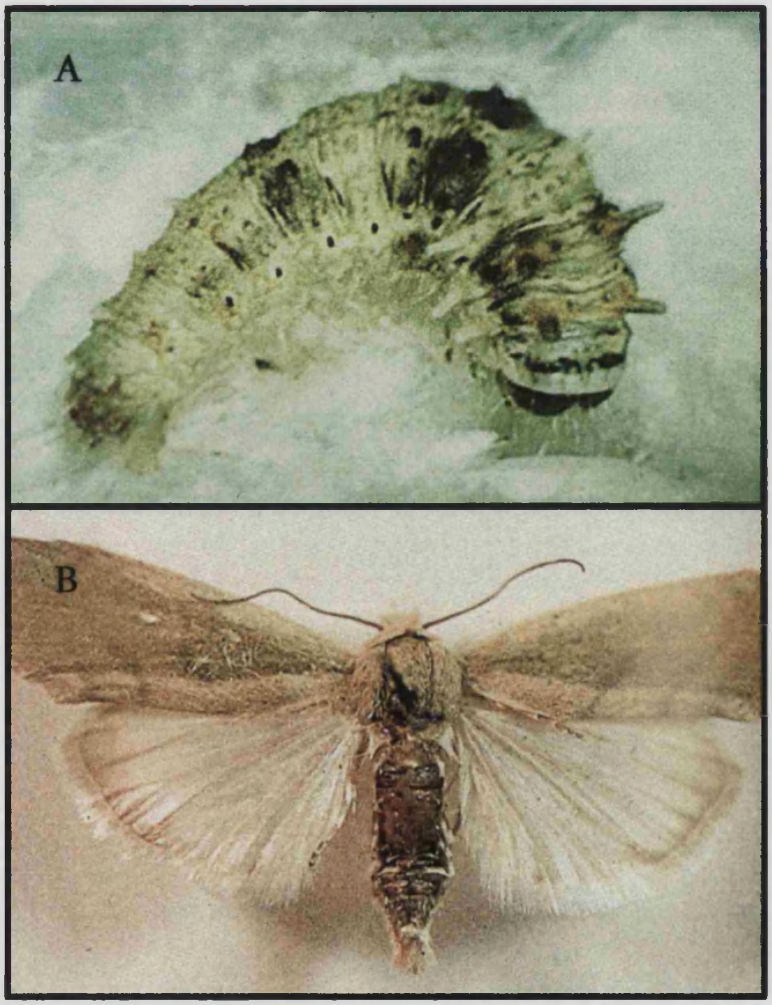

Fig. 11. Diferentes estadios de desarrollo de *E. insulana*.
(A) larva; (B) adulto.

1.6.3. Control de las especies diana

Para el control de *H. armigera* y *E. insulana* se han utilizado tradicionalmente diferentes tipos de insecticidas químicos tales como carbamatos, endosulfán, organofosfatos y piretroides, pero la aplicación excesiva e inadecuada ha llevado a la aparición de resistencia en *H. armigera* a estos productos (Kranthi *et al.*, 2001a; Kranthi *et al.*, 2001b; McCaffery, 1998). Se conocen casos de resistencia en *E. insulana* frente al DDT y endrín (http://www.pesticideresistance.org/DB/species_profile.php?arthropodid=372), así como en otras especies del género Earias muy emparentada filogenéticamente con ella, como *Earias vittella,* en la que los insecticidas químicos convencionales de amplio espectro han favorecido la aparición de resistencia (Kranthi *et al.*, 2001a).

Una alternativa a estos tipos de productos químicos de amplio espectro es la utilización de bioinsecticidas basados en las proteínas de *B. thuringiensis*, pero sin lugar a dudas la manera más efectiva de controlar ambas especie es mediante la utilización de las plantas Bt al disponer de una expresión continuada de toxina en la planta.

1.6.4. Toxicidad de *B. thuringiensis*

H. armigera es una especie relativamente poco susceptible a las toxinas de *B. thuringiensis* en comparación con otras especies muy emparentadas con ella (http://www.glfc.forestry.ca/bacillus) (Tabla 1). De entre todas, Cry1Ac se ha demostrado que es la más activa contra esta especie (Chakrabarti *et al.*, 1998; Liao *et al.*, 2002; Padidam, 1992). Este noctuido además presenta una elevada variabilidad intraespecífica en cuanto a su susceptibilidad a las toxinas de *B. thuringiensis*. Concretamente, en un estudio con diferentes poblaciones de *H. armigera* de China, la estima de la LC_{50} para Cry1Ac osciló entre 0.091 y 9.073 µg/ml (Wu *et al.*, 1999). Por lo tanto, diseñar buenas estrategias para el manejo de la resistencia y prevenir su evolución es una tarea muy importante en estas especies que poseen una elevada variabilidad poblacional. Otras toxinas con menor actividad contra esta especie son Cry1Aa, Cry1Ab, Cry2Aa y Cry2Ab (Chakrabarti *et al.*, 1998; Lambert *et al.*, 1996; Liao *et al.*, 2002) (Tabla 1). Cry2Aa no es tan tóxica contra *H. armigera* como lo es Cry1Ac (Chakrabarti *et al.*, 1998; Liao *et al.*, 2002), pero las toxinas del grupo de las Cry2A se consideran muy apropiadas para el control de ésta y otras plagas del algodón por poseer un modo de acción diferente

(English *et al.*, 1994; Jurat-Fuentes *et al.*, 2003; Karim y Dean, 2000a) y de esta forma poder evitar la aparición de resistencia cruzada.

Como se ha comentado anteriormente, no existen muchos datos acerca de la efectividad de las toxinas de *B. thuringiensis* contra especies del género Earias en la literatura científica. Para *E. vitella,* especie muy emparentada filogenéticamente con *E. insulana,* todas las toxinas del grupo Cry1A probadas mediante bioensayos resultaron ser activas, siendo la Cry1Ab la más toxica, seguida por Cry1Aa y por Cry1Ac (Kranthi *et al.*, 1999) (Tabla 2). Para *E. insulana* concretamente, no nos consta que existan datos de toxicidad a las proteínas Cry de *B. thuringiensis* previos a los presentados en este trabajo.

TABLA 1. Resumen de la toxicidades de *B. thuringiensis* en *H. armigera* determinadas mediante diferentes tipos de bioensayos y autores.

Toxina	Ensayo	Estadio	LC_{50}	Referencia
Cry1Aa	Dieta superficial	neonato	>720x10^6 cells/cm^2	Padidam *et al.*, 1992
	Incorporada en dieta	neonato	370 (140-950) ng/ml	Chackrabarti *et al.*, 1998
	Incorporada en dieta	neonato	>16000 ng/ml	Avilla *et al.*, 2005
Cry1Ab	Dieta superficial	neonato	54.2 (16.2-194.2) x10^6 cells/cm^2	Padidam *et al.*, 1992
	Dieta superficial	neonato	660 (430-810) ng/cm^2	Mandaokar *et al.*, 1998
	Incorporada en dieta	L1	1550 (470-3270) ng/ml	Chackrabarti *et al.*, 1998
	Dieta superficial	L1	692 (458-1071) ng/cm^2	Liao *et al.*, 2002
	Dieta superficial	neonato	1185 (837-2055) ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	>16000 ng/ml	Avilla *et al.*, 2005
Cry1Ac	Dieta superficial	neonato	4.7 (2.3-9.4) x 10^6 cells/cm^2	Padidam *et al.*, 1992
	Dieta superficial	L1	38 (24-55) ng/cm^2	Liao *et al.*, 2002
	Dieta superficial	L1	212 (167-280) ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	L1	72 (37-118) ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	20 (12-36) ng/ml	Chackrabarti *et al.*, 1998
	Incorporada en dieta	neonato	240 (180-310) ng/ml	Babu *et al.*, 2002
	Dieta superficial	L1	115 (82-159) ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	3500 (2700-4500) ng/ml	Avilla *et al.*, 2005
Cry1Ba	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
	Dieta superficial	neonato	no activa	Mandaokar *et al.*, 1998
Cry1Bb	Dieta superficial	L1	>4000 ng/cm^2	Liao *et al.*, 2002
Cry1Ca	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
	Dieta superficial	L1	>4000 ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	> 16000 ng/ml	Avilla *et al.*, 2005
Cry1Da	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
	Incorporada en dieta	neonato	>16000 ng/ml	Avilla *et al.*, 2005

Cry1Ea	Dieta superficial	L1	no activa	Liao *et al.*, 2002
	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
	Incorporada en dieta	neonato	>16000 ng/ml	Avilla *et al.*, 2005
Cry1Fa	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
	Dieta superficial	L1	4193 (2043-12053) ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	>16000 ng/ml	Avilla *et al.*, 2005
Cry1Ia	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
Cry1Ja	Incorporada en dieta	neonato	>16000 ng/ml	Avilla *et al.*, 2005
Cry2Aa	Dieta superficial	L1	149(117-189) ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	6300 (4900-8500) ng/ml	Avilla *et al.*, 2005
Cry2Ab	Dieta superficial	neonato	13 ng/cm^2	Kumar *et al.*, 2004
	Dieta superficial	L1	421 (98-1259) ng/cm^2	Liao *et al.*, 2002
Cry9A	Dieta superficial	L1	>4000 ng/cm^2	Liao *et al.*, 2002
	Incorporada en dieta	neonato	no activa	Chakrabarti *et al.*, 1998
Cry9C	Dieta superficial	neonato	>1350 ng/cm^2	Lambert *et al.*, 1996
	Incorporada en dieta	neonato	>16000	Avilla *et al.*, 2005
Cry32A	Incorporada en dieta	neonato	no activa	Balasubramanian *et al.*, 2002

TABLA 2. Resumen de las toxicidades de *B. thuringiensis* en *E. vitella*.

Toxina	Ensayo	Estadio	LC_{50}	Referencia
Cry1Aa	Superficie hoja	neonato	0.5999 (0.380-0.929) ng/cm^2	Kranthi *et al.*, 1999
Cry1Ab	Superficie hoja	neonato	0.447 (0.306-0.662) ng/cm^2	Kranthi *et al.*, 1999
Cry1Ac	Superficie hoja	neonato	0.88 (0.576-1.428) ng/cm^2	Kranthi *et al.*, 1999

2. OBJETIVOS

2. OBJETIVOS

2.1. Interacción de las toxinas Cry de *Bacillus thuringiensis* con dos especies plaga del algodón.

2.1.1. Establecimiento del modelo de unión de diferentes toxinas Cry a la membrana epitelial del intestino en poblaciones susceptibles de *Helicoverpa armigera.*

2.1.2. Utilización de *B. thuringiensis* para el control de la plaga del algodón *Earias insulana* (Boisduval) (Lepidoptera: Noctuidae).

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2.4. Determinación de las bases moleculares de la resistencia a las toxinas Cry1A de *B. thuringiensis* en *H. armigera.*

3. RESUMEN GENERAL

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3.1. Interacción de las toxinas Cry de *B. thuringiensis* con dos especies plaga del algodón.

3.1.1. Establecimiento del modelo de unión de diferentes toxinas Cry a la membrana epitelial del intestino en poblaciones susceptibles de *H. armigera.*

El uso extensivo de insecticidas químicos de amplio espectro ha repercutido en la aparición de resistencia a estos productos en campo en muchas especies plaga, entre las que cabe destacar *H. armigera* (Kranthi *et al.*, 2001a; Kranthi *et al.*, 2001b; McCaffery, 1998). Este noctuido es una especie relativamente poco susceptible a las toxinas de *B. thuringiensis* (http://www.glfc.forestry.ca/bacillus), pero de entre ellas la Cry1Ac es la más activa y ha sido utilizada tanto como base para numerosos formulados comerciales como para la obtención del algodón Bt de primera generación, cuya comercialización se inició en Australia y EEUU en 1996 (Perlak *et al.*, 2001).

La unión de la toxina al intestino medio de la larva es uno de los pasos más importantes y mejor caracterizado del mecanismo de acción de las toxinas de *B. thuringiensis,* y que ha sido la base de muchos trabajos (Schnepf *et al.*, 1998). Para nuestro trabajo con *H. armigera* se utilizaron las toxinas Cry1Ab y Cry1Ac marcadas con I^{125}, y vesículas de membrana (o BBMV, abreviatura del inglés "brush border membrane vesicles") para proponer un modelo general de unión para estas dos toxinas en el intestino de larvas de esta especie. Tras realizar ensayos *in vitro* de unión en suspensión con ambas toxinas marcadas con I^{125}, se determinó que Cry1Ab y Cry1Ac compartían sitios de unión en el intestino de la larva en las cuatro poblaciones de *H. armigera* estudiadas, aunque los parámetros de unión variaron entre dichas poblaciones. En todas ellas, Cry1Ac unió a las BBMV del insecto con mayor afinidad (menor K_d) que Cry1Ab. El valor de K_d obtenido para Cry1Ab confirmó la menor actividad de esta toxina en *H. armigera* respecto de Cry1Ac (alrededor de unas 20 veces).

Para completar el modelo general de unión en *H. armigera,* se utilizó una batería de toxinas Cry, y tras realizar ensayos de competencia por la unión con las toxinas Cry1Ab-I^{125} y Cry1Ac-I^{125}, se demostró que tanto Cry1Aa como Cry1Ab compartían sitios de unión con Cry1Ac, pero que esta

última toxina tenía sitios de unión propios no compartidos por las otras toxinas analizadas. Las otras toxinas Cry ensayadas no compitieron por los sitios de unión de las toxinas Cry1A, aunque sólo Cry1Ja lo hizo a concentraciones relativamente elevadas. El modelo de unión que se propone para *H. armigera* en este trabajo desaconseja la combinación de genes *cry1A* distintos en plantas de algodón para su protección contra el ataque de insectos, ya que existe la posibilidad de selección de alelos que confieran resistencia a más de una toxina Cry1A por alteración de sitios de unión comunes. Dado que *H. armigera* es una especie polífaga, las estrategias a seguir para evitar la aparición de resistencia cruzada en campo se aplicarían también a otros cultivos Bt que se pudieran desarrollaran en un futuro. Por tanto, la introducción del algodón Bt de segunda generación BollgardII®, donde se combina la expresión de las toxinas Cry1Ac y Cry2Ab, indudablemente conferirá una mayor protección al cultivo a largo plazo frente a la aparición de resistencia en *H. armigera,* ya que ambas toxinas no comparten sitios de unión en esta especie.

En *Heliothis virescens,* especie estrechamente emparentada con *H. armigera,* las toxinas del grupo Cry1A también comparten sitios de unión en el intestino de la larva, teniendo Cry1Ac sitios adicionales no compartidos (Jurat-Fuentes *et al.*, 2001; Lee *et al.*, 1995; Van Rie *et al.*, 1989). Los resultados obtenidos en *H. armigera* en este trabajo nos permitieron proponer un modelo general de unión para *H. armigera* similar al propuesto para *H. virescens* originalmente por Van Rie *et al.*, (1989).

Profundizando en el estudio del modelo de unión en *H. armigera*, se determinó el efecto inhibitorio de algunos azúcares y lectinas seleccionados en la capacidad de unión de Cry1Ab y Cry1Ac a BBMV preparadas a partir de intestinos de larvas de esta especie. En trabajos anteriores con otras especies de lepidópteros se demostró que la unión de Cry1Ac al intestino del insecto, pero no la de Cry1Ab, se veía inhibida por la acción del azúcar N-acetil galactosamina (GalNAc) (Knowles *et al.*, 1991; Luo *et al.*, 1997; Masson *et al.*, 1995). Nuestros resultados con *H. armigera* apoyan el efecto inhibitorio específico de este azúcar por Cry1Ac. El mismo patrón de inhibición fue hallado con el ácido siálico pero no con otros azúcares estudiados, como la N-acetil glucosamina (GlcNAc) y la manosa, indicando que la inhibición de GalNAc y del ácido siálico era específica. La aglutinina de soja (o SBA, del inglés soybean agglutinin), lectina con afinidad de unión por GalNAc, produjo un mayor efecto inhibitorio en la unión de Cry1Ac-I^{125} que de Cry1Ab-I^{125}. Por el contrario, la Concanavalina A (Con A), con afinidad de unión por residuos de manosa y glucosa, inhibió de igual forma la unión de

Cry1Ab-I^{125} que de Cry1Ac-I^{125}, indicando que estos residuos deben de estar próximos a los epitopos donde ambas toxinas se unen en el intestino de la larva, aunque no sean utilizados para la unión. Por tanto, asumiendo que los sitios de unión en las BBMV son estructuras complejas de proteínas de membrana glicosiladas, la unión de Cry1Ab y Cry1Ac al intestino del insecto podría producirse a través de epitopos diferentes del mismo receptor dificultando la unión de la toxina heteróloga a sitios cercanos.

3.1.2. Utilización de *B. thuringiensis* para el control de la plaga del algodón *E. insulana* (Boisduval) (Lepidoptera: Noctuidae).

El género Earias está ampliamente distribuido en el Viejo Mundo y Australasia, y algunas especies son plagas bastante importantes en algunos países productores de algodón. Concretamente, *E. insulana* (Boisduval), aunque puede alimentarse de otros tipos de cultivos (Abul-Nasr *et al.*, 1973), es una plaga importante del cultivo del algodón en algunas regiones de España (Durán *et al.*, 2000), Egipto (Hamed Amin *et al.*, 2001), Israel (Horowitz *et al.*, 1997), India y Pakistán (Kranthi *et al.*, 1999).

No existen datos en la literatura científica sobre la eficacia de toxinas individuales de *B. thuringiensis* en esta especie y, por tanto, éste es el primer trabajo en donde se ha determinado la actividad de 13 de las toxinas de *B. thuringiensis* más comunes activas contra lepidópteros y la capacidad de unión de algunas de ellas a BBMV preparadas a partir de intestinos de larvas del último estadio del insecto.

Un paso importante para evaluar el potencial insecticida de *B. thuringiensis* para el control de esta especie en el algodón es determinar la potencia relativa de las toxinas Cry. En este estudio, seis de las toxinas Cry ensayadas resultaron activas para larvas de *E. insulana.* En la población de *E. insulana* de Egipto, Cry1Ab y Cry9Ca resultaron con toxicidades similares, seguidas de Cry1Ba y Cry1Ac, y finalmente de Cry1Da y Cry1Ia. En la otra población estudiada (la de España) la toxina más activa fue la Cry9Ca, seguida en orden decreciente por Cry1Ac, Cry1Ba, y Cry1Da (Cry1Ab y Cry1Ia no se analizaron en esta población). Para todas las toxinas analizadas, la cepa española resultó ser más susceptible que la de Egipto. Esta variación en susceptibilidad a las toxinas de *B. thuringiensis* ya había sido descrita previamente en poblaciones separadas geográficamente pertenecientes a la misma especie en otros insectos (González-Cabrera *et al.*, 2001; Wu *et al.*, 1999). Sin embargo, la existencia de variación natural en la susceptibilidad entre poblaciones diferentes dentro de una misma especie a veces puede

confundirse con diferentes niveles de resistencia a las toxinas de *B. thuringiensis* (Ferré and Van Rie, 2002). En este estudio se pretendió determinar las diferencias de susceptibilidad existentes dentro de esta especie ya que podrían ser útiles para el control de esta especie mediante el algodón Bt en el futuro.

La exposición continuada a la toxina Cry1Ac (expresada en el algodón Bt de primera generación) podría provocar la aparición de insectos resistentes a esta toxina. En la mayoría de los casos mejor estudiados de resistencia en insectos, como se ha comentado anteriormente, se debe a alteraciones en los receptores de membrana del intestino medio del insecto (Ferré and Van Rie, 2002). Mediante ensayos de unión y competencia con la toxina Cry1Ac y las otras toxinas activas contra esta especie, marcadas con ^{125}I y con biotina, se determinó cuáles de ellas comparten sitios de unión en el intestino medio de *E. insulana* y, por tanto, podrían perder su capacidad tóxica si las poblaciones de *E. insulana* desarrollaran resistencia a Cry1Ac tras la alteración de su receptor.

En resumen, los resultados obtenidos en este trabajo muestran que *E. insulana* es una especie susceptible a Cry1Ab, Cry1Ac, Cry1Ba, Cry1Da, Cry1Ia, y Cry9Ca. Cry1Ab y Cry9Ca fueron significativamente más activas que Cry1Ac, la toxina actualmente utilizada en el algodón Bt. Desde el punto de vista del manejo de la resistencia, las toxinas más activas son buenas candidatas para utilizarse para el control de esta especie juntamente o en substitución de la Cry1Ac, excepto la Cry1Ab, ya que comparte sitios de unión con ésta en el intestino medio de la larva. La toxina Cry9Ca es particularmente interesante ya que, además de ser la más activa para *E. insulana*, tiene un amplio espectro de actividad que incluye también otras especies plaga importantes del algodón como *H. armigera*, *H. virescens*, y *Spodoptera littoralis* (Lambert *et al.*, 1996; Reed *et al.*, 2001; Van Frankenhuyzen *et al.*, 1997).

Mi contribución a este trabajo consistió en la preparación de las toxinas Cry de *B. thuringiensis* utilizadas para la realización de los bioensayos con *E. insulana*, su posterior purificación mediante FPLC y la realización de todos ensayos de unión a vesículas para determinar cuáles de las toxinas comparten sitios de unión en el intestino de la larva, así como en el análisis y discusión de los resultados.

3.2. Caracterización molecular y del espectro de acción de un nueva toxina Cry1I de *B. thuringiensis* para su posible utilización en el control biológico de plagas.

Durante los últimos 20 años, se han identificado más de 300 tipos de toxinas Cry y Cyt diferentes, y algunas de ellas en particular se han utilizado con éxito en programas para el control integrado de plagas (Schnepf *et al.*, 1998). Los cristales de proteínas Cry de *B. thuringiensis* se componen generalmente de protoxinas de unos 130-140 kDa (de los genes *cry1*), 65-70 kDa (de los *cry2*), y 70-130 (de los *cry3*), aunque existen algunas proteínas Cry que no forman parte del cristal paraesporal (entre ellas las Cry1I), por lo que no se han utilizado como ingrediente activo en los formulados comerciales basados en *B. thuringiensis*. Las toxinas Cry1I tienen un espectro de acción más amplio que la mayoría de toxinas Cry1, incluyendo a importantes especies de lepidópteros y coleópteros (Tailor *et al.*, 1992). La búsqueda de nuevas cepas de *B. thuringiensis* que contengan nuevos genes *cry* ha aumentado notablemente durante las últimas décadas, no sólo para hallar toxinas con un espectro de acción diferente, sino para poder incrementar el arsenal de toxinas Cry que puedan ser utilizadas para el control biológico en campo.

En el presente trabajo se ha aislado y expresado una nueva proteína de *B. thuringiensis* del grupo de las Cry1I, y posteriormente se ha determinado tanto su espectro tóxico de acción mediante bioensayos, sus propiedades de unión al intestino medio de larvas de insectos plaga y la relación con otras toxinas que se encuentran frecuentemente en los insecticidas basados en *B. thuringiensis*. El marco abierto de lectura (ORF, del inglés "opened reading frame") completo del nuevo gen *cry1Ia7* se clonó, expresó en células de *Escherichia coli* y se verificó, mediante SDS-PAGE, revelando una proteína de aproximadamente 81 kDa. El análisis de la secuencia aminoacídica de Cry1Ia7 mostró unas propiedades similares, aunque con diferencias en algunas posiciones, a las de otras proteínas del mismo grupo previamente descritas. Cry1Ia7 difirió de Cry1Ia1, por ejemplo, en cuatro aminoácidos del dominio II, aunque las regiones correspondientes a los tres dominios generalmente fueron muy similares en ambas toxinas.

Las toxinas del grupo Cry1I son especialmente interesantes por su amplio rango de huéspedes. En los bioensayos con Cry1Ia7, esta toxina no mostró actividad contra *H. armigera, Trichoplusia ni,* ni contra diferentes especies del género Spodoptera. Por otra parte, Cry1Ia7 sí resultó ser tóxica

para *Leptinotarsa decemlineata*, demostrando la actividad dual Lepidoptera-Coleoptera descrita por Tailor *et al.* (1992). Además Cry1Ia7 mostró actividad contra larvas de *Lobesia botrana*, cuya susceptibilidad no se había descrito previamente para las toxinas de este grupo. Por otra parte, Cry1Ia7 resultó ser activa contra *Plutella xylostella*, especie normalmente susceptible a las toxinas Cry1I (Choi *et al.*, 2000; Iriarte *et al.*, 1998; Schnepf *et al.*, 1998; Selvapandiyan *et al.*, 2001; Shin *et al.*, 1995).

Para determinar la posible utilización combinada de Cry1Ia7 con proteínas del grupo Cry1A que han sido comúnmente utilizadas en los cultivos Bt para evitar la aparición de resistencia en campo, se realizaron ensayos de unión y competencia con BBMV de *L. botrana* y *E. insulana*. Los resultados demostraron que Cry1Ia7 no comparte sitios de unión con Cry1Ab ni Cry1Ac y, por tanto, la aparición de resistencia a las toxinas Cry1A no produciría probablemente resistencia cruzada a Cry1Ia7, y viceversa, si estas toxinas se utilizaran conjuntamente para el control biológico de estas especies plaga. Si tenemos en cuenta las semejanzas encontradas el los modelos de unión para las toxinas Cry propuestos en diversas especies de insectos, los resultados obtenidos con *L. botrana* y *E. insulana* podrían ser extrapolados a otras especies no incluídas en este estudio.

El hecho de que las toxinas Cry1I se secreten al medio y no formen parte del cristal ha impedido su utilización como bioinsecticida de pulverización, aunque sus propiedades insecticidas podrían ser explotadas si estas toxinas se expresaran en plantas transgénicas. Alternativamente, los genes *cry1I* podrían expresarse y microencapsularse en especies del género Pseudomonas para ser utilizadas posteriormente como insecticidas convencionales, bien solas o en rotación con otros insecticidas basados en toxinas Cry1A de *B. thuringiensis*.

Mi contribución a este trabajo consistió en la producción y purificación de las toxinas Cry de *B. thuringiensis* utilizadas, en la realización de bioensayos con algunas de las especies plaga para determinar la toxicidad de Cry1Ia7, y en la preparación y realización de todos los ensayos de unión y competencia con BBMV preparadas a partir de intestinos de insectos, así como en el análisis y discusión de los resultados.

3.3. Análisis de la variabilidad genética en las poblaciones naturales de *H. armigera* susceptibles de la zona algodonera del sur de España mediante marcadores moleculares del tipo AFLP (Amplified Fragment Length Polymorphism).

H. armigera es una de las plagas más importantes del algodón en la región mediterránea, África y Australasia (Fitt, 1989). El control tradicional de esta especie se ha llevado a cabo mediante el uso de insecticidas químicos de amplio espectro, para los que esta especie ha desarrollado resistencia en numerosas ocasiones (Kranthi *et al.*, 2001a; Kranthi *et al.*, 2001b; McCaffery, 1998). Por esta razón se han desarrollado variedades de algodón protegidas contra el ataque de lepidópteros mediante la expresión de genes *cry* de *B. thuringiensis* (algodón Bt) en la planta. Esta metodología, comercializada en Australia y Estados Unidos desde 1996 (Krattiger, 1997; Perlak *et al.*, 2001), requiere la adopción de nuevas estrategias con el fin de evitar la aparición de resistencia. Estas variedades todavía no han sido aprobadas para su comercialización en España, uno de los pocos países productores de algodón de Europa, por lo que un estudio previo de la estructura poblacional de las especies plaga podría ayudar a entender la dinámica de dichas especies con tal de poder diseñar estrategias adecuadas para cada caso en particular. La estrategia comúnmente más aceptada consiste en la creación de refugios alrededor de la zona del cultivo Bt, plantados con cultivos no-Bt, bien del mismo o diferente cultivo donde la especie pueda sobrevivir y aparearse. Estas zonas libres de Bt, por tanto, servirían para diluir los alelos de resistencia, siempre que se tratara de un carácter con herencia recesiva.

Para determinar la estructura poblacional de *H. armigera* en la zona algodonera del sur de España se utilizaron marcadores moleculares del tipo AFLP. Para esto, se recogieron insectos de orígenes diferentes tanto de dentro como de fuera de la zona del algodón durante cuatro temporadas consecutivas (antes y después de la cosecha del algodón en los años 2003 y 2004). En total se estudiaron 475 insectos, 25 de cada uno de los cinco orígenes muestreados en cada una de las 4 temporadas de recolección (uno de los muestreos no pudo realizarse en la segunda temporada del 2003). Con las dos combinaciones de cebadores utilizadas para realizar las amplificaciones específicas en este trabajo se determinaron 104 bandas, todas ellas polimórficas, con tamaños comprendidos entre 100 y 500 pb.

El filograma resultante tras agrupar las 19 poblaciones mediante la distancia mínima de Nei (Nei, 1987), mostró un primer indicio de que las

poblaciones de una misma temporada se agrupaban entre sí. La naturaleza dominante de este tipo de marcadores impide una correcta estimación de las frecuencias alélicas, sesgando en muchos casos los resultados (Lynch y Milligan, 1994). Por esta razón se incluyó un análisis molecular de la varianza (AMOVA) ya que en este tipo de análisis no influye el carácter del marcador utilizado y permite estimar la varianza a varios niveles (Excoffier *et al.*, 1992). Para ello, se realizaron agrupaciones de las poblaciones según su origen independientemente de la temporada de muestreo, según la temporada independientemente del origen, y según la región dentro de cada temporada (dentro y fuera de la zona del algodón).

En ambos análisis, tanto la aproximación de Lynch y Milligan (1984) como el análisis de AMOVA determinaron que el mayor grado de variación se encontraba dentro de las poblaciones y que éstas presentaban un nivel moderado de diversidad genética (F_{ST} = 0.20). Las diferencias halladas entre los grupos de poblaciones establecidos sólo resultaron significantivas cuando dichos grupos se definían por la temporada de muestreo. Este resultado se respaldó con los datos de divergencia y flujo génico calculados entre todos los pares de poblaciones muestreados mediante diferentes aproximaciones.

Por tanto, la elevada diversidad hallada tras comparar poblaciones procedentes de un mismo origen o región pero capturadas en diferentes temporadas, junto con la estrecha relacion existente entre poblaciones procedentes de una misma temporada de muestreo, podría indicar que los individuos de las regiones andaluzas estudiadas podrían ser reemplazados completamente cada temporada por otros procedentes de zonas colindantes fuera del área de muestreo, lo que respalda la idea de que dichas poblaciones no están en equilibrio migración-deriva. A pequeña escala, las poblaciones muestreadas en una misma temporada obtuvieron valores de diversidad generalmente menores que los hallados entre poblaciones de diferentes temporadas, pero sin ningún tipo de estructura definida por la región de muestreo. Todos estos resultados, por tanto, sugieren que el flujo génico podría ser la fuerza conductora que explicaría la distribución genética en estas poblaciones a lo largo del tiempo. El comportamiento de las poblaciones de *H. armigera* andaluzas podrían compararse con el hallado en poblaciones de *Helicoverpa punctigera* del sureste de Australia por Duffield (2004). En estas poblaciones australianas, la mayor fuente de variabilidad no procede de las pupas de las temporadas o generaciones anteriores que han hibernado en la zona, sino de otras zonas alejadas al cultivo de origen, diluyendo la resistencia que pueda haberse generado en la zona tras los tratamientos insecticidas. De esta forma, como se ha demostrado que *H. armigera* puede tener una gran

capacidad de vuelo (Feng *et al.*, 2004; Feng *et al.*, 2005), los insectos de zonas alejadas podrían reemplazar a los que no hayan sobrevivido a los tratamientos insecticidas, con los que formarían una metapoblación, y podrían servir como refugios en el caso que el algodón Bt algún día se comercializara en España.

3.4. Determinación de las bases moleculares de la resistencia a las toxinas Cry1A de *B. thuringiensis* en *H. armigera*.

La resistencia a las toxinas Cry de *B. thuringiensis* se ha estudiado en muchos trabajos previos para diseñar estrategias adecuadas con la finalidad de evitar la aparición de resistencia dentro de programas de protección de cultivos. Aunque se trata de una especie polífaga, la importancia de *H. armigera* radica en ser una plaga relevante para el algodón y que ha desarrollado resistencia a muchos de los insecticidas químicos utilizados para su erradicación (Kranthi *et al.*, 2001a; Kranthi *et al.*, 2001b; McCaffery *et al.*, 1998). La alternativa al control de esta y otras muchas especies con insecticidas químicos de amplio espectro es la utilización de las toxinas Cry de *B. thuringiensis*. De entre ellas, se ha demostrado que Cry1Ac es la más activa contra *H. armigera* (Chakrabarti *et al.*, 1998; Liao *et al.*, 2002; Padidam, 1992), razón por la que se ha utilizado como componente activo en numerosos formulados comerciales y para ser expresada en el algodón Bt para el control de esta especie.

En un trabajo anterior a éste se demostró que la resistencia adquirida en ISOC (cepa de *H. armigera* resistente a Cry1Ac) tras ser comparada con una cepa de *H. armigera* susceptible (cepa ANGR) y casi isogénica con ISOC se explicaba por disminución en la afinidad de Cry1Ac por los sitios de unión al intestino medio de la larva en ISOC (Akhurst *et al.*, 2003). Para profundizar en las bases moleculares de la resistencia en ISOC, en el presente trabajo se realizaron experimentos de unión ligando-receptor en membrana con las toxinas Cry1Aa, Cry1Ab y Cry1Ac, y con BBMV preparadas a partir de intestinos de ambas cepas (técnica del "ligand blot"). Además también se utilizó la técnica de los "microarrays" para determinar las diferencias de expresión entre ANGR e ISOC. Para ello, se utilizó una genoteca de ADN complementario (ADNc) que contenía 4984 clones de ESTs (del inglés "Expressed Sequence Tags") obtenidos de intestinos de la cepa ANGR. Estos ESTs se amplificaron y purificaron antes de su impresión en diferentes portaobjetos (para formar los "microarrays" o chips de ADNc). Para determinar las diferencias de expresión entre las cepas ANGR e ISOC, se

marcó ADNc procedente de diferentes extracciones de intestinos de ambas cepas con dos tipos diferentes de fluoróforos (Cy3-dCTP y Cy5-dCTP) para poderse hibridar conjuntamente en los "microarrays". Se llavaron a cabo un total de seis experimentos (tres con una combinación de fluoróforos y otros tres con la combinación complementaria), y, posteriormente, se escasearon los resultados obtenidos y se normalizaron las intensidades antes de poder determinar las diferencias de expresión entre ambas cepas.

Los resultados de los experimentos de "ligand blot" demostraron que no existen diferencias aparentes de unión a las proteínas desnaturalizadas de las BBMV entre las toxinas Cry1A en ambas cepas, y que las tres toxinas Cry1A analizadas unieron de igual forma a cinco bandas de tamaños variables (48, 78, 100, 210 y 250 KDa aproximadamente) en el filtro. Estos resultados discrepan con los obtenidos por Akhurst *et al.* (2003) utilizando la misma cepa, donde Cry1Ac perdía la unión a las BBMV en ISOC pero no en ANGR. No obstante, se sabe que experimentos llevados a cabo mediante diferentes técnicas podrían diferir en los resultados obtenidos (Lee *et al.*, 1996a, Escriche *et al.*, 1995; Ferré *et al.*, 1991; Masson *et al.*, 1995b). Los análisis preliminares de las bandas obtenidas en el "ligand blot" mediante espectroscopia de masas desorción/ionización mediante láser asistida por matriz asociada a un analizador de tiempo de vuelo (MALDI-TOF), mostraron la coexistencia de diversas moléculas en la misma banda al no haber sido bien resueltas en el gel de SDS-PAGE unidimensional, lo cual podría estar enmascarando diferencias de unión debidas a otras proteínas de membrana. Entre ellas se encontraron algunas de las moléculas propuestas en la literatura como receptores para las toxinas de *B. thuringiensis* en diferentes especies de insectos tales como la aminopeptidasa-N y la fosfatasa alcalina (Banks *et al.*, 2001; Denolf *et al.*, 1997; Fernández *et al.* 2006; Gill *et al.*, 1995; Hua *et al.*, 2001; Ingle *et al.*, 2001; Jenkins and Dean, 2001; Jurat-Fuentes and Adang., 2004; Knight *et al.*, 1994; Lee *et al.*, 1996b; Liao *et al.*, 2005; Luo *et al.*, 1997; Masson *et al.* 1995a; Oltean *et al.*, 1999; Rajagopal *et al.*, 2003; Sangadala *et al.*, 1994; Valaitis *et al.*, 1995).

En los experimentos realizados con la técnica de los "microarrays" para determinar las diferencias de expresión entre las cepas ANGR e ISOC de *H. armigera*, nos centramos principalmente en diferencias de expresión (en escala logarítmica) mayores de 1.5 o menores de -1.5, que significaba diferencias de expresión mayores o menores, respectivamente, de 4.5 veces. Partimos de estos valores ya que nuestro objetivo en esta parte del trabajo era buscar diferencias grandes de expresión entre ambas cepas que pudieran estar relacionadas con la resistencia a las toxinas Cry en ISOC. En los resultados

obtenidos, la mayoría de los ESTs que presentaron diferencias relevantes entre ambas cepas no tenían una función conocida. Pero, entre ellos, destacaron cuatro ESTs con expresión reducida (más de 100 veces) en ISOC respecto de ANGR, uno de ellos (EST número 1595) con actividad transposasa predicha. Entre los ESTs sobreexpresados en ISOC destacó uno con más de 100 veces de expresión respecto a ANGR pero sin función conocida, candidato para ser estudiado en detalle con el fin de determinar las bases de la resistencia en ISOC. Además ISOC presentó sobreexpresadas diversas funciones relacionadas con proteólisis y peptidólisis, metabolismo de lípidos y carbohidratos, reparación del ADN y detoxificación, lo cual puede indicar que la presión de selección podría haber forzado al insecto a adaptarse a la exposición a la toxina Cry tras la modificación de su metabolismo basal.

Los resultados obtenidos en este trabajo, conjuntamente con los obtenidos por Akhurst *et al.* (2003), podrían indicar que la resistencia a Cry1Ac adquirida por la cepa ISOC se debe principalmente a la disminución en la afinidad de unión por Cry1Ac, pero que otros cambios adicionales podrían estar participando en la resistencia final formando un mecanismo complejo, ya que algunos genes han modificado su expresión tras la selección con Cry1Ac.

4. CONCLUSIONES

4. CONCLUSIONES

1. El estudio de cuatro poblaciones susceptibles de *H. armigera* ha permitido demostrar que existe al menos un sitio de unión común para las toxinas Cry1Aa, Cry1Ab, Cry1Ac y Cry1Ja de *Bacillus thuringiensis* y que éste no es compartido por Cry1Ba, Cry1Ca, Cry1Da, Cry1Fa, Cry2Aa, Cry2Ab, y Cry9Ca. Por tanto, para proporcionar una mayor protección al cultivo se aconseja la combinación de dos o más toxinas activas contra esta especie pero que no compartan sitios de unión en el intestino del insecto. De esta forma, en caso de aparecer resistencia a una de las toxinas utilizadas debido a pérdida del sitio de unión, no conllevará la aparición de resistencia cruzada a la/s otra/s.

2. La unión de Cry1Ac, pero no la de Cry1Ab, al intestino de *H. armigera* parece estar mediada por el azúcar N-acetil galactosamina. Por tanto, la unión de ambas toxinas al intestino de la larva se produciría tanto a través de epitopos comunes como de epitopos diferentes dentro del mismo receptor multimérico.

3. Por primera vez se ha ensayado una batería de toxinas individuales de *B. thuringiensis* con *E. insulana,* y el resultado ha sido que esta especie es susceptible a Cry1Ab, Cry1Ac, Cry1Ba, Cry1Da, Cry1Ia, and Cry9Ca. Además, Cry1Ab y Cry9Ca resultaron ser más activas que Cry1Ac, la toxina más comúnmente utilizada en el algodón Bt, por lo que su utilización podría conferir una mayor protección al cultivo contra esta especie.

4. La toxina Cry1Ab de *B. thuringiensis* comparte sitios de unión con Cry1Ac en *E. insulana*, pero no Cry1Ba, Cry1Ia, Cry9Ca por lo que estas tres últimas toxinas podrían utilizarse conjuntamente con Cry1Ac para el control biológico de esta especie y conferir una mayor protección al cultivo.

5. La nueva toxina Cry1Ia resultó tóxica para las especies de lepidópteros *E. insulana*, *Lobesia botrana, Plutella xylostella*, y para el coleóptero *Leptinotarsa decemlineata.* No obstante, se demostró que no tiene actividad insecticida contra las especies de lepidópteros *Bombyx mori, H. armigera, Manduca sexta, Spodoptera exigua,*

Spodoptera frugiperda, *Spodoptera littoralis*, and *Trichoplusia ni*, ni para el díptero *Tipula oleracea*.

6. Cry1Ia no comparte sitios de unión con Cry1Ab y Cry1Ac en *E. insulana* y *L. botrana*, y, por tanto, podrían ser combinadas con Cry1Ia para proporcionar una mayor protección contra el ataque de estas especies plaga. En vista de las similitudes halladas en otros modelos de unión entre diferentes especies de insectos, el modelo de unión obtenido para estas toxinas con estas dos especies es probable que sea aplicable también a otras especies.

7. La elevada variabilidad genética hallada en las poblaciones de *H. armigera* de la zona algodonera andaluza del sur de España se agrupa por la temporada de muestreo. La estructura poblacional de esta especie podría considerarse como una metapoblación que se reemplaza temporada tras temporada, después del cuello de botella provocado por los tratamientos insecticidas aplicados en la zona, por insectos procedentes de zonas alejadas al cultivo de origen. Este hecho apoya la idea de que estas poblaciones no están en equilibrio migración-deriva y que el flujo génico es el agente que explica la distribución de la diversidad genética en estas poblaciones a lo largo del tiempo.

8. Los resultados obtenidos con la cepa ISOC de *H. armigera*, conjuntamente con los obtenidos anteriormente, indican que la resistencia adquirida a la toxina Cry1Ac por esta cepa se debe principalmente a una disminución en la afinidad por la unión a Cry1Ac. Además, se han detectado otros genes candidatos que pueden estar contribuyendo parcialmente a la resistencia final en ISOC al modificar su expresión respecto a la cepa susceptible ANGR, tras la selección con Cry1Ac.

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Interaction of *Bacillus thuringiensis* Toxins with Larval Midgut Binding Sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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In 1996, Bt-cotton (cotton expressing a *Bacillus thuringiensis* toxin gene) expressing the Cry1Ac protein was commercially introduced to control cotton pests. A threat to this first generation of transgenic cotton is the evolution of resistance by the insects. Second-generation Bt-cotton has been developed with either new *B. thuringiensis* genes or with a combination of *cry* genes. However, one requirement for the "stacked" gene strategy to work is that the stacked toxins bind to different binding sites. In the present study, the binding of ^{125}I-labeled Cry1Ab protein (^{125}I-Cry1Ab) and ^{125}I-Cry1Ac to brush border membrane vesicles (BBMV) of *Helicoverpa armigera* was analyzed in competition experiments with 11 nonlabeled Cry proteins. The results indicate that Cry1Aa, Cry1Ab, and Cry1Ac competed for common binding sites. No other Cry proteins tested competed for either ^{125}I-Cry1Ab or ^{125}I-Cry1Ac binding, except Cry1Ja, which competed only at the highest concentrations used. Furthermore, BBMV from four *H. armigera* populations were also tested with ^{125}I-Cry1Ac and Cry1Ab to check the influence of the insect population on the binding results. Finally, the inhibitory effect of selected sugars and lectins was also determined. ^{125}I-Cry1Ac binding was strongly inhibited by *N*-acetylgalactosamine, sialic acid, and concanavalin A and moderately inhibited by soybean agglutinin. In contrast, ^{125}I-Cry1Ab binding was only significantly inhibited by concanavalin A. These results show that Cry1Ac and Cry1Ab use different epitopes for binding to BBMV.

Helicoverpa armigera (Hübner) is one of the most important insect pests in many cotton-producing countries, including Australia, India, and China. Larvae cause important economical losses to fiber and vegetable crops and have proven to be difficult to control by conventional means, in part because many pest populations have already evolved resistance to chemical insecticides (20, 21, 27). A rather recent alternative has been the introduction of cotton expressing a *Bacillus thuringiensis* insecticidal protein (Bt-cotton). *B. thuringiensis* is a gram-positive bacterium that produces crystalline proteins (delta-endotoxins, Cry toxins, or Cry proteins) during its sporulation phase of growth and has been used as a microbial insecticide for many years. Cry proteins have a narrow and specific spectrum of action against different pests, including coleopteran, dipteran, and lepidopteran species (34). Among the advantages of Bt crops are increased crop productivity, reduced production costs, selectivity and specificity against the target pests, conservation of biodiversity, and no demonstrated threat to the environment and human health (15, 29).

Heliothine species are rather tolerant to Cry toxins compared to many other lepidopteran pests (http://www.glfc.forestry.ca/bacillus). Despite the high number of Cry proteins discovered to date (>100) (5) (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html), just a few have proven to be effective for their control. Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab are the most toxic Cry proteins against *H. armigera* and *H. zea* and, along with Cry1Fa, against *Heliothis virescens* and *H. punctigera* (reviewed in reference 24). The introduction of the *cry1Ac* gene in selected lines of cotton has increased the productivity and has reduced the number of conventional insecticide applications (14). Bollgard cotton (Monsanto Co., Chesterfield, Mo.), a first-generation Bt-cotton that expresses Cry1Ac, has been commercialized since 1996 (32). In 2002 there were about 4.6 million Ha planted with Bt-cotton worldwide, and this figure is expected to increase significantly in the coming years as the adoption of this technology continues to expand in large established markets (such as China and Australia) and is commercially approved in new countries (such as India) (15).

A potential problem associated with this first generation of transgenic cotton expressing Cry1Ac is the possibility that the insect populations may evolve resistance to this toxin. In contrast to applications with chemical insecticides or with *B. thuringiensis* conventional products, the constitutive expression of the toxin in Bt-cotton allows very few escapes and thus exerts a strong selection pressure on the target population. For this reason, alternatives to Cry1Ac-cotton have been developed, such as Bt-cotton expressing other *B. thuringiensis* genes (a hybrid *cry1Ab/cry1Ac* gene, a *vip3* gene) or a combination of the *cry1Ac* gene with other genes (either *cry2Ab* or *cry1F*) (14). Starting in 2002, the first of such second-generation Bt-cotton, producing the Cry1Ac and Cry2Ab toxins, has been approved for commercial planting in Australia (15). The combined expression of these two toxins not only aims at preserving the effectiveness of Bt cotton in terms of delaying the evolution of resistance but also renders a more effective product against the major pests of this crop by combining the action of the two toxins.

One requirement for the "stacked" gene strategy to work is that the "stacked" toxins have a different mode of action (9, 10). Some cases of multiple Cry toxin resistance and cross-resistance have been shown to be due to alteration of a com-

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mon binding site (17, 23, 36). Altered binding is the best-characterized mechanism of resistance to Cry toxins and generally confers high resistance levels (10). By means of the study of the interaction of Cry proteins with the larval midgut, the aim of the present study was to determine the adequacy of the second generation Bt-cotton varieties expressing *cry* genes and to recommend or discourage new combinations of *cry* genes based on their predicted usefulness for insect resistance management. We have used ^{125}I-Cry1Ab and ^{125}I-Cry1Ac toxins and brush border membrane vesicles (BBMV) to propose a general model for Cry1Ab and Cry1Ac binding to specific sites in the larval midgut of *H. armigera*. We have determined the inhibitory effect of selected sugars and lectins on Cry1Ab and Cry1Ac binding and whether midgut binding sites are shared between these toxins or by any of nine other Cry proteins selected for being among the most active toxins against lepidopterans. Furthermore, we have tested BBMV from four different *H. armigera* populations to check the influence of the insect population on the binding site model.

MATERIALS AND METHODS

Production and purification of *B. thuringiensis* Cry proteins. The recombinant *B. thuringiensis* strains used in the present study (Cry protein they produced) were as follows: EG1273 (Cry1Aa), EG7077 (Cry1Ab), EG11070 (Cry1Ac), EG11916 (Cry1Ba), EG1081 (Cry1Ca), EG7300 (Cry1Da), EG11069 (Cry1Fa), and EG7279 (Cry1Ja) (Ecogen, Inc., Langhorne, Pa.) and EG7543 (Cry2Aa1) and EG7699 (Cry2Ab2) (Monsanto Co., Chesterfield, Mo.). Purified and trypsin activated Cry9Ca (the Lys mutant) (22) was kindly provided by Jeroen Van Rie (Bayer BioScience N.V., Ghent, Belgium).

Recombinant *B. thuringiensis* strains were grown in CCY medium (35) supplemented with the appropriated antibiotic for 48 h at 29°C. Spores and crystals were separated by centrifugation at 9,700 × *g* for 12 min and then washed four times with 1 M NaCl–10 mM EDTA. The pellet was finally suspended in 10 mM KCl. Crystal solubilization was carried out in carbonate buffer (50 mM Na_2CO_3, 0.1 M NaCl; 10 mM dithiothreitol; pH 10.5) for 1 h with constant shaking. After centrifugation to eliminate insoluble material, protoxin activation was carried out with trypsin (type I from bovine pancreas; Sigma Chemical Co., St. Louis, Mo.), and the completion of the reaction was checked by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Trypsin activated Cry proteins were purified by anion-exchange chromatography with the MonoQ HR5/5 column by fast-protein liquid chromatography (Pharmacia, Uppsala, Sweden). Protein was quantified by the Bradford assay (3) with bovine serum albumin (BSA) as a standard.

Iodination of Cry1A toxins. Purified Cry1Ab and Cry1Ac toxins were labeled with ^{125}I by the method of chloramine-T (37). Specific activities of the labeled proteins were analyzed by an enzyme-linked immunosorbent sandwich assay (37). The specific activities for ^{125}I-Cry1Ab and ^{125}I-Cry1Ac were 2.9 and 24 mCi/mg, respectively.

Insect populations, midgut isolation, and BBMV preparation. One laboratory *H. armigera* population and three field populations were used. The laboratory population had been maintained in the laboratory for several years on an artificial diet. The field populations were from the provinces of Barcelona (Catalonia region, Northeastern Spain) and Seville and Córdoba (both from the Andalusia region, Southern Spain). The Andalusian populations were from the cotton growing area of Spain but not the Catalan population. Insects were reared at 25°C, in 60% relative humidity, and with a 16-8 (light-dark) photoperiod. Midguts were dissected from last-instar (L5) larvae, washed in ice-cold MET buffer (250 mM mannitol, 17 mM Tris-HCl, 5 mM EGTA [pH 7.5]), frozen in liquid nitrogen, and kept at −80°C until required. BBMV were prepared by the $MgCl_2$ precipitation method (39), and the protein concentration was determined by the method of Bradford (3) with BSA as a standard.

Binding assays. Binding assays were performed at room temperature in a final volume of 0.1 ml of binding buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl [pH 7.4], 0.1% BSA). Bound ligand was separated from free ligand by centrifugation at 11,000 × *g* for 10 min, followed by two washes with 0.5 ml of cold binding buffer (12). Radioactivity in the pellet was measured directly in the microtubes in a gamma counter (Compugamma 1282; LKB). Appropriate conditions for carrying out the binding assays were determined in preliminary experiments. The incubation times were 1 h for ^{125}I-Cry1Ab and 30 min for ^{125}I-Cry1Ac, and the amount of labeled toxin in the 0.1-ml assay mixture was 1 ng for ^{125}I-Cry1Ab and 0.14 ng for ^{125}I-Cry1Ac. The appropriate BBMV concentration to be used in the competition assays was determined for each BBMV preparation.

Homologous and heterologous competition experiments were done by incubating the labeled Cry toxin in the presence of increasing amounts of nonlabeled competitor and the appropriate BBMV concentration. For the ^{125}I-Cry1Ab assays, the BBMV protein concentration was 0.04 mg/ml for the laboratory population. For the ^{125}I-Cry1Ac assays, the BBMV protein concentration was 0.045 mg/ml for the laboratory population, 0.02 mg/ml for the Barcelona population, and 0.03 mg/ml for the Seville and Córdoba populations. All binding experiments were repeated two to three times.

Inhibition of ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding by sugars and lectins. All sugars and lectins were from Sigma. The sugars tested were *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), *N*-acetylneuraminic acid (sialic acid), and α-D-mannose. The lectins tested were soybean agglutinin (SBA, which binds GalNAc), wheat germ agglutinin (WGA, which binds, from higher to lower affinity, GlcNAc, sialic acid, and GalNAc), and concanavalin A (ConA, which binds α-D-mannose and, with lower affinity, α-D-glucose). Inhibition experiments with sugars were performed as in the binding assays described above but with a preincubation of the labeled toxin with the sugar for 45 min at room temperature, prior to the start of the assay with the addition of the BBMV. With lectins, the same protocol was used except that the preincubation step was done with BBMV and the assay was started with the addition of the labeled toxin. Inhibition experiments were replicated two to three times.

Data analysis. The analyses of binding data to obtain the dissociation constants and the concentration of binding sites was performed by using the LIGAND program (28). Graphic representations and curve fittings were done with the Graphpad Prism version 3.02 for windows (Graphpad Software, San Diego, Calif. [www.graphpad.com]).

RESULTS

Binding of ^{125}I-Cry1Ac to BBMV from different populations of *H. armigera*. BBMV from a laboratory population and three field populations were tested for their binding characteristics to ^{125}I-Cry1Ac. The results of competition experiments were qualitatively similar in all four cases, either with Cry1Ac as a homologous competitor or with Cry1Ab as a heterologous competitor (Fig. 1). The homologous competition assay indicated saturable binding of ^{125}I-Cry1Ac, since increasing concentrations of nonlabeled Cry1Ac decreased the fraction of ^{125}I-Cry1Ac binding to the BBMV. The heterologous competition assay showed that Cry1Ab competed for ^{125}I-Cry1Ac binding, indicating that these two toxins share the same binding sites. However, there was always some ^{125}I-Cry1Ac binding that could not be displaced by Cry1Ab even at high concentrations.

Quantitative analysis of the competition curves showed that both curves could only be fitted to a single-site model equation. The estimated dissociation constants (K_d) and concentrations of binding sites (R_t) for the four populations are shown in Table 1. K_d values were in the ranges of 0.92 to 4.2 nM for ^{125}I-Cry1Ac and 10 to 43 nM for Cry1Ab. R_t values ranged from 19 to 59 pmol/mg of BBMV protein for ^{125}I-Cry1Ac and from 13 to 23 pmol/mg of BBMV protein for Cry1Ab. The K_d values for ^{125}I-Cry1Ac were always lower than the K_d values estimated for Cry1Ab, indicating a lower affinity of Cry1Ab for Cry1Ac binding sites. The R_t values for ^{125}I-Cry1Ac were either equal to or higher than their respective R_t values for Cry1Ab.

The effect of other Cry proteins as heterologous competitors on ^{125}I-Cry1Ac binding was tested with BBMV from the laboratory population and a field population (Barcelona). Cry1Aa and Cry1Ja were tested with both BBMV, and the results were essentially identical (Fig. 2), with both proteins competing for

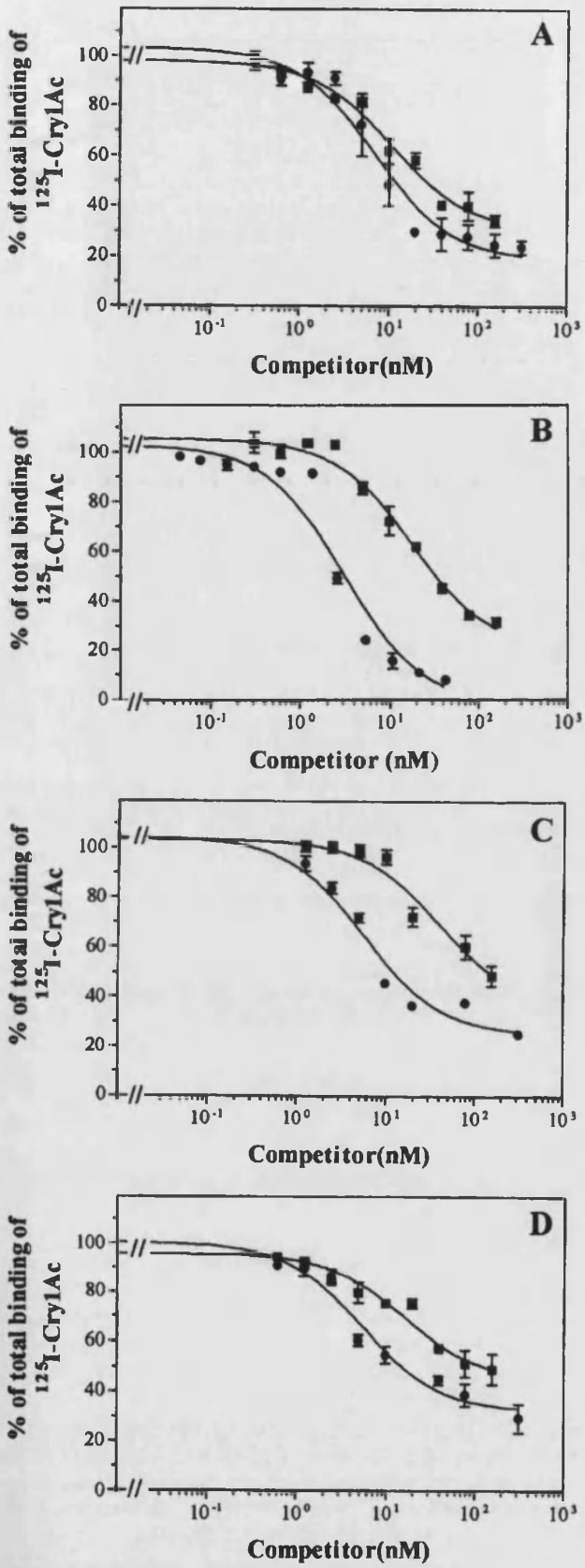

FIG. 1. Binding of ^{125}I-Cry1Ac at increasing concentrations of Cry1Ac (●) and Cry1Ab (■) to BBMV from four different populations of *H. armigera*: laboratory (A), Barcelona (B), Seville (C), and Córdoba (D).

TABLE 1. Dissociation constants (K_d) and concentration of binding sites (R_t) for Cry1Ab and Cry1Ac binding to BBMV from different populations of *H. armigera*[a]

Population	Mean binding parameters of Cry toxins ± SEM for:			
	Cry1Ac		Cry1Ab	
	K_d (nM)	R_t (pmol/mg)	K_d (nM)	R_t (pmol/mg)
Laboratory	1.5 ± 0.4	22 ± 4	10 ± 2	15 ± 3
Barcelona	0.92 ± 0.12	59 ± 4	17 ± 4	23 ± 5
Seville	2.9 ± 0.7	53 ± 9	43 ± 8	13 ± 9
Córdoba	4.2 ± 0.9	19 ± 4	34 ± 8	19 ± 4

[a] Binding parameters for Cry1Ac were obtained from homologous competition data. Binding parameters for Cry1Ab were obtained from heterologous competition data by using ^{125}I-Cry1Ac as the labeled ligand. The results are the means of two experiments.

^{125}I-Cry1Ac binding sites only at relatively high concentrations. The rest of the proteins (Cry1Ba, Cry1Ca, Cry1Da, Cry1Fa, Cry2Aa, Cry2Ab, and Cry9Ca) did not compete for ^{125}I-Cry1Ac binding sites (Fig. 3).

Binding of ^{125}I-Cry1Ab to *H. armigera BBMV*. Homologous and heterologous competition experiments with ^{125}I-Cry1Ab were performed with BBMV from the laboratory population (Fig. 4). The homologous competition curve indicated saturable binding, and its quantitative analysis estimated a K_d = 26 ± 5 nM and an R_t = 62 ± 11 pmol/mg of BBMV protein. From all of the heterologous Cry proteins tested (Cry1Aa, Cry1Ac, Cry1Ba, Cry1Ca, Cry1Da, Cry1Fa, Cry1Ja, Cry2Aa, Cry2Ab, and Cry9Ca), only Cry1Aa, Cry1Ac, and Cry1Ja competed for ^{125}I-Cry1Ab binding, although the latter only did it at very high concentrations (Fig. 4). Cry1Ac completely displaced ^{125}I-Cry1Ab binding, indicating that all ^{125}I-Cry1Ab binding sites are recognized by Cry1Ac. In contrast, at the highest concentration tested, Cry1Aa was not able to completely displace ^{125}I-Cry1Ab binding.

^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding inhibition by sugars and lectins. Different sugars and lectins were tested for their ability to inhibit ^{125}I-Cry1Ab and ^{125}I-Cry1Ac binding to BBMV. The results indicated a different binding inhibition pattern of these toxins (Fig. 5). ConA was the only product that strongly inhibited ^{125}I-Cry1Ab binding (62%). SBA and high concentrations of GalNAc and GlcNAc (250 mM) had a minor influence on this toxin binding (from 11 to 19% inhibition). WGA, sialic acid, and mannose had no significant effect. However, ^{125}I-Cry1Ac binding was strongly inhibited by ConA, GalNAc, and sialic acid (from 60 to 81%) and moderately inhibited by SBA (35%). WGA, GlcNAc, and mannose had only a minor influence on the binding inhibition of this toxin.

DISCUSSION

Reported data on the susceptibility of *H. armigera* to individual Cry proteins generally agree on Cry1Ab and Cry1Ac being among the most toxic ones (along with Cry2Aa and Cry2Ab), although their relative potencies varied among the different studies (4, 24). Because one of the aims of the present study was to propose a general model for Cry1Ab and Cry1Ac binding to specific sites in the larval midgut of this species, we first checked the influence of working with different populations on the binding results. The qualitative results obtained

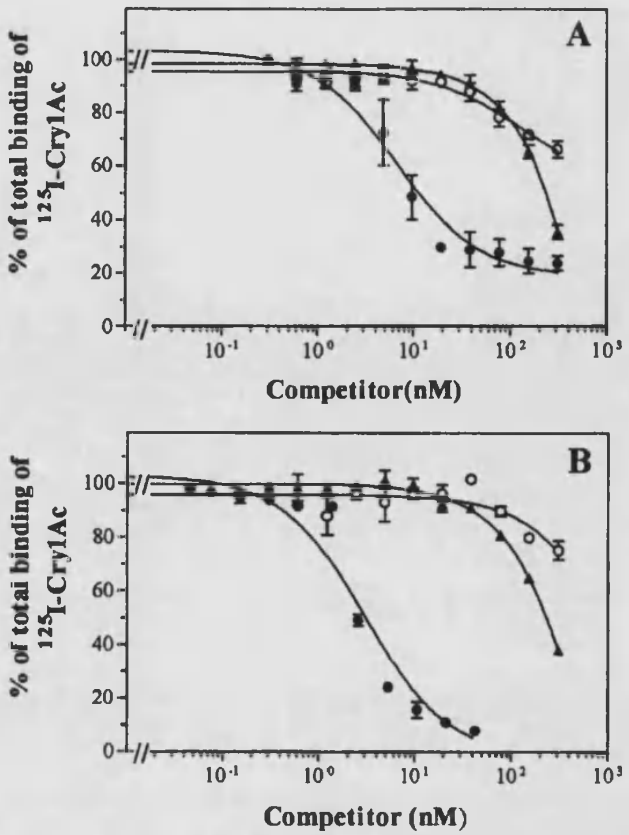

FIG. 2. Binding of ^{125}I-Cry1Ac at increasing concentrations of Cry1Ac (●), Cry1Aa (▲), and Cry1Ja (○) to BBMV from the laboratory (A) and Barcelona (B) populations. The homologous competition curve is the same as in Fig. 1 and is displayed here as a reference.

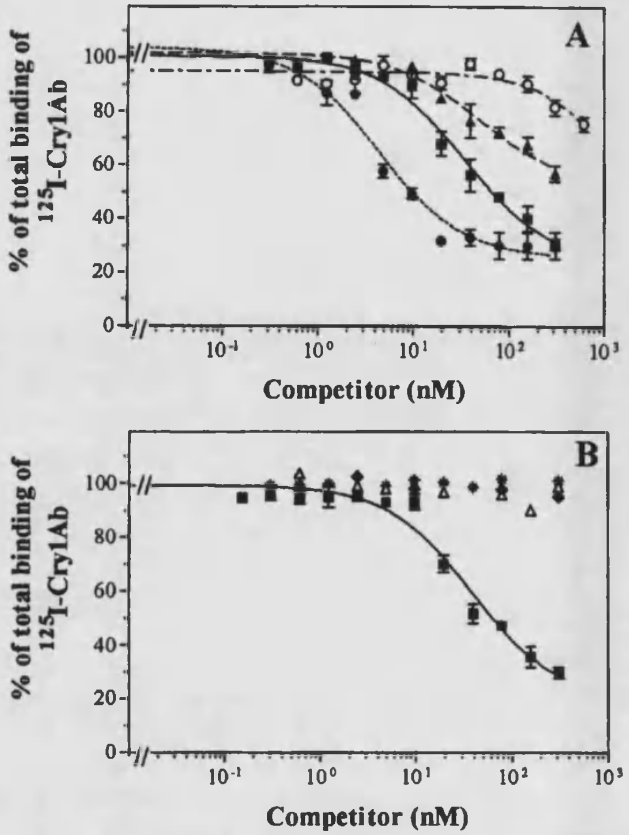

FIG. 4. Binding of ^{125}I-Cry1Ab to BBMV from the laboratory population at increasing concentrations of competitor. (A) Cry1Ab (■, solid line), Cry1Aa (▲, broken line), Cry1Ac (●, dotted line), and Cry1Ja (○, dashed-dotted line). (B) Cry1Ab (■, solid line), Cry1Fa (△), Cry2Ab (✱), and Cry9Ca (◆). Heterologous data with Cry1Ba, Cry1Ca, Cry1Da, and Cry2Aa are not shown, but they were essentially similar to those displayed.

with BBMV from the four populations were essentially identical, although quantitative estimates gave K_d and R_t values slightly different between populations (the greatest difference was found in the K_d for ^{125}I-Cry1Ac). In all populations Cry1Ac bound with higher affinity (lower K_d) than Cry1Ab did.

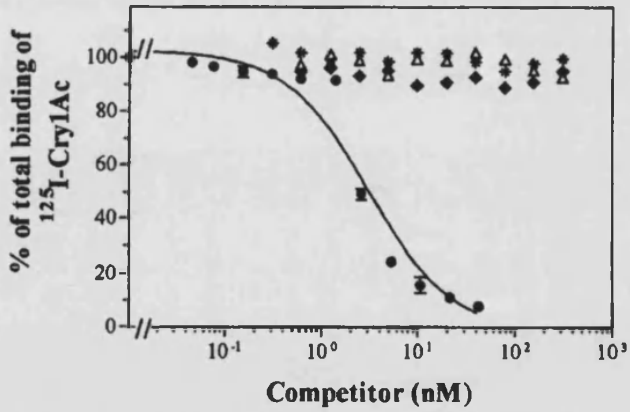

FIG. 3. Binding of ^{125}I-Cry1Ac at increasing concentrations of Cry1Ac (●), Cry1Fa (△), Cry2Ab (✱), and Cry9Ca (◆) to BBMV from the laboratory population. The homologous competition curve is the same as in Fig. 1 and 2 and is displayed here as a reference. Heterologous data with Cry1Ba, Cry1Ca, Cry1Da, and Cry2Aa are not shown, but they were essentially similar to those displayed.

The K_d value for Cry1Ab obtained from homologous competition data confirmed its significantly lower affinity than Cry1Ac (~20-fold). Therefore, our binding results would agree with Cry1Ac being more toxic than Cry1Ab to *H. armigera* larvae, although it is well known that there is not always a direct relationship between binding affinity and toxicity (7, 38).

To propose a general model for binding of Cry proteins to specific sites in the larval midgut of *H. armigera*, we have performed heterologous competition experiments with ^{125}I-Cry1Ab and ^{125}I-Cry1Ac labeled toxins and a set of 11 nonlabeled Cry proteins. These Cry proteins were selected for being among the most active against lepidopterans, although only Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab have been reported to be toxic for *H. armigera* (4, 24) (to our knowledge, Cry1Ja has never been tested). Only four of them competed for Cry1Ab and Cry1Ac binding sites: Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Ja. The simplest model that fits these results would be a single binding site shared by Cry1Ab and Cry1Ac, to which Cry1Aa and Cry1Ja also bind but not the other toxins. However, this model cannot explain why Cry1Ab does not completely compete for binding of ^{125}I-Cry1Ac (Fig. 1) or why the ^{125}I-Cry1Ab versus Cry1Aa heterologous curve was far from reaching the bottom plateau of the homologous curve at the highest concentration tested (Fig. 4A). It is worth noting the

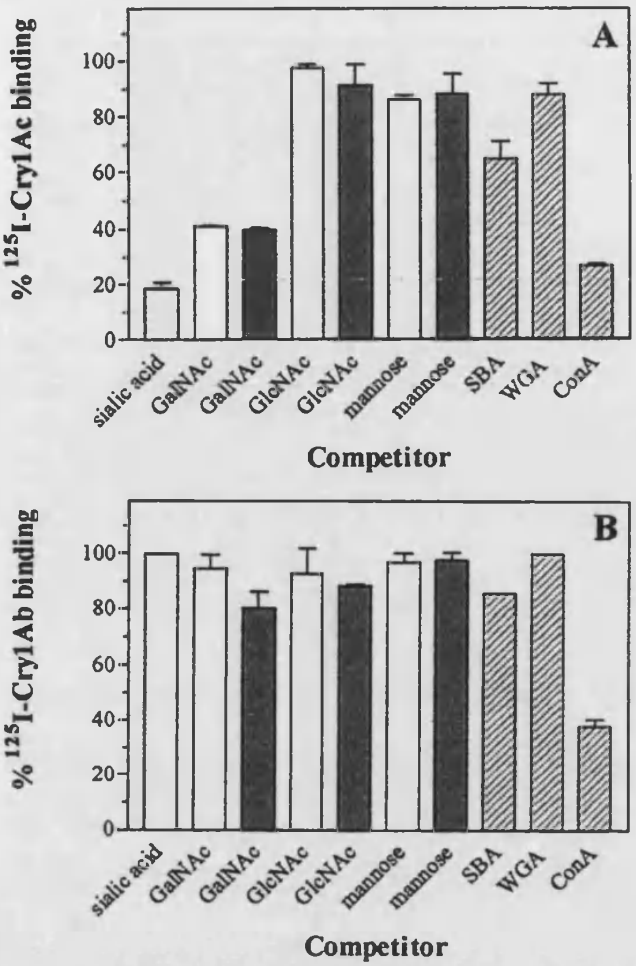

FIG. 5. Effect of sugars and lectins on ^{125}I-Cry1Ac (A) and ^{125}I-Cry1Ab (B) binding to *H. armigera* BBMV. Inhibition experiments with sugars were performed with a preincubation of the labeled protein with the sugar at 25 mM (□) or 250 mM (▩) for 45 min at room temperature prior to the start of the assay with the addition of the BBMV. With lectins (at 50 μg/ml [▨]), the preincubation step was done with BBMV and the assay was started with the addition of the labeled protein.

striking similarities of our heterologous competition results with those previously published for *H. virescens* with the Cry1A proteins (37). In this species, a three-site model was proposed based on the qualitative data from the competition curves. In this model, one population of binding sites (population A) is shared by the three toxins, a second population (B) binds Cry1Ab and Cry1Ac but not Cry1Aa, and a third population (C) has only affinity for Cry1Ac. The fact that the quantitative analysis of the data fitted a one-site model could be explained, assuming that the affinity of Cry1Ab and Cry1Ac for their respective populations of binding sites was similar and, thus, the occurrence of more than one binding site could not be inferred by the quantitative treatment of the data. Our results with *H. armigera* could also be explained by a three-site model, which we cannot discard as a plausible model for our data. The fact that neither Cry1Ab nor Cry1Aa could completely compete for binding of ^{125}I-Cry1Ac (Fig. 1 and 2) suggests that Cry1Ac has at least two different binding sites, one of them not being recognized by the heterologous proteins (equivalent to *H. virescens* population of binding sites C). In the reciprocal experiment, Cry1Ac completely competed for binding of ^{125}I-Cry1Ab (Fig. 4A), which would indicate that all binding sites of the latter are recognized by Cry1Ac. The fact that Cry1Aa did not completely compete for binding of ^{125}I-Cry1Ab (Fig. 4A) suggests two different binding sites for Cry1Ab (equivalent to *H. virescens* binding site populations A and B). We are aware that this three-site model could still be a simplification of the actual toxin-binding interactions that take place in vivo.

It is interesting that in all lepidopteran species tested so far, Cry1Ac and Cry1Ab always share binding sites in radioligand competition experiments, sometimes even an apparently single binding site (2, 6, 7, 8, 18, 37, 38). It has been known for some time that Cry1Ac binding to BBMV is inhibited by GalNAc (19, 25, 26); however, Cry1Ab binding has been found not to be inhibited by this sugar (25, 26). Our results show that this is also the case with *H. armigera*, since ^{125}I-Cry1Ac binding was strongly inhibited by GalNAc, whereas ^{125}I-Cry1Ab was minimally affected. This same pattern of inhibition was also found with sialic acid, indicating that Cry1Ac, but not Cry1Ab, requires sugar residues to bind to BBMV in *H. armigera*. This binding inhibition is specific, since other sugars, such as GlcNAc and mannose, had little or no effect on the binding of either toxin. SBA, a lectin that has affinity for GalNAc, produced more inhibition of ^{125}I-Cry1Ac than ^{125}I-Cry1Ab binding. In contrast, ConA, which binds specifically to mannose and glucose residues, inhibited ^{125}I-Cry1Ac and ^{125}I-Cry1Ab binding similarly, suggesting that these sugar residues are close to the epitopes to which these toxins bind, although mannose residues themselves would not be used for binding.

The fact that Cry1Ab and Cry1Ac share binding sites in radioligand competition experiments seems to be in contradiction to the binding inhibition results with sugars. Furthermore, results from ligand blots have generally shown that, under denaturing conditions, Cry1Ac and Cry1Ab bind to different BBMV proteins (16, 30, 31). This paradox can be explained by proposing that binding sites in BBMV are oligomeric complexes of glycosylated membrane proteins (30). Binding of Cry1Ac and Cry1Ab could take place through different epitopes of the multimeric receptor and at the same time hinder binding of the heterologuos toxin by impeding access to a nearby site. This does not exclude the possibility that both toxins could also share identical epitopes, but in this case full binding might require anchorage of the Cry protein to both the shared and the nonshared epitopes. Support for the latter situation can be found in the biochemical analysis of resistant strains of *Plutella xylostella* for which, for the common Cry1A binding site, some mutations have been found to preclude binding of only Cry1Ab and others to preclude binding of more than one Cry1A toxin (2, 33, 36, 40).

Although Cry1Fa is not very toxic to *H. armigera* (24) and there are as yet no data reported for the toxicity of Cry1Ja, from an academic standpoint it is interesting to discuss the results obtained with these two proteins in competition experiments with the Cry1A proteins. In the seven lepidopteran species tested thus far, Cry1Ja competes for binding with Cry1Ac (13, 16). Similarly, in two species tested, Cry1Fa competes for binding with Cry1A proteins (11, 16). Based on these results, the alteration of a common receptor has been pro-

posed to be the main mechanism of cross-resistance to these toxins in strains of *H. virescens* (16) and *P. xylostella* (2, 11, 13) that had been selected with Cry1A mixtures. Our results with these two toxins and BBMV from *H. armigera* contrast with those published for other species. Cry1Fa does not compete at all (this may contribute to its low toxicity to this species) and Cry1Ja only competes at very high concentrations, indicating very low affinity for the Cry1A binding sites and little or no biological significance of this competition in *H. armigera*.

The lack of competition of Cry2Ab protein for the Cry1Ac binding sites is relevant since the *cry2Ab* gene has been introduced, in combination with the *cry1Ac* gene, in the so-called second generation of Bt cotton (14). The purpose of using "stacked" *cry* genes in Bt plants is not only to broaden the pest spectrum but also to be used as a strategy for resistance management whenever the Cry proteins they produce do not share the same mechanism of action (9, 10). According to our results, Cry2Ab does not share binding sites with Cry1Ac. Since binding site alteration is generally the mechanism that confers higher levels of resistance and cross-resistance to Cry toxins (10), it is unlikely that single mutations in field populations will be found that, by affecting the binding site for one of the "stacked" toxins, would confer resistance to the second Cry toxin in these plants. A result that supports our prediction is the one reported recently for an Australian population of *H. armigera*, which was selected for Cry1Ac resistance (1). The population was cross-resistant to Cry1Ab but not to Cry2Aa or Cry2Ab, and the resistant insects had lost the capacity of binding Cry1Ac. This result also supports the biological relevance of the binding data obtained in our study.

The model for Cry toxin binding proposed for *H. armigera* strongly discourages combination of *cry1A* genes in plants to be protected against this pest because of the possibility of selecting for alleles that could confer resistance to more than one Cry1A toxin by the alteration of the common binding site. According to the model, other Cry proteins active against this pest, such as Cry2Aa or Cry2Ab, can be used in combination with either Cry1Ac or Cry1Ab without the threat of selecting for resistance alleles conferring major protection to these two families of toxins. Since *H. armigera* is a polyphagous pest, this recommendation not only applies to "stacked" genes in Bt-cotton but also to other crops that could be developed in the future either with a combination of *cry* genes or simply with just one *cry* gene targeting insect populations with a previous history of exposure to *B. thuringiensis* sprays.

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Use of *Bacillus thuringiensis* Toxins for Control of the Cotton Pest *Earias insulana* (Boisd.) (Lepidoptera: Noctuidae)

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Thirteen of the most common lepidopteran-specific Cry proteins of *Bacillus thuringiensis* have been tested for their efficacy against newly hatched larvae of two populations of the spiny bollworm, *Earias insulana*. At a concentration of 100 μg of toxin per milliliter of artificial diet, six Cry toxins (Cry1Ca, Cry1Ea, Cry1Fa, Cry1Ja, Cry2Aa, and Cry2Ab) were not toxic at all. Cry1Aa, Cry1Ja, and Cry2Aa did not cause mortality but caused significant inhibition of growth. The other Cry toxins (Cry1Ab, Cry1Ac, Cry1Ba, Cry1Da, Cry1Ia, and Cry9Ca) were toxic to *E. insulana* larvae. The 50% lethal concentration values of these toxins ranged from 0.39 to 21.13 μg/ml (for Cry9Ca and Cry1Ia, respectively) for an *E. insulana* laboratory colony originating from Egypt and from 0.20 to 4.25 μg/ml (for Cry9Ca and Cry1Da, respectively) for a laboratory colony originating from Spain. The relative potencies of the toxins in the population from Egypt were highest for Cry9Ca and Cry1Ab, and they were both significantly more toxic than Cry1Ac and Cry1Ba, followed by Cry1Da and finally Cry1Ia. In the population from Spain, Cry9Ca was the most toxic, followed in decreasing order by Cry1Ac and Cry1Ba, and the least toxic was Cry1Da. Binding experiments were performed to test whether the toxic Cry proteins shared binding sites in this insect. ^{125}I-labeled Cry1Ac and Cry1Ab and biotinylated Cry1Ba, Cry1Ia, and Cry9Ca showed specific binding to the brush border membrane vesicles from *E. insulana*. Competition binding experiments among these toxins showed that only Cry1Ab and Cry1Ac competed for the same binding sites, indicating a high possibility that this insect may develop cross-resistance to Cry1Ab upon exposure to Cry1Ac transgenic cotton but not to the other toxins tested.

Bacillus thuringiensis (Berliner) is a soil bacterium that produces a diversity of Cry proteins that are selectively toxic against a wide variety of insect pests (6). Synthetic *cry* genes from *B. thuringiensis*, modified for plant-preferred codon usage, have been introduced in a number of major crops (referred to as Bt crops) such as maize, cotton, and potato to make them insect resistant (28). Bt cotton was commercially released in the United States in 1996 and subsequently in several countries including Argentina, Australia, China, Colombia, Indonesia, Mexico, South Africa, and India (42). Cotton is currently the third most important transgenic crop in terms of surface area (after soybean and maize), involving nine million hectares in 2004 (11% of global genetically modified area). The countries that devote the largest area to Bt cotton are the United States and China, where more than half of their planted cotton is the result of biotechnological engineering (23). The European Union has recently opened its market to several products, some of them derived from Bt cotton, under the regulation on genetically modified food and feed (EC regulation no. 1829/2003; EC regulation no. 258/97-Art.5). However, no variety of Bt cotton has yet been approved for commercial planting in Europe.

The primary pests targeted by Bt cotton technology in North America are the tobacco budworm, *Heliothis virescens*, the cotton bollworm, *Helicoverpa zea*, and the pink bollworm, *Pectinophora gossypiella*. Throughout the rest of the world, *Helicoverpa armigera* is a primary pest with high resistance to organophosphate and pyrethroid insecticides that causes crop losses comparable to those caused by *H. virescens* in North America (29). According to the susceptibility of the above-described species to different lepidopteran-specific *B. thuringiensis* toxins, Cry1Ac cotton was selected as the best choice for commercial release. The second generation of Bt cotton combines Cry1Ac with a second *B. thuringiensis* toxin (Cry2Ab) and provides growers with a product that offers a broader spectrum of pest control and reduced chances of insects developing *B. thuringiensis* resistance (12, 45). Therefore, most commercially planted insect-resistant cotton contains Cry1Ac (in China, Bt cotton has been transformed to express a Cry1Ab-Cry1Ac hybrid toxin), which undoubtedly will pose an important selection pressure on the lepidopteran populations in the cotton ecosystem.

The genus *Earias* is widely distributed in the Old World and Australasia, and some are pests of considerable importance in many of cotton-growing countries of Africa and Asia. The spiny bollworm, *Earias insulana* (Boisduval), has an extremely wide range and is found throughout most of Africa and the Mediterranean region and eastwards to India, China, and Southeast Asia (38).

This species is an important component of the lepidopteran pest complex of cotton in some regions in Spain (7), Egypt (18), Israel (21), India, and Pakistan (25). Although it is a pest of cotton, it can also grow on other alternative host plants (2). Spiny bollworm causes damage by attacking terminal shoots, flower buds, and green bolls. The most serious damage to cotton is caused when larvae bore into the bolls, destroying the

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fiber, consuming seeds, and producing putrefaction due to the accumulation of feces and fungus. In some regions, if the attack is not controlled, *E. insulana* larvae can destroy all the cotton bolls in the field.

Virtually no quantitative data are available on the efficacy of single purified *B. thuringiensis* Cry proteins against *E. insulana*. In the present study, the insecticidal activity of 13 of the most common lepidopteran-specific Cry proteins was determined in terms of 50% lethal concentration (LC_{50}) for neonate larvae of *E. insulana*. Assessment of the relative potency of these individual *B. thuringiensis* proteins is an important step in the determination of their insecticidal potential for control of this pest in cotton. Furthermore, since continuous exposure to Cry1Ac may result in the development of resistance to this toxin, we have addressed the possibility of Cry1Ac-resistant insects becoming resistant to other *B. thuringiensis* toxins. Considering that most cases of high levels of resistance to Cry proteins have been due to the alteration of a midgut membrane receptor (12), competition experiments between Cry1Ac and other active Cry proteins were performed to determine which toxins share a target site and therefore which toxins could lose their insecticidal properties if populations of *E. insulana* with an altered Cry1Ac binding site become common.

MATERIALS AND METHODS

Insects. Two populations of *E. insulana* were used in this study. One colony was established in Spain from pupae obtained from a continuous laboratory culture maintained since 2001 at the Plant Protection Institute, Giza, Egypt. A second colony was started with field-collected larvae from cotton fields in Córdoba, Spain, during the summer of 2002. These colonies were continuously maintained in our laboratory in a growth chamber at 27 ± 1°C and 60% relative humidity, with a photoperiod of 14 h of light and 10 h of dark and with an artificial diet (16).

***Bacillus thuringiensis* Cry proteins.** The following Cry proteins were produced in recombinant *B. thuringiensis* strains (strain names are given in parentheses) expressing just one type of Cry protein: Cry1Aa3 (EG1273), Cry1Ab3 (EG7077), Cry1Ac4 (EG11070), Cry1Ba (EG19916), Cry1Ca2 (EG1081), Cry1Da (EG7300), Cry1Ea (EG11901), Cry1Fa1 (EG11096), and Cry1Ja1 (EG7279) (all obtained from Ecogen Inc., Langhorne, Pa.) Cry2Aa1 (EG7543) and Cry2Ab2 (EG7699) (supplied by Monsanto Co., Chesterfield, Mo.). Protoxin solubilization, trypsin activation, and toxin chromatography purification were performed as previously described (10). Cry1Ia7 was produced in recombinant *Escherichia coli* cultures in 2× tryptone yeast extract medium at 37°C and with constant shaking until exponential growth was achieved. The expression of the protein was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) and was purified by nickel columns. Cry1Ia7 was not trypsin activated, because this resulted in a loss of toxic activity for *E. insulana* larvae. Purified and activated Cry9Ca toxin (Lys mutant) was obtained from Bayer BioScience (N.V. Gent, Belgium). Protein concentration was determined by the Bradford method (5) using bovine serum albumin as a standard.

Insect bioassays. Insect bioassays involved incorporating the toxin protein into the insect artificial diet (30). First, the susceptibility of *E. insulana* larvae to each Cry toxin tested was determined at a high protein concentration (100 μg/ml) by incorporating the toxin into the diet that was fed to 25 neonate larvae. A second experiment involved determining the LC_{50} for active Cry proteins. The concentration range used for each Cry protein was determined in preliminary bioassays. The toxin was mixed with the artificial diet of the insect when it reached 50°C and then dispensed into 24-multiwell plates. A total of 30 neonate larvae were treated with each protein concentration, and a range of five concentrations (ranging from 40 μg/ml to 0.10 μg/ml) was used for each toxin. The bioassay was performed three times. Control insects were fed artificial diet without toxin. The multiwell plates were incubated at 25°C and 60% relative humidity with a 14-h light/10-h dark photoperiod. Mortality was recorded after 6 days. Concentration-mortality data were subjected to probit regression analysis (13) in the POLO-PC program (27). To assure that Cry proteins which showed no toxicity to *E. insulana* were not degraded, several of these proteins were also bioassayed against larvae of known susceptible species, namely, *Spodoptera exigua*, *Plutella xylostella*, and *Lobesia botrana*.

Toxin purification and labeling. For binding assays, trypsin-activated toxins (except Cry1Ia) were further purified by anion-exchange chromatography with the Mono Q HR 5/5 column using a fast protein liquid chromatograph (Pharmacia, Uppsala, Sweden). Cry1Ab and Cry1Ac were labeled with ^{125}I by the chloramine-T method (47). Specific activities of the radio-iodinated toxins were analyzed by a sandwich enzyme-linked immunosorbent assay (47). The specific activities for ^{125}I-labeled Cry1Ab (^{125}I-Cry1Ab) and ^{125}I-Cry1Ac were 2.9 mCi/mg and 1.8 mCi/mg, respectively. Cry1Ba, Cry1Ia, and Cry9Ca labeling was performed by biotinylation (Amersham Biosciences, N.J.) according to the manufacturer's instructions.

Midgut isolation and BBMV preparation. Final-instar larvae (L5) were dissected to obtain the whole insect midguts, which were immediately frozen in liquid nitrogen and stored at −80°C until required. Brush border membrane vesicles (BBMV) were prepared by the $MgCl_2$ precipitation method (48).

Binding experiments with ^{125}I-Cry1Ac and ^{125}I-Cry1Ab. Binding experiments with *E. insulana* BBMV and ^{125}I-Cry1Ac were performed as previously described (10) using the following conditions adapted for the spiny bollworm: 0.14 ng of ^{125}I-Cry1Ac, 0.05 mg/ml BBMV, and a 1-h incubation time at room temperature in a final volume of 0.1 ml binding buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4, 0.1% bovine serum albumin). Competition experiments were carried out with increasing concentrations of unlabeled Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ia, and Cry9Ca. Radioactivity incorporated into the BBMV after washing twice with cold binding buffer was measured directly in the microtubes in which the assays were performed by using a gamma counter (Compugamma 1282; LKB).

^{125}I-Cry1Ab binding and competition assays with unlabeled Cry1Ab and Cry1Ac were performed as described above for ^{125}I-Cry1Ac under the appropriate conditions (1 ng of ^{125}I-Cry1Ab and 0.15-mg/ml BBMV concentration).

Binding data analyses to obtain the dissociation constants (K_d) and the concentrations of binding sites (R_t) were performed from the homologous competition experiments using the LIGAND program (31). Graphic representations and curve fitting were performed using the Graphpad Prism v.4.0 for Windows package (Graphpad Software, San Diego, Calif.).

Binding experiments with biotinylated toxins. Binding experiments with the biotinylated toxins were carried out by incubating 25 μg of BBMV with the appropriate amount of labeled toxin (10 ng for Cry1Ba, 14 ng for Cry1Ia, and 20 ng for Cry9Ca). An excess of at least 400-fold of unlabeled toxin was added in the homologous and heterologous competition experiments. After centrifuging the binding mixture, the pellet in the microtube containing the toxin bound to the BBMV was suspended with 10 μl electrophoresis buffer and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then electrotransferred onto a Hybond nitrocellulose membrane (Amersham) and blocked with 3% ECL blocking agent (Amersham) in TPBS buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4, 0.1% Tween 20). Biotinylated toxin bound to the BBMV was detected by incubating the membrane with streptavidin conjugated to alkaline phosphatase (Roche Diagnostics, Ind.) in TPBS according to the manufacturer's recommendations. The membrane was developed with an NBT/BCIP solution (Roche) in Genius 3 color buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5).

RESULTS

Toxicity of Cry toxins. *E. insulana* larvae showed different degrees of susceptibility to 13 of the most common lepidopteran-specific Cry proteins produced by *B. thuringiensis*. At the concentration tested (100 μg of toxin per milliliter of artificial diet), the percent mortality obtained with six of these Cry toxins (Cry1Ca, Cry1Ea, Cry1Fa, Cry1Ja, Cry2Aa, and Cry2Ab) did not differ significantly from the mortality of the control larvae, reared on a toxin-free diet, for the Egyptian and Spanish insect colonies assayed. Consequently, when a Cry toxin caused no mortality in *E. insulana*, it was attributed to the lack of toxic activity of that particular Cry toxin or a reduced feeding rate on toxin-contaminated diet. No mortality was observed for Cry1Aa, Cry1Ja, and Cry2Aa, but they caused an inhibition of growth. By the end of the toxicity test, all the treated larvae remained in the first instar, whereas most of the

TABLE 1. Toxicity of the active Cry proteins to neonate larvae of *E. insulana* from the Egyptian insect colony[a]

Protein	LC_{50} (μg/ml)	Relative potency	95% fiducial limits of relative potency		Slope ± SE	Intercept ± SE
			Lower	Upper		
Cry1Ia	21.13	0.052	0.04	0.06	3.25 ± 0.35	0.71 ± 0.47
Cry1Da	4.94	0.22	0.16	0.31	1.66 ± 0.16	3.85 ± 0.12
Cry1Ac	1.09	1.00			1.87 ± 0.17	4.92 ± 0.06
Cry1Ba	1.03	1.11	0.84	1.42	1.53 ± 0.17	4.98 ± 0.07
Cry9Ca	0.39	2.78	2.32	3.35	3.67 ± 0.31	6.49 ± 0.14
Cry1Ab	0.45	2.41	1.80	3.23	1.29 ± 0.13	5.44 ± 0.07

[a] Parameters were obtained from the POLO-PC program (27). The χ^2 value was not significant ($P > 0.05$) for a goodness-of-fit test for each regression. Slopes could not be fitted in parallel. The relative potency was expressed as the ratio of the LC_{50} value for each Cry protein to the LC_{50} value for Cry1Ac (39).

control larvae had molted to the third instar. The toxins Cry1Ca, Cry1Ja, and Cry2Aa were confirmed to be active against known susceptible species (*S. exigua*, *P. xylostella*, and *L. botrana*). Susceptible species were not available to confirm the integrity of the Cry1Ea, Cry1Fa, and Cry2Ab proteins, so we cannot exclude the possibility that they were degraded. All other Cry proteins (Cry1Ab, Cry1Ac, Cry1Ba, Cry1Da, Cry1Ia, and Cry9Ca) were toxic against *E. insulana* for both populations and resulted in larval mortality which increased with increasing toxin concentrations. In all cases, χ^2 values generated in goodness-of-fit tests indicated that the probit model was appropriate for each toxin and insect colony tested (Tables 1 and 2). Probit regression lines could not be fitted in parallel, and so the relative potencies (RP) were expressed as the ratio of the LC_{50} values for each active Cry protein to the LC_{50} value for the Cry1Ac standard (39). The LC_{50} value of Cry1Ac toxin for *E. insulana* was selected as a reference because it is the usual toxin produced in transgenic cotton.

In the population from Egypt, the RP of all the Cry toxins active against *E. insulana* indicated that Cry9Ca and Cry1Ab were the most potent toxins, with RP values of 2.78 and 2.41, respectively (Table 1). Cry1Ba toxin was also highly toxic for *E. insulana*, with an LC_{50} of 1.03 μg protein per milliliter of diet, similar to that of Cry1Ac based on the overlap of 95% confidence limits. Cry1Da and Cry1Ia were found to be 4.5 and 19.2 times, respectively, less toxic than Cry1Ac (Table 1).

In the population from Spain, the RP values indicated that Cry9Ca was significantly more toxic than Cry1Ac by a factor of 1.33, whereas Cry1Ba and Cry1Da were 1.53 and 15.69 times less toxic than Cry1Ac. The concentration-mortality relationship and the relative potencies for Cry1Ab and Cry1Ia toxins could not be determined because the Spanish colony succumbed due to a bacterial infection. The toxins assayed showed different activities for the two experimental insect populations. The estimated RP values for Cry1Ac, Cry1Ba, and Cry9Ca toxins were 3.97, 2.54, and 1.97 times significantly more toxic in the population from Spain than in the population from Egypt, respectively. However, the RP of the Cry1Da toxin was similar in both populations.

Binding of Cry proteins to BBMV of *E. insulana* from Egypt. To determine if binding sites were shared by more than one toxin, competition binding assays among the active toxins were performed. Using ^{125}I-labeled Cry1Ac, competition assays indicated that Cry1Ab was the only toxin that competed for Cry1Ac binding sites (Fig. 1). The fact that there was some ^{125}I-Cry1Ac binding that could not be competitively displaced by unlabeled Cry1Ab (even at the highest concentration used) suggests that there is a second binding site which is specific for Cry1Ac and to which Cry1Ab does not bind. The homologous competition curve (^{125}I-Cry1Ac versus unlabeled Cry1Ac) fitted a one-site model, which indicates that the affinities of the two proposed binding sites for Cry1Ac must be similar. Quantitative estimates gave a K_d of 1.9 ± 1.2 nM and an R_t of 19 ± 3 pmol/mg for Cry1Ac binding sites. The other toxins tested (Cry1Ba, Cry1Ia, and Cry9Ca) did not compete for the Cry1Ac binding sites.

Competition experiments using ^{125}I-labeled Cry1Ab confirmed the above-described results of a shared binding site for the two toxins. The total competition of Cry1Ac indicates that Cry1Ac competes for all binding sites of Cry1Ab (Fig. 2). Quantitative estimates gave a K_d of 6 ± 2 nM and an R_t of 16 ± 2 pmol/mg for Cry1Ab binding sites. The lower affinity of this toxin compared to that of Cry1Ac is in agreement with the relative positions of the competition curves of these two toxins, in which Cry1Ac is always a better competitor than Cry1Ab (Fig. 1 and 2).

Binding of biotinylated Cry1Ba, Cry1Ia, and Cry9Ca and competition with their respective unlabeled homologs revealed

TABLE 2. Toxicity of the active Cry proteins to neonate larvae of *E. insulana* from the Spanish insect colony[a]

Protein	LC_{50} (μg/ml)	Relative potency	95% fiducial limits of relative potency		Slope ± SE	Intercept ± SE
			Lower	Upper		
Cry1Da	4.25	0.06	0.09	0.04	1.93 ± 0.15	−1.22 ± 0.10
Cry1Ba	0.40	0.65	0.87	0.50	2.34 ± 0.19	0.92 ± 0.10
Cry1Ac	0.27	1.00			1.55 ± 0.17	0.89 ± 0.10
Cry9Ca	0.20	1.33	1.01	1.74	2.71 ± 0.28	1.90 ± 0.20

[a] Parameters were obtained from the POLO-PC program (27). The χ^2 value was not significant ($P > 0.05$) for a goodness-of-fit test for each regression. Slopes could not be fitted in parallel. The relative potency was expressed as the ratio of the LC_{50} value for each Cry protein to the LC_{50} value for Cry1Ac (39).

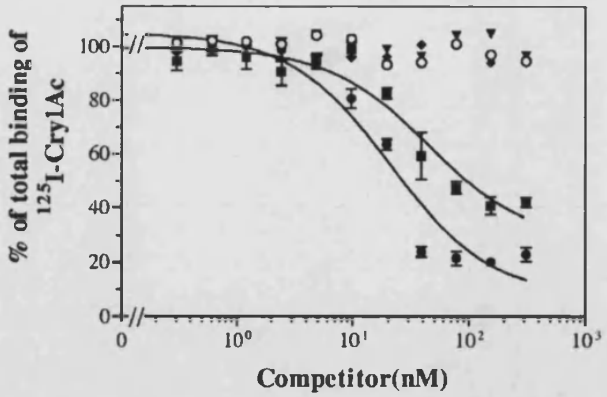

FIG. 1. ^{125}I-Cry1Ac binding to *E. insulana* BBMV at increasing concentrations of the following unlabeled competitors: Cry1Ab (■), Cry1Ac (●), Cry1Ba (◆), Cry1Ia (○), and Cry9C (▼). Data for the competing toxins represent the means of three experiments, and error bars are the standard errors of the means. Data for the noncompeting toxins were replicated twice, and error bars are not shown for clarity.

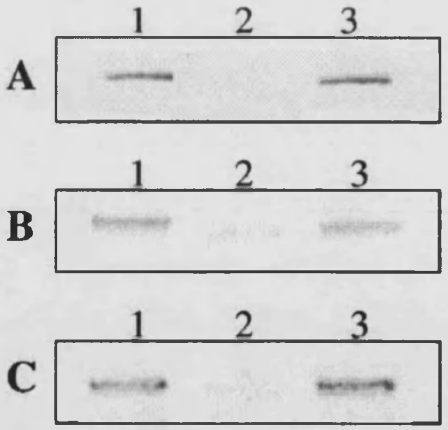

FIG. 3. Binding of biotinylated toxins to *E. insulana* BBMV. (A) Binding of biotin-labeled Cry1Ba alone (lane 1) and in the presence of an excess of unlabeled Cry1Ba (lane 2) or unlabeled Cry1Ac (lane 3). (B) Binding of biotin-labeled Cry9Ca alone (lane 1) or in the presence of an excess of unlabeled Cry9Ca (lane 2) or unlabeled Cry1Ac (lane 3). (C) Binding of biotin-labeled Cry1Ia alone (lane 1) and in the presence of an excess of unlabeled Cry1Ia (lane 2) or Cry1Ac (lane 3).

that binding of these toxins to BBMV from *E. insulana* was specific (Fig. 3). These labeled toxins were tested with unlabeled Cry1Ac as a competitor, and in all three cases, no competition was observed, confirming the results obtained using ^{125}I-Cry1Ac.

Of the active toxins against *E. insulana*, Cry1Da was the only toxin for which direct binding could not be tested due to the high nonspecific binding obtained using both ^{125}I-Cry1Da and biotin-labeled Cry1Da.

DISCUSSION

This is the first report on the insecticidal properties of individual *B. thuringiensis* Cry proteins against the spiny bollworm, *E. insulana*. Previous studies on the toxicity of *B. thuringiensis* isolates have examined the response of *E. insulana* to mixtures of toxins produced by each isolate (1, 4, 22, 32–34, 41, 43). Furthermore, the susceptibility of *E. insulana* to the Cry1Ac protein has been reported in assays performed using the MV-PII (Dow Agrosciences, Calif.) bioinsecticide, which contains this protein expressed in and encapsulated by transgenic *Pseudomonas fluorescens* (36, 49).

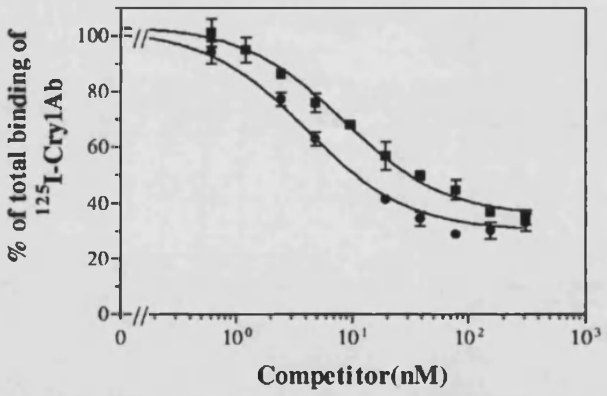

FIG. 2. ^{125}I-Cry1Ab binding to *E. insulana* BBMV at increasing concentrations of unlabeled Cry1Ab (■) and Cry1Ac (●). Data are the means of three experiments, and error bars represent the standard errors of the means.

Information on the insecticidal spectrum, potency, and mode of action of individual Cry proteins is critical to identify the most appropriate gene(s) for use in the development of *B. thuringiensis*-based control agents and insect-resistant transgenic plants. Among the Cry proteins known, only a limited number were included in this study. These Cry proteins were chosen because their activity towards species of the order Lepidoptera is well established. In our study, only 6 of the 13 Cry proteins assayed were toxic for *E. insulana* larvae. Cry9Ca was the most potent toxin for both colonies of *E. insulana*. For the colony from Egypt, Cry1Ab showed similar toxicity to Cry9Ca, followed jointly by Cry1Ba and Cry1Ac and finally by Cry1Da and Cry1Ia. In the population from Spain, the most active toxin was Cry9Ca, followed in decreasing order by Cry1Ac, Cry1Ba, and Cry1Da (Cry1Ab and Cry1Ia were not tested).

The occurrence of differential susceptibility to *B. thuringiensis* toxins has been demonstrated for numerous other insect pests, including other species of the genus *Earias*. A report on the related species *Earias vitella* indicated that Cry1Ab was more toxic than Cry1Aa and Cry1Ac (25). Our results with *E. insulana* showed that for the population from Egypt, Cry1Ab was also the most toxic among the assayed Cry1A proteins, with a relative potency 2.41 times higher than that of Cry1Ac. In contrast, the Cry1Aa toxin caused no mortality in our assays but resulted in larval growth inhibition, probably due to reduced feeding rates on toxin-contaminated diet, in both populations.

Variation in susceptibility to *B. thuringiensis* toxins has been reported among geographically distinct populations of a given species (15). In studies of 15 species of insects, only *P. xylostella* populations exhibited major differences in susceptibility that can be attributed to previous exposure to *B. thuringiensis* in the field (44). However, it is sometimes difficult to distinguish natural variation among susceptible populations from low to

moderate resistance (12, 24). The present study did not address resistance per se because the relationship between the history of exposure and susceptibility was not examined. However, we report here on the baseline susceptibility to different individual Cry proteins in two different geographical populations of *E. insulana*, which might be a useful reference to measure changes in susceptibility given possible future exposure to Bt cotton crops.

Some of the proteins that showed activity against *E. insulana* (Cry1Ab and Cry1Ac) are present in strain HD1 of *B. thuringiensis* var. *kurstaki* (which contains Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa), which is the active ingredient of many formulated bioinsecticides for the control of cotton pests including *E. insulana* (11). Evidently, the activity of the strain HD1 against *E. insulana* must be due to the presence of one or all of these proteins. A field population systematically treated with HD1-based insecticides may eventually result in the appearance of insects resistant to these Cry1A toxins.

The protection against *E. insulana* conferred by Cry1Ac in Bollgard cotton has been proven in field trials in southern Spain (35). Cry1Ac expressed in cotton provided better protection against *E. insulana* feeding damage than the conventional chemical insecticides. In transgenic plants, the efficacy of a determined Cry protein on a susceptible target insect is determined by the expression level required for effective control. Cry1Ac levels in Bollgard cotton declined steadily as the growing season progressed, ranging from 57.1 μg/g (dry weight) at 53 days after planting to 6.7 μg/g at 116 days after planting in fruit and from 163.4 μg/g at 53 days after planting to 34.5 μg/g at 116 days after planting in terminal foliage (17). If the results of our study are extrapolated for comparison with the expression level of Cry1Ac in transgenic plants, 1 μg of protein/ml of diet is equivalent to 6.23 μg of protein/g (dry weight) of diet (1 ml of diet had a dry weight of 160.4 mg). Therefore, the LC_{50} of Cry1Ac would be equivalent to 6.8 μg/g in the *E. insulana* population from Egypt and 1.7 μg/g in the population from Spain. The levels of Cry1Ac are sufficient to control *E. insulana* during the entire growing season; however, the relationship between Cry1Ac and its activity in the plant will likely be influenced by non-*B. thuringiensis* plant factors which, along with Cry1Ac, may be affected by the type and age of the plant tissue in question (17).

A possible risk in the use of transgenic plants is the potential development of pest resistance. One strategy to delay the development of resistance is the use of transgenic crops simultaneously expressing two or more insecticidal proteins with different modes of action (40). For this strategy to work, the two insecticidal proteins must not share key steps in the mode of action. Since the alteration of binding to midgut receptors seems to be the most important mechanism of resistance to *B. thuringiensis* toxins, determination of the binding sites of the active toxins can give us information on the possible risk of insects becoming resistant to more than one toxin by a change in a single receptor. Our results with labeled toxins show that among the toxins tested, Cry1Ab was the only one that shared common binding sites with Cry1Ac. This is a feature observed in all lepidopterans (3, 8, 9, 14, 19, 20), and it explains the basis of many cases of resistance to more than one *B. thuringiensis* toxin (12). It is interesting that Cry1Ac, in addition to the shared sites, also seems to have binding sites not shared with Cry1Ab in *E. insulana*, a feature not particularly common among lepidopteran species. To our knowledge, this model of Cry1Ac having binding sites shared with Cry1Ab and binding sites not shared with this toxin has only been proposed in two other insect species, *H. virescens* (47) and *H. armigera* (10).

In conclusion, the results obtained in this work show that *E. insulana* is susceptible to Cry1Ab, Cry1Ac, Cry1Ba, Cry1Da, Cry1Ia, and Cry9Ca. Cry1Ab and Cry9Ca were significantly more active than Cry1Ac, the toxin currently used in Bt cotton. From a resistance management standpoint, the most active proteins are good candidates for use in the control of this pest in addition to, or combination with, Cry1Ac, except for Cry1Ab, which shares binding sites with Cry1Ac. Cry9Ca is of particular interest since besides being the most active protein, it has a wide spectrum of toxicity that includes other important cotton pests, such as *H. armigera*, *H. virescens*, and *Spodoptera littoralis* (26, 37, 46).

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Molecular and Insecticidal Characterization of a Cry1I Protein Toxic to Insects of the Families Noctuidae, Tortricidae, Plutellidae, and Chrysomelidae

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The most notable characteristic of *Bacillus thuringiensis* is its ability to produce insecticidal proteins. More than 300 different proteins have been described with specific activity against insect species. We report the molecular and insecticidal characterization of a novel *cry* gene encoding a protein of the Cry1I group with toxic activity towards insects of the families Noctuidae, Tortricidae, Plutellidae, and Chrysomelidae. PCR analysis detected a DNA sequence with an open reading frame of 2.2 kb which encodes a protein with a molecular mass of 80.9 kDa. Trypsin digestion of this protein resulted in a fragment of ca. 60 kDa, typical of activated Cry1 proteins. The deduced sequence of the protein has homologies of 96.1% with Cry1Ia1, 92.8% with Cry1Ib1, and 89.6% with Cry1Ic1. According to the Cry protein classification criteria, this protein was named Cry1Ia7. The expression of the gene in *Escherichia coli* resulted in a protein that was water soluble and toxic to several insect species. The 50% lethal concentrations for larvae of *Earias insulana*, *Lobesia botrana*, *Plutella xylostella*, and *Leptinotarsa decemlineata* were 21.1, 8.6, 12.3, and 10.0 μg/ml, respectively. Binding assays with biotinylated toxins to *E. insulana* and *L. botrana* midgut membrane vesicles revealed that Cry1Ia7 does not share binding sites with Cry1Ab or Cry1Ac proteins, which are commonly present in *B. thuringiensis*-treated crops and commercial *B. thuringiensis*-based bioinsecticides. We discuss the potential of Cry1Ia7 as an active ingredient which can be used in combination with Cry1Ab or Cry1Ac in pest control and the management of resistance to *B. thuringiensis* toxins.

Bacillus thuringiensis is a spore-forming bacterium that has been isolated from many different natural habitats (32). The main interesting characteristic of this bacterium is that during sporulation, it produces one or sometimes more crystalline protein inclusions that exhibit high insecticidal activity upon ingestion. *B. thuringiensis*-susceptible species range across a wide variety of insects belonging to the orders Lepidoptera, Diptera, Coleoptera, and Hymenoptera (19) and include other types of invertebrates, such as nematodes and mites (31). The protoxins that form the crystal are dissolved in the alkaline insect midgut and are then proteolytically activated to yield a toxic fragment (18, 45). The activated toxin binds to specific receptors on the brush border membrane of gut epithelial cells and is partially inserted into the membrane, generating pores. This results in colloid osmotic lysis of gut epithelial cells followed by the death of the insect (17, 36).

The crystal is composed of Cry and Cyt proteins, in different combinations and proportions. To date, these proteins have been classified in 49 Cry groups and 2 Cyt groups and in different subgroups depending on their amino acid sequence homologies (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/toxins2.html). Over the past 20 years, more than 300 different Cry and Cyt proteins have been identified, and several of them have been successfully employed in biological insecticides in integrated pest management programs (36). Generally, crystals are composed of protoxins of 130 to 140 kDa (corresponding to the expression of *cry1* genes), 65 to 70 kDa (from *cry2* genes), and 70 or 130 kDa (from *cry3* genes). However, not all insecticidal proteins produced by *B. thuringiensis* clump together in the crystal. Some *B. thuringiensis* strains also secrete insecticidal proteins during the vegetative growth phase; these are called VIP proteins (10). In addition, some *cry* genes, named *cry1I* genes (formerly *cryV* genes), encode proteins of around 70 to 81 kDa that do not accumulate in the crystal (5, 12, 23, 34, 35, 37, 39, 41, 43, 46); these have been classified as Cry1I proteins due to their similarity with those in the Cry1 group (6). Their lack of involvement in the crystal structure has prevented these proteins from being included as active ingredients of *B. thuringiensis*-based insecticides. However, the effectiveness of Cry1I in protecting transformed plants from insect attack has been demonstrated (25, 29, 38). *cry1I* genes are usually located approximately 500 bp downstream of other *cry1* genes, but *cry1I* genes sometimes may not be expressed due to the lack of an upstream promoter-like sequence (23). In fact, *cry1I* genes are usually either silent or expressed in the vegetative phase and secreted into the growth suspension (23, 37, 41, 46). Cry1I proteins have a broader host range than most other Cry1 proteins, and the hosts include important species of lepidopteran and coleopteran pests (43). Strains containing

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novel *cry1I* genes have been evaluated as a source of new proteins with a broad host range (39, 41).

The intensive use of *B. thuringiensis*-based insecticides has already given rise to resistance in field populations of the diamondback moth, *Plutella xylostella* (11). Since *B. thuringiensis*-transgenic crops (Bt crops) generally express one or two types of Cry proteins, the development of pest resistance is viewed as a major threat for this technology. The search for novel *B. thuringiensis* strains harboring new *cry* genes has received considerable attention during the last 2 decades, not only to find toxins with novel activity spectra but also to increase the arsenal of toxins that can be used for resistance management (11, 12). The most common mechanism of resistance is the reduction of binding of the toxin to its specific midgut receptor(s). This may also confer cross-resistance to other toxins that share the same receptor (11). It is therefore important to study toxin binding properties to determine the utility of novel toxins and the possibility of their being used appropriately in combination with other commercially used *B. thuringiensis* toxins. In the present paper, we characterize the expression, toxicity spectrum, and binding site characteristics of a novel Cry1I protein from a Spanish strain, *B. thuringiensis* HU4-2, originally described by Martínez et al. (33). This strain was selected for study because it contains a wide variety of *cry* genes and has a broad spectrum of activity against lepidopteran pest species (33).

MATERIALS AND METHODS

***Bacillus thuringiensis* strains.** Strain HU4-2 was isolated from a dust sample originating from a maize grain silo in the Spanish province of Huesca as part of a countrywide screening program involving the isolation and characterization of *B. thuringiensis* strains suitable for use in biological control (21). Strain HU4-2 was classified as *B. thuringiensis* serovar *aizawai* (33) and deposited in the Spanish collection of type cultures (accession number CECT5950). Recombinant *B. thuringiensis* strains EG7077, EG11070, EG11916, and EG1081 (from Ecogen Inc., Langhorne, Pa.) expressing single proteins (Cry1Ab, Cry1Ac, Cry1Ba, and Cry1Ca, respectively) were used for comparative bioassays and binding experiments.

Preparation of parasporal crystals from strain HU4-2. Single colonies from Luria-Bertani (LB) plates were inoculated in 500 ml of CCY sporulation medium (42) and grown for 3 days until lysis was complete. A 1/5 volume of 5 M NaCl was added to the culture medium, which was then mixed. Spores and crystals were harvested by centrifugation at 15,000 × *g* for 20 min. The pellet was washed twice with sterile bi-distilled water, resuspended in sterile Milli-Q water, and finally stored at −20°C until used. Crystals were purified by ultracentrifugation in a sucrose discontinuous gradient as previously described (44). Briefly, the spore-crystal mixture was sonicated for 20 s in a Soniprep 150 MSE apparatus (Curtin Matheson Scientific) and immediately loaded onto centrifuge tubes containing two layers of sucrose solutions at 67% and 79% (wt/vol). After centrifugation at 70,000 × *g* for 16 h, the interphase containing the crystals was recovered with a Pasteur pipette, mixed with bi-distilled sterile water to a final volume of 200 ml, and centrifuged again (15,000 × *g*, 15 min). This step was repeated twice, and the crystal pellet was finally resuspended in sterile bi-distilled water. Crystal purity was checked by phase-contrast microscopy at a magnification of ×400, and the crystal samples were stored at −20°C until required.

Production, purification, and analysis of crystal proteins. Single-protein-expressing strains were grown for 48 h at 29°C in CCY medium (42) supplemented with the appropriate antibiotic (3 μg/ml of chloramphenicol for EG11070 and EG1081, 10 μg/ml of tetracycline for EG7077, and 25 μg/ml of erythromycin for EG11916). After centrifugation to concentrate the spores and crystals, crystal protein solubilization and trypsin activation were carried out as described previously (9). For binding analyses, Cry1Ab and Cry1Ac were further purified by anion-exchange chromatography with a MonoQ HR5/5 column by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden).

For the production of the Cry1I-type protein, fresh *Escherichia coli* BL21(DE3) cells were transformed with pPC-ire1 plasmid (see "Identification, cloning, and sequencing of the novel *cry* gene" below). Cells were grown overnight in LB with kanamycin (50 μg/ml) at 37°C and used to inoculate 750 ml of tryptone-yeast extract (2× TY) culture medium (26). The culture was grown at 37°C until the optical density at 600 nm was 0.5 to 0.6 and then incubated at 25°C for 45 min. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) was added and the incubation continued for 2 h at 25°C. Cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.5) (0.5 volume) was added to the culture, and cells were recovered by centrifugation (16,000 × *g*, 15 min). The pellet was resuspended in cold PBS (1/10 volume), centrifuged, and stored at −80°C. Cells were thawed on ice with 1/33 volume of cold binding buffer (40 mM imidazole, 4 M NaCl, 160 mM Tris-HCl, pH 7.9; Novagen, Darmstadt, Germany) and sonicated for 60 s in 15-s pulses. Protein purification was performed using a His Bind purification kit (Novagen) according to the manufacturer's instructions. Finally, the buffer was changed to carbonate (50 mM $NaCO_3$, 100 mM NaCl, pH 11.3) with a Sephadex G-25 prepacked column (Amersham Biosciences, Uppsala, Sweden) and the protein solution stored at 4°C until used. This protein was trypsin activated for binding assays but used as protoxin for insect bioassays.

Protein quantification was performed with the Bradford assay (3) using bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide gel, 100:1 acrylamide/bisacrylamide ratio) was run at 50 mA for 1 h in a mini-Protean III apparatus (Bio-Rad, Hercules, CA) as previously described (24). Gels were stained with a solution containing 50% (vol/vol) ethanol, 10% (vol/vol) acetic acid, and 0.1% (wt/vol) Coomassie brilliant blue R250 for 40 min and destained with a solution containing 6.75% (vol/vol) glacial acetic acid and 9.45% (vol/vol) ethanol. Protein sizes were determined by comparison with a broad-range protein marker (Bio-Rad).

To investigate the presence of insecticidal proteins in the growth medium, samples were taken every 12 h from a culture of the HU4-2 strain grown in CCY medium at 28°C over a total period of 72 h. Samples were purified and concentrated by centrifugation in tubes with a polyethersulfone membrane with a pore size corresponding to a cutoff of 10 kDa (Vivascience, Hannover, Germany). Noninoculated medium was used as the negative control. Proteins in the supernatant were analyzed by 10% SDS-PAGE and bioassayed as described below.

Identification, cloning, and sequencing of the novel *cry* gene. The presence of a *cry1I*-type gene in the *B. thuringiensis* HU4-2 strain was detected by PCR. A general primer pair [1I(98)Fw and 1I(98)Rv] recognizing both *cry1Ia* and *cry1Ib* genes was used in combination with oligonucleotides specifically recognizing *cry1Ia* [primers 1Ia(10)Fw and 1Ia(11)Rv)] or *cry1Ib* [primers 1Ib(8)Fw and 1Ib(9)Rv)] (Table 1). These primers were selected because no other toxin sequences from the Cry1I group had been published at the moment that the amplifications were performed. Template DNA was obtained directly from a loopful of cells from an overnight LB plate, suspended in 100 μl of sterile water, and boiled for 10 min. Five microliters of this suspension was added to 20 μl of the PCR mix containing 0.25 mM deoxynucleoside triphosphates, 1 mM $MgCl_2$, 0.6 to 1 mM of each primer, and 1 U of *Taq* DNA polymerase (Amersham Biosciences). Amplification was performed using an Eppendorf Mastercycler thermal cycler with the following program: a 3-min denaturation step at 95°C; 30 amplification cycles of 1 min at 95°C, 1 min at 45 to 50°C, and 1 min at 72°C; and a final extension step of 10 min at 72°C.

PCR products were sequenced and, according to the sequence information obtained, several primers were designed to specifically amplify the whole gene (Table 1). PCR with all possible primer combinations was performed under the conditions described above, and for positive reactions, primers were modified for NheI site inclusion. Three independent PCRs were carried out with primers MUTFw and 1I(E)Rv, and the amplified products were cloned into pGEM-T Easy vector (Promega, Madison, WI). The resulting plasmids were named pPC-ire1, pPC-ire2, and pPC-ire3. These three cloned amplicons were sequenced.

Nucleotide and amino acid sequence analysis. Sequence homology was determined using the NCBI nucleotide-nucleotide BLAST and protein-protein BLAST online services at http://www.ncbi.nlm.nih.gov/BLAST. Protein alignments were performed by use of ClustalW from the European Bioinformatics Institute at http://www.ebi.ac.uk/services/.

Bioassays. The activity of the Cry1I-type protein obtained from the recombinant *E. coli* strain was tested against 10 lepidopteran species, including 1 of the family Bombycidae (*Bombyx mori*), 6 of Noctuidae (*Earias insulana*, *Helicoverpa armigera*, *Spodoptera exigua*, *Spodoptera frugiperda*, *Spodoptera littoralis*, and *Trichoplusia ni*), 1 of Sphingidae (*Manduca sexta*), 1 of Tortricidae (*Lobesia botrana*), and 1 of Plutellidae (*P. xylostella*). This protein was also tested against two species from other insect orders, namely, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and *Tipula oleracea* (Diptera: Tipulidae). The Cry1I-type

TABLE 1. PCR oligonucleotides designed and used for cloning the *cry1Ia7* gene

Primer	Sequence (5′–3′)	Positions	Source[c]
1I(98)Fw	CACTAAAAAATGAAACAGATATAGA	74–98[a]	Van Rie, pers. comm.
1I(98)Rv	CCACATATTCATATACTGAGTGRTT	1057–1080[a]	Van Rie, pers. comm.
1Ia(10)Fw	TGTCTGAGTATGAAAATGTAGA	134–155[c]	Van Rie, pers. comm.
1Ia(11)Rv	GTTTTAATTGGATACATTTG	841–860[c]	Van Rie, pers. comm.
1Ib(8)Fw	TCTGAGCATGAGAGTATTGA	136–155[b]	Van Rie, pers. comm.
1Ib(9)Rv	GTGGTTTTAATAGGATATACAA	842–862[b]	Van Rie, pers. comm.
1I(A)Fw	GTATGAATAAAATTATATCTG	300–320[c]	This study
1I(B)Rv	GCAACAAATGTAAATTTGCAGC	928–949[c]	This study
1I(C)Fw	TTCTCTACCATAGAGTCTGC	1318–1337[c]	This study
1I(D)Rv	TTATAGTCTAAGTCCTCTCC	2134–2153[c]	This study
1I(E)Rv	CTACATGTTACGCTCAATATGGA	2138–2160[a]	This study
1I(F)Rv	GCAGACTCTATGGTAGAG	1320–1337[c]	This study
1I(G)Fw	GGTCTAAATAACTTGAGGGG	1078–1097[c]	This study
1I(H)Rv	GAAGAGAAGTTCCAAGCACC	2221–2240[c]	This study
MUTFw	GGGCTAGCATGAAACTAAAGAATCC[d]	1–17[a]	This study

[a] The positions given correspond to the *cry1Ia7* gene.
[b] The positions given correspond to the *cry1Ib1* gene.
[c] The positions given correspond to the *cry1Ia1* gene.
[d] The underlined nucleotides represent the NheI site.
[e] Van Rie, pers. comm., personal communication with J. Van Rie (Bayer, Belgium).

protein was not trypsin activated because we were most interested in determining the toxicity spectrum of the Cry1Ia7 protein in its nonprocessed form.

Cry1I-type protein concentrations for bioassays were adjusted by diluting the protein stock with 50 mM $NaCO_3$, 100 mM NaCl, pH 11.3. A preliminary test was performed to determine the specificity of this protein at a relatively high protein concentration (100 μg/ml). For the resulting susceptible species, five different protein concentrations were prepared to determine the mortality responses. Different bioassay methods were chosen depending on the insect tested. For *E. insulana*, *H. armigera*, *L. botrana*, *S. exigua*, *S. frugiperda*, *S. littoralis*, and *T. ni*, bioassays were performed with neonate larvae by incorporating the protein into an artificial diet (30). A diet surface contamination assay and neonate larvae were used for *M. sexta*. Bioassays with *B. mori*, *L. decemlineata*, *P. xylostella*, and *T. oleracea* were carried out by dipping leaf disks prepared from white mulberry, potato, cabbage, or lettuce, respectively, into the protein solution (21). *B. mori* and *P. xylostella* were tested with larvae in the second and third instars, respectively, whereas for *L. decemlineata* and *T. oleracea*, neonate larvae were used. Positive controls for the bioassays were included in the study by use of the same conditions as described above but with the following toxins: Cry1Ab for *B. mori*, *L. botrana*, *M. sexta*, and *P. xylostella*; Cry1Ac for *E. insulana* and *H. armigera*; Cry1Ba for *L. decemlineata*; Cry1Ca for *S. exigua*, *S. frugiperda*, and *S. littoralis*; and a crystal suspension of *B. thuringiensis* serovar *israelensis* for *T. oleracea*. Cry1A, Cry1Ba, and Cry1Ca proteins were trypsin activated because, in this state, they represent the toxins with the highest known insecticidal activities against the tested insect species. Negative controls for all the insects tested were included using the same conditions but without any toxin. For each insect species, at least 25 larvae were tested per concentration, and the bioassays were repeated at least three times. Bioassays were conducted at 25°C, 60 to 70% rH, and a 16:8 (light/dark [h]) photoperiod. For all insects bioassayed, mortality was evaluated after 5 days, except for *P. xylostella*, which was scored after 48 h. Concentration-mortality data for the four most susceptible insect species were analyzed by Probit analysis (28). Additionally, to test for toxins present in the growth medium, purified protein fractions recovered from growth medium supernatant that had been subjected to SDS-PAGE were bioassayed at concentrations of 8 to 11 μg/ml against *L. botrana*, as described above.

Midgut isolation and preparation of BBMV. Midguts of *E. insulana* and *L. botrana* were dissected from final instar larvae, washed with MET buffer (250 mM mannitol, 17 mM Tris-HCl, 5 mM EDTA, pH 7.5), frozen in liquid nitrogen, and kept at −80°C until used. Brush border membrane vesicles (BBMV) were prepared by the $MgCl_2$ precipitation method (49).

Toxin labeling and binding assays with *E. insulana* and *L. botrana* BBMV. Trypsin-activated Cry1Ia7 and trypsin-activated and fast protein liquid chromatography-purified Cry1Ab and Cry1Ac were labeled with a protein biotinylation kit (Amersham Biosciences) according to the manufacturer's instructions. Binding assays were carried out in a final volume of 0.1 ml binding buffer (PBS [1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4], 0.1% BSA) by incubating the biotinylated protein with the appropriate amount of BBMV (25 μg for both insect species) for 1 h at room temperature. The amounts of biotinylated protein were 20 ng for Cry1Ab, 50 ng for Cry1Ac, and 140 ng for Cry1Ia7 when *E. insulana* BBMV were used and 30 ng for Cry1Ab and 140 ng for Cry1Ia when *L. botrana* BBMV were used. The same binding conditions were used for competition assays, but an excess of at least 400-fold of unlabeled protein was added to the reaction mixture. After BBMV incubation, toxin bound to BBMV was recovered by centrifugation at 11,000 × *g* for 10 min at 4°C followed by two washes with 0.5 ml of cold binding buffer, as described elsewhere (16). The final pellet was solubilized in 10 μl sample buffer (24) and boiled for 10 min, and the proteins were separated by 10% SDS-PAGE. Proteins were electrotransferred onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences). The membrane was blocked overnight with a solution containing 3% ECL blocking agent (Amersham Biosciences), 0.1% BSA, and 0.1% Tween 20 in PBS; incubated for 1 h with a streptavidin-AP conjugate (Roche Diagnosis, Indiana); and washed three times with 0.1% BSA, 0.1% Tween 20 in PBS. Toxin bound to the BBMV was revealed by incubating the membrane with a Nitro Blue Tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) solution (Roche Diagnostics).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been filed in the GenBank database under accession number AF278797.

RESULTS

Analysis of the proteins produced by the HU4-2 strain. The purified crystals from HU4-2 produced a three-band pattern corresponding to peptides with sizes ranging between 130 and 145 kDa. A band corresponding to the expected molecular mass of a Cry1I-type protein was not detected as a component of the crystal (Fig. 1). However, a protein of ~75 kDa was observed following SDS-PAGE of purified samples of the growth medium of the HU4-2 strain (data not shown) and was subjected to insect bioassay as described below.

Cloning and nucleotide sequence of the *cry1I* gene. The *cry1I* general primer pair, 1I (98)Fw and 1I(98)Rv, produced an amplification fragment of 983 bp (data not shown), whereas *cry1Ia*- and *cry1Ib*-specific primers (Table 1) did not show any amplification, suggesting the presence of a new *cry1I* gene. Three independent PCRs performed with the primers MUTFw and 1I(E)Rv resulted in the same 2,200-bp fragment being amplified in all of them. The amplified fragments were independently cloned, and plasmids pPC-ire1, pPC-ire2 and pPC-ire3 were obtained. All of these fragments showed iden-

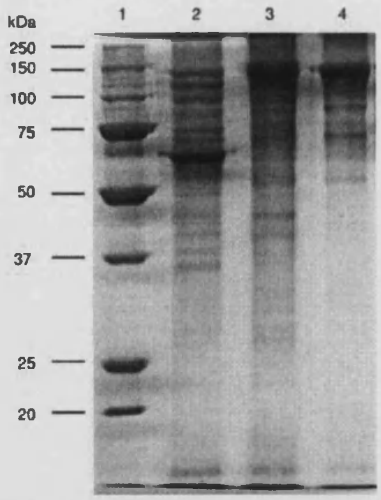

FIG. 1. SDS-PAGE of parasporal crystals obtained from the commercial biopesticides Dipel (lane 2) and Xentari (lane 3) and from the strain HU4-2 (lane 4) and purified by ultracentrifugation in a sucrose gradient as described in Materials and Methods. Molecular masses of the protein markers (lane 1) are given on the left.

tical sequences. The cloned fragments contained an open reading frame in the nucleotide sequence, and these data were deposited into GenBank. Comparison with other available sequences indicated 97.2% identity to *cry1Ia1*, 93.6% to *cry1Ib1*, 92.5% to *cry1Ic1*, 90% to *cry1Id1*, and 93.7% to *cry1Ie*.

Deduced amino acid sequence of Cry1Ia7. A fragment from pPC-ire1 encoding amino acids 1 to 719 of the Cry1I-type protein was cloned into pET28b(+) vector (Novagen) to construct pPC-ire1I plasmid for T7 polymerase-driven overexpression. The Cry1I-type polypeptide has a predicted molecular mass of 80.9 kDa, which is in agreement with the 80-kDa band observed by SDS-PAGE analysis of the expression and purification procedure (Fig. 2A). Trypsin digestion of this protein resulted in a fragment of ca. 60 kDa, which is typical of activated Cry1 proteins (Fig. 2B). Alignment of the deduced amino acid sequence with those of other known Cry1I proteins showed that the Cry1I-type protein had 96.1% sequence identity to Cry1Ia1, 92.8% to Cry1Ib1, 89.6% to Cry1Ic1, 89% to Cry1Id1, and 93% to Cry1Ie1. According to current classification criteria based on amino acid sequence similarity (6), the name Cry1Ia7 was assigned to the novel polypeptide. Cry1Ia7 had from 28 to 47 amino acid differences from other Cry1I proteins, and these appeared randomly distributed throughout the peptide sequence (Fig. 3). Some amino acid substitutions were found in the five conserved blocks identified. Block 2 was the most variable one, with one to six amino acid substitutions, and block 5 was the most conserved. The N-terminal domain contains features such as a positively charged stretch of amino acids (from M-1 to Q-10) followed by relatively hydrophobic residues (from S-11 to A-16) and a more-polar region (from K-17 to K-44). This region is highly conserved in Cry1I proteins, as confirmed in the amino acid sequence analysis (Fig. 3), and may function as a secretion signal peptide in *Bacillus* species (40). Thus, in *B. thuringiensis*, the mature putative protein would start at amino acid 46 in the predicted amino acid sequence and may be secreted as a protein with a theoretical molecular mass of ~75 kDa.

Cry1Ia7 insecticidal activity. The Cry1Ia7 protoxin was active against the lepidopterans *E. insulana*, *L. botrana*, and *P. xylostella* and the coleopteran *L. decemlineata* (Table 2). However, Cry1Ia7 was not toxic at the highest doses assayed (100 μg/ml) to the lepidopterans *B. mori*, *H. armigera*, *M. sexta*, *S. exigua*, *S. frugiperda*, *S. littoralis*, and *T. ni* or to the dipteran *T. oleracea*. Cry1Ia7 50% lethal concentration (LC_{50}) values were calculated for the four susceptible species and then compared to the LC_{50} values of activated Cry proteins known to be active against these species (Table 2). With an LC_{50} of 10.0 μg/ml,

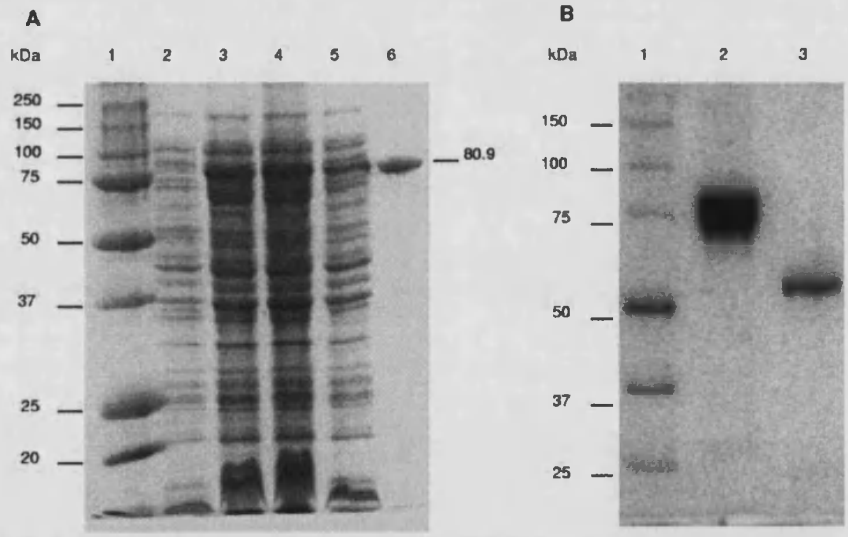

FIG. 2. SDS-PAGE showing the expression and purification of Cry1Ia7. (A) Lanes: 1, molecular mass markers; 2, *E. coli* BL21(DE3) native strain; 3, *E. coli* transformed with plasmid pPC-ire1 after IPTG-induced expression of the cloned *cry1Ia7* gene; 4, pellet of the culture that expressed Cry1Ia7 after sonication; 5, supernatant of this culture after sonication; 6, Cry1Ia7 protein after nickel affinity column purification and Sephadex column buffer exchange. (B) Cry1Ia7 protein after purification (lane 2) and after trypsin digestion (lane 3). Molecular sizes of the markers (lanes 1) are given in kDa.

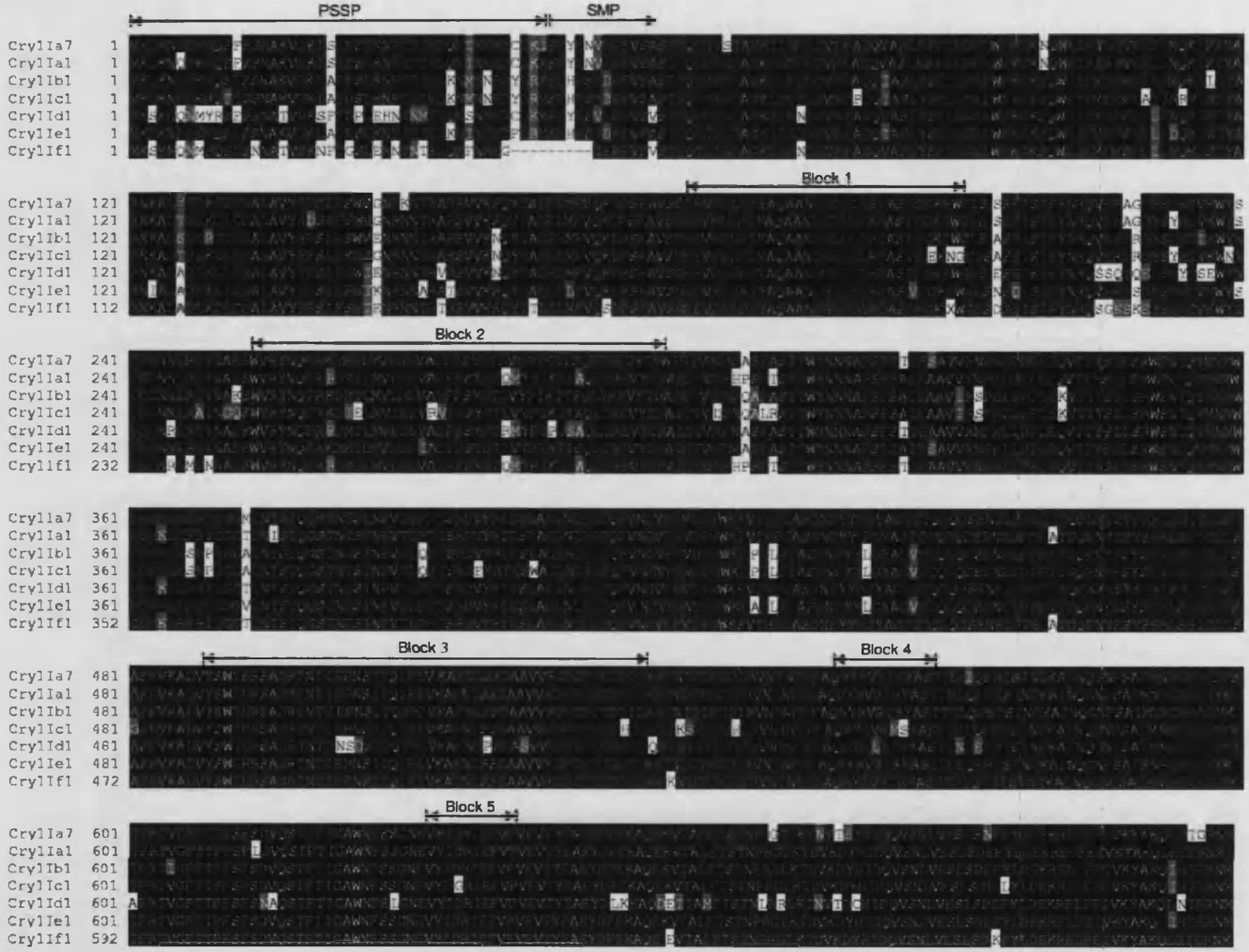

FIG. 3. Comparison of the deduced amino acid sequences of Cry1I proteins. Conserved amino acid blocks for Cry1I proteins, predicted secretion signal peptides (PSSP), and starts of mature proteins (SMP) are shown. Letters highlighted in black are conserved amino acids, and those in gray are semiconserved.

TABLE 2. Toxicity of Cry1Ia7 against first-instar larvae of *L. botrana*, *E. insulana*, *P. xylostella*, and *L. decemlineata*[a]

Treatment for indicated species	Regression line value		LC_{50} (μg/ml)	Distribution value		Relative potency	Confidence limit (95%)	
	Slope ± SE	$a^{\downarrow}$ ± SE[b]		χ^2	df		Lower	Upper
L. botrana								
Cry1Ab	1.87 ± 0.20	4.71 ± 0.12	1.4	0.30	3	1		
Cry1Ia7	2.93 ± 0.19	2.65 ± 0.20	8.6	0.75	3	0.18	0.13	0.23
E. insulana								
Cry1Ac	1.87 ± 0.17	4.92 ± 0.06	1.1	0.09	3	1		
Cry1Ia7	3.25 ± 0.35	0.71 ± 0.47	21.1	1.27	3	0.05	0.04	0.06
P. xylostella								
Cry1Ab[c]	1.90 ± 0.20	ND	0.08	ND	ND	ND	ND	ND
Cry1Ia7	2.74 ± 0.25	2.01 ± 0.26	12.3	1.62	3	ND	ND	ND
L. decemlineata								
Cry1Ba	1.32 ± 0.09	3.35 ± 0.14	17.9	1.33	3	1		
Cry1Ia7	1.32 ± 0.09	3.68 ± 0.12	10.0	1.22	3	1.79	1.2	2.4

[a] Relative potencies were calculated with respect to positive control Cry1 proteins that have been trypsin activated. ND, not determined.
[b] $a^{\downarrow}$, intercept of the regression line.
[c] Data are from reference 14.

Cry1Ia7 was 1.8-fold more active than Cry1Ba against *L. decemlineata* but less active than the control Cry1A proteins chosen for being among the most toxic ones for these species. Against *L. botrana*, Cry1Ia7 (LC_{50} of 8.6 μg/ml) was sixfold less active than activated Cry1Ab. Against *E. insulana*, Cry1Ia7 (LC_{50} of 21.1 μg/ml) was 19-fold less active than activated Cry1Ac. Against *P. xylostella*, Cry1Ia7 (LC_{50} of 12.2 μg/ml) was around 150-fold less active than activated Cry1Ab.

Bioassay of the ~75-kDa protein component purified from the HU4-2 growth medium resulted in 47 to 53% mortality of *L. botrana* larvae that had consumed 8 to 11 μg protein/ml, which is comparable with the insecticidal activity of Cry1Ia7 expressed in *E. coli*.

Binding experiments with *E. insulana* and *L. botrana* BBMV. Biotinylated Cry1Ia7 bound to BBMV from the two insect species, and this binding was specific, since it was completely displaced by the addition of an excess of the same unlabeled toxin (Fig. 4, lanes 6 and 7, for *L. botrana* and Fig. 5B, lanes 2 and 3, for *E. insulana*). Heterologous competition experiments were also performed with biotinylated Cry1Ia7 and an excess of unlabeled Cry1A toxins. In these experiments, neither Cry1Ab nor Cry1Ac displaced Cry1Ia7 binding to BBMV (Fig. 4, lane 8, for *L. botrana*, and Fig. 5B, lanes 4 and 5, for *E. insulana*). Similarly, experiments with biotinylated Cry1Ab and biotinylated Cry1Ac (the former only with *E. insulana* BBMV) were carried out, and the binding was also shown to be specific (Fig. 4, lanes 3 and 4, and Fig. 5A, lanes 2, 3, 7, and 8). Cry1Ia7 could not displace Cry1Ab binding to *L. botrana* BBMV (Fig. 4, lane 5), nor could it displace Cry1Ab or Cry1Ac binding to *E. insulana* BBMV (Fig. 5A, lanes 4 and 9, respectively). Cry1Ac could displace Cry1Ab binding in *E. insulana* (Fig. 5A, lane 5), in agreement with previous data (20). Taken together, these results indicate that Cry1Ia7 binds to sites different from those of Cry1Ab in both species. In the case of *E. insulana*, where Cry1Ac was also included in the analysis, this toxin shared binding sites with Cry1Ab but not with Cry1Ia7.

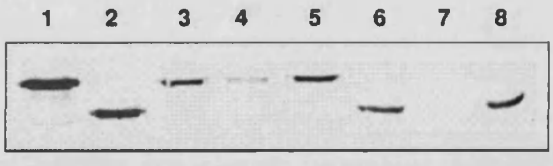

FIG. 4. Binding and competition experiments with biotinylated Cry1Ab and Cry1Ia7 with *L. botrana* BBMV. Lanes: 1, biotinylated Cry1Ab (control without BBMV); 2, biotinylated Cry1Ia7 (control without BBMV); 3 to 5, binding of biotinylated Cry1Ab to BBMV (lane 3) and in the presence of an excess of unlabeled Cry1Ab (lane 4) or Cry1Ia7 (lane 5); 6 to 8, binding of biotinylated Cry1Ia7 to BBMV (lane 6) and in the presence of an excess of unlabeled Cry1Ia7 (lane 7) or Cry1Ab (lane 8).

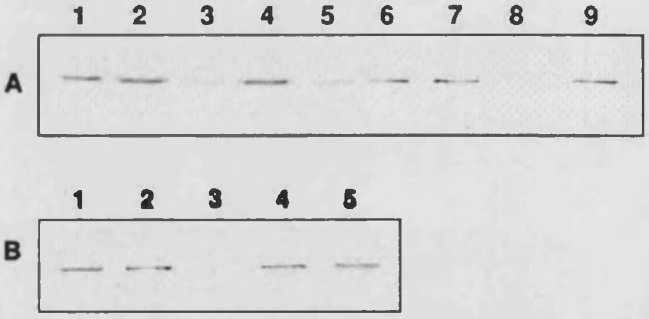

FIG. 5. Binding and competition experiments with biotinylated Cry1Ab, Cry1Ac, and Cry1Ia7 with *E. insulana* BBMV. (A) Lanes: 1, biotinylated Cry1Ab (control without BBMV); 2 to 5, binding of biotinylated Cry1Ab to BBMV either without further addition (lane 2) or in the presence of an excess of unlabeled Cry1Ab (lane 3), Cry1Ia7 (lane 4), or Cry1Ac (lane 5); 6, biotinylated Cry1Ac (control without BBMV); 7 to 9, binding of biotinylated Cry1Ac to BBMV either without further addition (lane 7) or and in the presence of an excess of unlabeled Cry1Ac (lane 8) or Cry1Ia7 (lane 9). (B) Lanes: 1, biotinylated Cry1Ia7 (control without BBMV); 2 to 5, binding of biotinylated Cry1Ia7 to BBMV either without further addition (lane 2) or and in the presence of an excess of unlabeled Cry1Ia7 (lane 3), Cry1Ab (lane 4), or Cry1Ac (lane 5).

DISCUSSION

A novel gene encoding a protein of the Cry1I group has been cloned and sequenced. The new protein has interesting insecticidal properties, particularly with regard to its wide host range, a feature of some proteins in this group. Moreover, its binding sites are different from those of the Cry1A toxins commonly found in Bt crops and *B. thuringiensis*-based insecticides.

Our studies did not detect the protein (predicted molecular mass of 81 kDa) by SDS-PAGE analysis after the crystal was formed in HU4-2 cells. However, the *cry1Ia7* gene was identified in this strain, which contains an important level of diversity of additional *cry* genes. Sometimes the *cry* gene complex of a strain is not reflected in the protein content of the crystal (33), because not all *cry* genes are expressed or because not all Cry proteins take part in crystal formation, and some may be secreted into the growth medium (10, 23). In fact, our results indicated a significant contribution of 130- to 145-kDa proteins in the parasporal crystal that may correspond to the expression of other *cry1* genes, but not *cry1I*, which code for proteins of approximately 80 kDa (6). This could be due to the lack of significant expression of this gene or to the fact that the protein is secreted into the medium (23, 37, 41, 46).

The complete open reading frame of the new *cry1Ia7* gene was cloned and expressed in *E. coli* cells. SDS-PAGE analysis of the purified protein generated in *E. coli* revealed a major peptide of ca. 81 kDa which is similar to other proteins of the Cry1I group (5, 12, 41, 43). Cry1I proteins produce inclusion bodies when they are expressed in *E. coli*. However, these proteins do not participate in crystal formation, since they are secreted into the medium during the vegetative growth phase of the bacterium (5, 23, 37). Cry1Ia7 also produced inclusion bodies in *E. coli* after being induced by IPTG at 37°C, but at a lower temperature (25°C), the protein produced is soluble. Lower growth temperatures, a different culture medium (2× TY instead of LB), and a lack of overexpression could be some of the reasons for this difference. Amino acid sequence analysis of Cry1Ia7 showed characteristics similar to those of other secreted proteins from the same group. However, Cry1Ia7 was different at several positions from the reported Cry1I proteins (46). Moreover, minor changes in amino acid sequences can produce important variations in the host range or insecticidal properties of these toxins. Cry1Ia1 and Cry1Ia2 differ by a single amino acid in domain II and exhibit differing insecticidal activity spectra (12). Cry1Ia7 differs from Cry1Ia1 by four amino acids which are scattered throughout block II, so we may have expected modifications in the toxicity and specificity of the encoded Cry protein. However, these changes in the sequence did not appear to modify the activity spectrum. The regions corresponding to the three domains of Cry1Ia7 were highly similar to those of Cry1Ia1. Loop regions in domain II have been reported to be involved in receptor binding and toxicity (36), and the three potential loop regions in domain II of Cry1Ia7 matched those of Cry1Ia1. Similar results were obtained by Choi et al. (5), who observed no amino acid differences in loop regions of Cry1Id1 compared with Cry1Ia3, although the latter protein exhibited a significantly higher activity against *B. mori*. Amino acid substitutions in regions adjacent to the loops of domain II might also affect the protein conformation, and thus the toxicity could also be altered. In addition, domain III has been reported to be involved in receptor binding (7), and some of the amino acid substitutions in the corresponding region of Cry1Ia7 could therefore correlate with differences in receptor binding and toxicity.

Cry1I toxins are particularly interesting from an agricultural perspective because of their wide host range. Other proteins, such as Cry1B, Cry1C, and Cry2A, also exhibit host ranges spanning more than one insect order (1, 4, 48, 50). Cry1I, Cry1B, Cry1C, and Cry2A protein groups include the only native proteins which, independent of their differential proteolytic processing, are toxic for insects of different orders. Cry1I proteins were initially characterized by their dual activity towards Lepidoptera and Coleoptera (43). The first protein reported by Tailor et al. (43) had dual activity against *L. decemlineata* and *Ostrinia nubilalis*, but the activity of the remaining Cry1I-type proteins that have been characterized subsequently has been restricted to lepidopteran species (5, 12, 23). Cry1Ic2 is the only protein for which insecticidal activity against a beetle, *Diabrotica virgifera*, has been reported (34). Cry1Ia7 protein has been found not to be toxic against *H. armigera*, *T. ni*, and *Spodoptera* spp. However, Cry1Ia7 was toxic against *P. xylostella*, an insect which is usually susceptible to Cry1I proteins (5, 23, 37, 39, 41). Nevertheless, Cry1Ia7 showed activity against *L. decemlineata*, supporting the dual activity against Lepidoptera and Coleoptera described by Tailor et al. (43). Moreover, Cry1Ia7 was toxic to *L. botrana* larvae, whose susceptibility had not been previously reported in the Cry1I group.

Despite the fact that binding site competition studies involving Cry1A toxins are numerous in the literature (8, 9, 11, 15, 16, 47), this study is the first involving a Cry1I toxin. Cry1A proteins compete for common binding sites in all species studied, and this forms the basis of cross-resistance or multiple resistance among these toxins (2, 9, 13, 27). To determine the compatibility, in terms of resistance management, of Cry1Ia7 with other Cry1A proteins widely used in Bt crops, we performed heterologous binding assays with BBMV from *L. botrana* and *E. insulana*. The results showed that Cry1Ia7 does not compete for Cry1Ab or Cry1Ac binding sites. It is therefore unlikely that development of resistance to Cry1A toxins would confer cross-resistance to Cry1Ia7 or vice versa (11). In view of the similarities found in binding site models among insect species, the results obtained with *L. botrana* and *E. insulana* seem likely to apply to other species.

The fact that Cry1I proteins are secreted and not crystallized impedes their use in biopesticide spray applications. Nevertheless, their interesting insecticidal characteristics can be successfully exploited if Cry1I proteins are expressed in transgenic plants (25, 29, 38). Alternatively, *cry1I* genes could be cloned in *Pseudomonas* spp., thus expressing the Cry1I proteins and microencapsulating them in the bacterial cell wall (22). Microencapsulated Cry1I could be used in spray applications alone or in rotations with *B. thuringiensis*-based insecticides containing Cry1A toxins.

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Artículo enviado a: Molecular Ecology

Genetic variation of *Helicoverpa armigera* populations around the cotton growing area in southern Spain as revealed by amplified fragment length polymorphism (AFLP).

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Running title: Spanish *Helicoverpa armigera* genetic structure

ABSTRACT

Amplified fragment length polymorphisms (AFLPs) were used to determine the population genetic structure and variation of *Helicoverpa armigera* in the cotton growing region in the south of Spain. Individuals were collected, before and after the cotton harvest in 2003 and 2004, from three separate areas where cotton is planted (Seville), and from strawberry greenhouses (Huelva), outside the cotton growing area. The two primer combinations used in this study gave 104 AFLP bands that were polymorphic along all 475 samples analyzed. A neighbour-joining phylogram of the 19 populations showed two major groups subdivided in two other groups that clearly separated populations by season. Results of population genetic structure revealed high levels of diversity within all populations studied and low levels of genetic diversity among all populations (F_{ST} = 0.20). Differences among groups were found significant only when populations were grouped by season (populations collected in the same season). Pairwise F_{ST} and Nm (to get an estimation of gene flow) between all populations were also calculated. The high level of diversity and the relationships found in *H. armigera* populations in the south of Spain indicate that individuals from one place are completely replaced season after season and provide support for considering these populations away from migration-drift equilibrium. As populations are not defined by their origin and all diversity is replaced season after season, the results indicate that insects from other crops or fields are replacing those after insecticide treatments and all populations of insects could be considered as a single metapopulation.

INTRODUCTION

Helicoverpa armigera is one of the most important cotton pests in the Mediterranean area, Africa and Australasia (Fitt 1989). It has been controlled traditionally by conventional means with chemical insecticides including synthetic pyrethroids and organophosphates, and has evolved resistance to many of them (Kranthi et al. 2001a; Kranthi et al. 2001b; McCaffery 1998). Bt cotton (transgenic cotton varieties expressing *Bacillus thuringiensis* proteins) has been commercially available since 1996 as an alternative to chemical pesticides in Australia and the United States (Krattiger 1997), and has been adopted by other countries later on. Although cotton is planted in southern Spain and Greece, commercial planting of Bt cotton has not been approved in these countries. Bt cotton is the second most important Bt crop around the world, with 9.8 millions hectares planted in 2005 (James 2005). In Spain, the only Bt crop which is currently commercialized is maize which constituted 11 % of all planted maize in the country (James 2005). In industrial countries, the introduction of transgenic crops expressing *B. thuringiensis* toxins has been accompanied by the adoption of several measures to avoid the evolution of resistant insects in the field. The most common strategy to avoid the evolution of resistance is designing refuges surrounding the area where transgenic crops expressing high doses of toxin are planted. This surrounding area will delay substantially the evolution of resistance in insects which had survived to Bt crops. Ideally, resistance is conferred by rare, recessive alleles (Ferré and Van Rie, 2002), and most resistant adults from Bt crops mate with susceptible adults living in the refuges. As a polyfagous pest, *H. armigera* can feed on alternative crops such as pulses and other vegetables (Trivedi et al. 2005) and these areas could also be used as natural refuges in case Bt cotton would be planted in the area and no *B. thuringiensis* formulates were used for insect control.

An understanding of the genetic structure and diversity in *H. armigera* natural populations is fundamental to properly design and interpret the behavior of this species, and also to create suitable strategies in order to prevent the appearance of resistance to *B. thuringiensis* toxins in the field. It is known that *H. armigera* is a polyphagous pest and has also been reported to follow quite long migratory movements (Feng et al. 2004; Feng et al. 2005). This capability of dispersion could allow this species to fly easily from one to another relatively distant area, either increasing the variability of the species after mating there or replacing them if some pesticide treatments had been applied. In the present work, amplified fragment length polymorphisms (AFLP) were used to determine the population structure and differentiation in several populations collected from southern Spain. AFLP markers have been widely used to determine population structure and differentiation (Mueller and Wolferbarger 1999). There is some concern on whether allele frequency-based techniques are appropriate for calculating population parameters for dominant markers (Wong et al. 2001). The dominant nature of AFLP markers has precluded direct estimations of allele frequencies and can also bias calculations of genetic diversity and population structure (Lynch and Milligan 1994). For this reason, analysis of molecular variance (AMOVA) (Excoffier et al. 1992) has been included in our study as this analysis is not influenced by the dominance trait.

We have collected insects from several origins either inside (in three areas) or outside (two areas) cotton growing region in Andalusia (southern Spain), twice a year (before and after the cotton harvest) in two consecutive years (2003 and 2004) which constituted four sampling seasons. The main goal of this work was determining *H. armigera* population genetic structure for the future of Bt cotton planting in southern Spain and to check whether areas surrounding transgenic plants could be used as natural refuges if insects survived after their exposure to Bt cotton.

MATERIALS AND METHODS

Insect populations

H. armigera individuals were taken from five different areas from the cotton growing and surrounding (non-cotton) region in southern Spain. Samples were collected in two consecutive years (2003 and 2004), the first batch before and the second one after the cotton harvest (before and after summer, respectively), giving samples from four different seasons (seasons 1 and 2, from 2003 before and after cotton harvest, and seasons 3 and 4, from 2004 before and after cotton harvest, respectively). Three of the five populations analyzed were collected from areas within the cotton-growing region in the province of Seville: Alcalá del Río (AR) and Los Palacios (LP) from cotton fields, and Carmona (C) from chickpea and cotton fields, and other two populations were collected from strawberry greenhouses in two areas in Huelva, around 100 km apart from the region where cotton is planted: Moguer (M) and Lepe (L). We analyzed 25 insects from each area and season, giving a total of 475 samples as insects from one area (L) could not be collected in the second season (2003 after cotton harvest).

DNA extraction and quantification

DNA was extracted from *H. armigera* adults (half insect) with the DNeasy Tissue kit (Qiagen Inc., Valencia, CA), following the manufacture's instructions. DNA concentration and quality of the samples was determined by spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) (A_{260} nm/A_{280} nm) and by 1% agarose gel electrophoresis.

AFLP methodology

AFLP analysis was performed according to Vos et al. (1995), with the following modifications. After DNA digestions with 5 U EcoRI (Pharmacia, Uppsala, Sweden) and 5 U Mse I (NewEngland BioLabs Inc., Herts, United Kingdom) for 2 h at 37 °C, samples were incubated at 65 °C for 20 minutes to inactivate the enzymes. Preamplification was carried out with the AFLP Pre-amp Primer Mix I (Invitrogen, Carisbad, CA). To select the best combination of primers for specific amplifications, twenty-five primer pair combinations containing three selective nucleotide at their 3'-end were tested in two samples from each population, and only two of them (*Eco*RI-ACT/ *Mse*I-CTG and *Eco*RI-CAG/ *Mse*I-AGG) (Invitrogen) were selected for being the ones that gave more information in all insects analyzed. Resulting amplicons were run on an ABI 3700 Capillary sequencer (Applied Biosystems, Foster City, CA) and edited with the GeneScan Analysis Software (Applied Biosystems) using a ROX 500 (ABI) as a size standard. Genographer v1.6 software (Benham 2001) was used to visualize, normalize the intensities and score the AFLP markers when they were clearly defined.

Data analysis

Presence/absence matrices were built with data derived from all samples and markers for further statistical analysis. Since AFLP bands are inherited as dominant markers, genotypes are not directly available for this kind of data. For this reason, AFLP data were analyzed with different methodologies. First, *AFLP-SURV* 1.0 software (Vekemans et al. 2002, available at http://www.ulb.ac.be/sciences/lagev) was used to determine genetic diversity within populations and population genetic structure after estimating allele frequencies, and assuming Hardy-Weinberg (H-W) equilibrium, with the method of Lynch and Milligan (1994). A permutation test was also carried out with *AFLP-SURV* 1.0 using 1000 random permutations to test for significance of the global F_{ST} statistics (proportion of total genetic variability attributed to the genetic differences between populations) calculated. Pairwise distances between populations were calculated using

Nei's minimum genetic distance, Dm, (Nei 1987) with POPULATIONS 1.2.28 program (Langella 2002; available at http://www.pge.cnrs-gif.fr/pge/bioinfo/populations/index.php?lang=en). A neighbor-joining (Saitou and Nei, 1987) phylogram was created from Dm matrices with POPULATIONS 1.2.28 program and visualized with MEGA 3.1 software (Kumar et al. 2004; available at http://www.megasoftware.net/mega.html).

Analysis of molecular variance (AMOVA) is based on phenetic distance measures (Excoffier et al. 1992), and was used as an alternative approach to study population structure and their components of variance. This methodology is appropriate to partition genetic variation at different levels (for instance, intrapopulational, intraregional, and interregional). Variation within and among populations grouped by origin and season was also studied with the AMOVA procedure. Firstly, populations were grouped according to the origin where they were collected irrespective of the season. Secondly, populations were grouped according to sampling season irrespective of their origin. Finally, samples were grouped according to their region, within each season, into two separate groups, one with the samples taken from the cotton growing region and the second one with the ones collected outside. Variance components were tested statistically by nonparametric procedures using 1000 permutations. All AMOVA tests were conducted using the ARLEQUIN 3.1 version program (Schneider et al. 2000). Genetic structure and F_{ST} by seasons, origin and regions within seasons were also studied with the method of Lynch and Milligan (1994), based on allele frequencies, with the *AFLP-SURV* 1.0 program and then compared with the results obtained with the AMOVA. F_{ST} values were further tested for their significance as before (using 1000 random permutations).

Population pairwise F_{ST} between all populations were also calculated with the Slatkin's (Slatkin 1995) and Reynold's (Reynolds et al. 1983) distance statistics, and a permutation test (using 1000 random replicates) was included to test for significance of the pairwise F_{ST} between all populations. The number of migrants exchanged between each pair of populations was also calculated to get an estimation of the population genetic divergence ($M=N_m$ for haploid data) as migration-drift equilibrium could not be assumed. Both pairwise F_{ST} and N_m values were calculated with the ARLEQUIN 3.1 version program (Schneider et al. 2000).

RESULTS

Overall, 475 *H. armigera* adults were collected from five separated areas before and after the cotton harvest seasons in 2003 and 2004. Only two primer combinations were selected among the initial screening which produced a total of 104 polymorphic bands among all the samples analyzed with sizes ranging from 100 to 500 bp. All banding patterns were unique for each individual. The number of polymorphic loci ranged from 37 (in M1) to 72 (in L3) (Table 1) among the populations studied (most of them with values between 45 and 55), and with a global percentage of polymorphism around 55 %. When the percentage of polymorphic loci was calculated by origin irrespective of the season, the resulting values were similar (57.98 % for AR, 57.2 % for C, 57.46 % for LP, 52.78 % for M, and 60.33 % for L). Furthermore, populations sampled in the same season showed similar numbers of polymorphic loci, the highest one being found in the third season (year 2004 before the cotton harvest) with values between 65 and 72 (Table 1).

The average genetic diversity within all 19 populations (Hw) was 0.1931 (Table 2), with values of heterozygosity within populations (Hj) ranging from 0.107 (in M1) to 0.286 (in LP3) (Table 1). Genetic diversity values were higher within than among populations (Table 2), an initial

indication that there were more differences within the populations than among them. The global F_{ST} (0.20), calculated with Lynch and Milligan's method, was found to be significantly larger than zero ($p < 0.05$) by using a permutation test with 1000 random replicates. This value corresponds to populations with a moderate level of divergence.

Nei's minimum genetic distance (Nei 1987) was used to calculate distances among AFLP patterns. The resulting distance matrix for the 475 AFLP patterns grouped in 19 populations was used to construct a phylogram showing the relationships among all of them. The resulting tree (Fig. 1) revealed a clear differentiation in two major groups, one including samples from the first and fourth sampling seasons (2003 before and 2004 after the cotton harvest, respectively), and the other one composed by samples from the two other seasons (2003 after and 2004 before the cotton harvest). Within these two major groups, populations grouped by season and not by geographical origin. A phylogram based on the Dm distance for all the 475 samples considered as separate individuals was also constructed (results not shown), but it did not show any clear structure by origin, region, season or year.

Partitioning of AFLP variance within populations, within populations among groups and among groups, by means of their origin, season or region within season was also performed with the AMOVA procedure to determine the different components of the total variance in the study (Table 3). Results of F_{ST} estimated with AMOVA (Table 3) were then compared with previous data obtained with Lynch and Milligan's method (1984) based on the same groups (Table 2). In all the groupings considered for AMOVA analyses, the within populations component of variance was the highest one (with values ranging from 63.43 to 92.71 %, depending on the groups considered and number of samples analyzed, Table 2). These results indicate that all *H. armigera* populations sampled were very heterogeneous. The influence of the origin was first studied with AMOVA analysis and the results showed that the fixation indexes were significantly larger than zero ($P < 0.001$), both among populations of the same origin irrespective of the seasons (populations within compared groups) and within populations (corresponding to 39.03 % and 68.15 % of the total variance, respectively), but it was not significant among populations of different origin (Table 3). The influence of sampling season was also studied, and the fixation indexes calculated for all the components of the genetic variance were significant ($P < 0.001$). These results indicate that populations sampled in the same season were not statistically different (and the percentage of variation due to sampling season was 27.87 %, Table 3). Since the variance due to sampling season was found to be significant, an AMOVA procedure was used to determine whether samples collected in the cotton and non-cotton regions within each season were homogenous or could be considered as genetically separated areas. In the second sampling season (2003 after the cotton harvest), the comparison between cotton and non-cotton region with AMOVA could not be performed since one of the two populations in the later group (L2) was missing. The results in the three seasons studied (2003 and 2004 before cotton harvest, and 2004 after cotton harvest) showed that the fixation indexes among regions (cotton versus non-cotton) were not statistically significant ($p \geq 0.05$, Table 3). The remaining components of variance, variation among populations within the same region and variation within populations, were significant ($p < 0.001$) when comparing cotton and non-cotton regions. For all the seasons, although variance among populations within regions was significant, it only contributed from 5.36 % to 15.58 % of variation among samples (Table 3), and the largest proportion of variance was always found within populations.

Population genetic structure analyses including F_{ST} calculations based on allele frequencies were also carried out with the same groups defined for AMOVA analyses. All F_{ST} calculated were

significant in all groups (with $P < 0.01$ in groups of population from the same origin and from the same season, and $P < 0.05$ in groups of populations from different regions), but error values were very high in all groups performed except the one grouping populations by season. The highest value ($F_{ST} = 0.1774$, Table 2) was also found when populations were grouped by sampling season. This value indicated that the highest diversity was found in populations among seasons, and it is in agreement with the previous results obtained with the AMOVA analyses. In this grouping, the expected heterozygosity among groups was also the highest among all the analyses, a value very close to the one found when the 19 populations were studied separately.

Pairwise F_{ST} between all 19 populations were also calculated to obtain an estimate of their genetic divergence (Table 4, lower hemimatrix). Although migration-drift equilibrium could not be assumed for these data, since all sampled populations seemed to be recolonized anew every season, estimates of the number of migrants exchanged between pairs of populations (N_m) were included to get an additional estimate of population divergence (Table 4, upper hemimatrix). All F_{ST} values were significant by a permutation test with 1000 random replicates ($P < 0.05$) except for populations pairs AR2 - C2, AR2 - LP2, AR4 - C4, AR4 - LP4, and C4 - LP4 (Table 4, lower hemimatrix), all of them being from the same season. Correspondingly, pairs of populations with non-significant F_{ST} values also had the highest estimated N_m values (Table 4, upper hemimatrix) (in four of them an infinite number of migrants would be exchanged between population pairs if migration-drift equilibrium had been assumed). These results indicate that these five pairs of populations could not be considered as separate populations. They were also found to be at a minimum genetic distance (using Nei's Dm) as shown in the phylogram for all the populations (Fig. 1).

DISCUSSION

The AFLP analysis of *H. armigera* populations collected from five different origins in four seasons, before and after the cotton harvest, during 2003 and 2004 revealed substantial levels of genetic variation. All 104 bands obtained with the two primer combinations used were polymorphic across samples, with an average of 55 % polymorphic loci within samples. The total expected heterozygosity in the study was 0.2408 (Table 2), more than twice the value obtained in previous studies with *H. armigera* natural populations using allozymes and RAPD markers (Daly and Gregg 1985; Nibouche et al. 1998; Zhou et al. 2000); within populations values were quite variable (Table 1), and constituted 80 % of the total heterozygosity in the samples.

Populations grouped according to the season of sampling and did not show any further divergence due to geographical origin (Fig. 1). F_{ST} data obtained with the Lynch and Milligan's method (1984) to estimate population genetic structure showed that all 19 populations were significantly different among them, with moderate levels of population divergence. When the populations were grouped by season, F_{ST} value was found significant. Furthermore, F_{ST} in the other groups performed by means of geographical origin or region within season were also found significant but with very high error values (Table 2). In contrast, partitioning of AFLP variance components with AMOVA procedures with the same groups as before indicated that differences in populations among the defined groups were significant only when populations were grouped by sampling season irrespective of their origin or region. This result provides further support for considering these populations away from migration-drift equilibrium. This corresponds to a local extinction-recolonization dynamics that indicates that *H. armigera* should be treated as a

metapopulation when inferences are to be made on structure and putative evolutionary trends at short and medium time-scales (Hanski 1998; Harrison and Hastings 1996).

Pairwise population F_{ST} showed that some pairs have from very low to moderate level of divergence, and smaller values are normally for pairs from the same season. Some F_{ST} values obtained between population pairs from the same season were not significant, but these pairs were not defined by belonging to the same region, either inside or outside the cotton growing area, giving further support to the metapopulation structure of this pest in southern Spain. These results at a smaller spatial scale, when considered along the above mentioned differences at short temporal scales, strongly suggest that gene flow from external sources is the driving force explaining the distribution of genetic diversity in these populations along time.

Furthermore, because of its pest nature, *H. armigera* populations are often exposed to severe selection pressure imposed by insecticide treatments (Avilla et al. 2006; Fitt 1989), and these could indicate that its populations are replaced season after season by insects from other non-treated areas. In the Andalusian region, there are three generations of this species per year, the first one being less important in number but more devastating (Avilla et al. 2006). For the control of *H. armigera* in cotton in southern Spain, endosulphan is the pesticide recommended, either alone or combined with metomile, or complemented with others pesticides (Avilla et al. 2006). In strawberry greenhouses in Huelva, an integrated production system is established which combines the use of *B. thuringiensis* formulates and other chemical pesticides (Boletín Oficial de la Junta de Andalucía, núm. 21, pág. 2188-2195). It is generally considered that high levels of gene flow may retard the evolution of insecticide resistance, because local populations are transfused with susceptible individuals. In contrast, high levels of gene flow could also help spreading resistance into susceptible populations if resistant insects had higher fitness costs under no selective pressure (Slatkin 1987). This is not normally the case since resistant insects have been associated with lower fitness cost when compared with susceptible populations (Bird and Akhurst 2004; Bird and Akhurst 2005; Carriére et al. 2001a; Carriére et al. 2001b).

H. armigera has the potential to migrate, but can also diapause as pupae in the soil close to the host plant after harvest, and emerge from overwintering in the following season. Previous studies with Australian *H. armigera* populations showed that migration could occur between relatively geographically distant areas due to their inherent capability of movement (Scott et al. 2005a; Scott et al. 2005b; Scott et al. 2006). Furthermore, this migration was demonstrated to be very variable depending on the season and area analyzed, and thus having different implications when trying to design appropriate strategies such as to avoid the evolution of resistance. Since the genetic structure and potential of resistance of Heliothis species may vary from one region to another, it is essential to determine which insects are to be targeted in control strategies, either those from the local population or migrant individuals.

The behavior of *H. armigera* populations from southern Spain in our study could be compared with that found in south-eastern Australian *Helicoverpa punctigera* populations (Duffield 2004). In these populations, the major source of variation seems not to come from local overwintering pupae which have survived from previous generations or seasons in the host crop. Both Heliothine species are capable of traveling hundreds of kilometers on high altitude winds, and in Australia, *H. punctigera* breeds during winter on flowering plants inland and moves to other parts of the country when vegetation dies off in late winter/spring (Duffield 2004). Consequently, the source of variability in south-eastern *H. punctigera* populations comes from other places far away

from the original crop field, diluting the risk of evolution of resistant to occur. All *H. armigera* populations in southern Spain studied could be considered as a metapopulation. Genetic structure in andalusian *H. armigera* populations is rebuilt every season after insecticide treatments pressure causes a bottleneck in the populations, and their source of variability seems to come from insects either inside or outside the cotton growing area, but not from overwintering pupae which have survived from a previous season. This phenomenon determines the strategies to be taken for the control of this species in case Bt cotton would be introduced in Spain.

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FIGURE LEGENDS

Figure 1. Neighbor-joining phylogram of the 19 *Helicoverpa armigera* populations with AFLP markers based on Nei's minimum genetic distance (Dm) (Nei, 1987). The numbers given after

population names represent the sampling season (1 and 2, before and after cotton harvest 2003, and 3 and 4, before and after cotton harvest 2004, respectively).

TABLES

TABLE 1: Summary of population genetic variation in *Helicoverpa armigera* using AFLP data. Samples were taken in two consecutive years before (Pre) and after (Post) cotton harvest, which were considered as 5 different collection seasons. Sample sizes were 25 individuals in all cases. Gene frequencies were calculated with Lynch & Milligan's method (1984).

POPULATION	Area	Season	Abbreviation	Number of polymorphic loci	Gene diversity within populations (Hj ± S.E*)
Alcalá del Río	cotton	2003-Pre	AR1	54	0.20 ± 0.02
		2003-Post	AR2	51	0.177 ± 0.018
		2004-Pre	AR3	67	0.227 ± 0.017
		2004-Post	AR4	51	0.163 ± 0.017
Carmona	cotton	2003-Pre	C1	54	0.20 ± 0.02
		2003-Post	C2	51	0.179 ± 0.018
		2004-Pre	C3	65	0.213 ± 0.017
		2004-Post	C4	50	0.162 ± 0.017
Los Palacios	cotton	2003-Pre	LP1	46	0.156 ± 0.018
		2003-Post	LP2	49	0.179 ± 0.019
		2004-Pre	LP3	72	0.286 ± 0.019
		2004-Post	LP4	54	0.177 ± 0.017
Moguer	Non cotton	2003-Pre	M1	37	0.107 ± 0.012
		2003-Post	M2	54	0.166 ± 0.017
		2004-Pre	M3	70	0.270 ± 0.019
		2004-Post	M4	42	0.128 ± 0.015
Lepe	Non cotton	2003-Pre	L1	52	0.168 ± 0.017
		2004-Post	L3	72	0.282 ± 0.019
		2004-Pre	L4	57	0.23 ± 0.02

* S. E.: Standard error of Hj's mean.

TABLE 2. Genetic structure of 5 *Helicoverpa armigera* populations. Samples were taken in two consecutive years before (Pre) and after (Post) cotton harvest. Total gene diversity (H_T), the average gene diversity within and among populations (H_w and H_b, respectively) and F_{ST} were calculated by the Lynch & Milligan's method (1984). All F_{ST} values were significant with a permutation test with 1000 random permutations.

POPULATION GROUPS	Number of groups (size)	H_T	H_W	H_b	F_{ST}
All populations	19 (25)	0.2408	0.19310	0.048	0.20**
	S.E[b]		0.01118	0.004	0.06
Populations from the same origin (AR/C/LP/M/L)[a]	5 (100[c])	0.2235	0.220	0.0036	0.02**
	S.E[b]		0.014	0.0013	0.4
Populations from the same season (2003-Pre/2003-Post/ 2004-Pre/2004-Post)	4 (125[d])	0.2395	0.20	0.0427	0.1774**
	S.E[b]		0.02	0.000000	0.000000
Populations 2003-Pre, from different regions (cotton/ non cotton)	2 (75,50)[e]	0.1807	0.16	0.0172	0.09*
	S.E[b]		0.03	0.000000	0.14
Populations 2004-Pre, from different regions (cotton/ non cotton)	2 (75, 50)[e]	0.2655	0.258	0.0072	0.03*
	S.E[b]		0.016	0.000000	0.06
Populations 2004-Post, from different regions (cotton/ non cotton)	2 (75, 50)[e]	0.1994	0.18	0.0164	0.08*
	S.E[b]		0.02	0.000000	0.1

[a] AR (Alcalá del Río); C (Carmona); LP (Los Palacios); M (Moguer); L (Lepe)
[b] S.E: Standard error of the mean
[c] Population size in L was 75
[d] Population size in 2003-Post was 100
[e] Size of first and second group separated by comas
* $P < 0.05$
** $P < 0.01$
*** $P < 0.005$

TABLE 3. Summary of analyses of molecular variance for *Helicoverpa armigera* populations from southern Spain with AFLP markers. All fixation indices were significantly greater than zero ($P < 0.05$) with a permutation test with 1000 random replicates, except those indicated.

Groups	Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation index
Populations from the same origin (AR/C/LP/M/L)[a]	σ^2 among	4	239.023	-1.17381	-7.17	-0.07165 *ns*
	σ^2 among populations within groups	14	2393.423	6.39178	39.02	0.36408***
	σ^2 within	456	5090.880	11.16421	68.15	0.31852***
Populations from the same season (2003-Pre/2003-Post/2004-Pre/2004-Post)	σ^2 among	3	1890.764	4.90461	27.87	0.27867***
	σ^2 among populations within groups	15	741.682	1.53125	8.70	0.12061***
	σ^2 within	456	5090.880	11.16421	63.43	0.36567***
Populations 2003-Pre, different regions (cotton/ non cotton)	σ^2 among	1	99.685	1.09594	9.32	0.09317 *ns*
	σ^2 among populations within groups	3	101.787	0.96924	8.24	0.09086***
	σ^2 within	120	1163.760	9.69800	82.44	0.17556***
Populations 2004-Pre, different regions (cotton/ non cotton)	σ^2 among	1	52.387	0.29615	1.94	0.01938 *ns*
	σ^2 among populations within groups	3	103.853	0.81818	5.36	0.05461***
	σ^2 within	120	1699.600	14.16333	92.71	0.07294***
Populations 2004-Post, different regions (cotton/ non cotton)	σ^2 among	1	125.733	1.05007	7.80	0.07804 *ns*
	σ^2 among populations within groups	3	188.187	2.09678	15.58	0.16901***
	σ^2 within	120	1237.120	10.30933	76.61	0.23386***

[a] AR (Alcalá del Río); C (Carmona); LP (Los Palacios); M (Moguer); L (Lepe)

ns $P \geq 0.05$

* $P < 0.05$

** $P < 0.01$

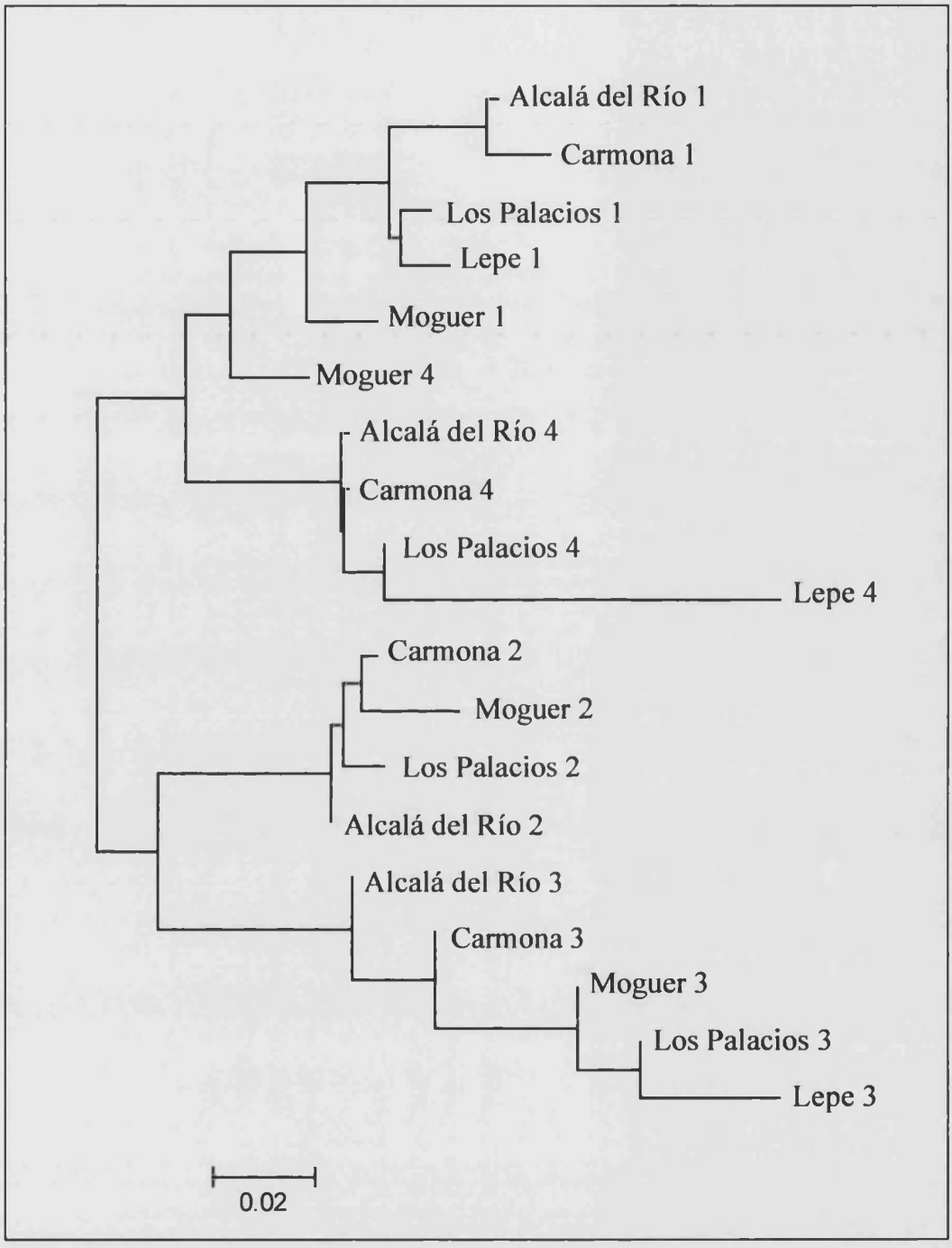
*** $P < 0.005$

TABLE 4. Population pairwise genetic distances. Pairwise F_{ST} (lower hemimatrix) and M values (M= Nm for haploid data, M= $2Nm$ for diploid data) (upper hemimatrix) were calculated with ARLEQUIN 3.1 version program (Schneider et al. 2000). All F_{ST} values were significant by a permutation test ($P < 0.05$) except those indicated (*). Population name abbreviations: AR (Alcalá del Río); C (Carmona); LP (Los Palacios); M (Moguer); L (Lepe). The numbers given represent their sampling season (1 and 2, before and after cotton harvest 2003, and 3 and 4, before and after cotton harvest 2004, respectively).

	AR1	AR2	AR3	AR4	C1	C2	C3	C4	LP1	LP2	LP3	LP4	M1	M2	M3	M4	L1	L3	L4
AR1		0,854	1,127	1,108	11,364	0,773	0,836	1,019	3,892	0,749	0,800	1,159	1,482	0,625	0,802	1,199	3,772	0,596	0,719
AR2	0,369		1,773	1,320	0,729	inf	1,725	1,173	0,903	405,635	1,078	1,161	1,082	6,501	1,220	1,129	0,971	0,820	0,558
AR3	0,307	0,220		1,293	1,048	1,614	14,676	1,222	1,185	1,156	6,073	1,300	1,124	1,442	9,140	1,710	1,126	3,040	0,735
AR4	0,311	0,275	0,279		0,944	1,172	1,183	inf	1,156	1,100	0,827	inf	1,571	0,951	0,927	1,778	1,229	0,662	1,258
C1	0,042	0,407	0,323	0,346		0,649	0,734	0,859	2,985	0,641	0,762	0,995	1,097	0,536	0,756	1,092	2,505	0,548	0,713
C2	0,393	-0,005*	0,237	0,299	0,435		1,696	1,070	0,764	11,783	1,052	1,048	0,893	6,480	1,219	0,935	0,854	0,827	0,514
C3	0,374	0,225	0,033	0,297	0,405	0,228		1,133	0,809	1,523	4,988	1,140	0,938	1,494	9,465	1,102	0,912	4,130	0,560
C4	0,329	0,299	0,290	-0,011*	0,368	0,319	0,306		0,997	1,009	0,787	inf	1,325	0,859	0,894	1,570	1,103	0,638	1,156
LP1	0,114	0,357	0,297	0,302	0,143	0,396	0,382	0,334		0,779	0,707	1,113	4,297	0,700	0,712	1,988	9,246	0,515	0,665
LP2	0,400	0,001*	0,248	0,313	0,438	0,041	0,247	0,331	0,391		0,971	0,997	0,929	4,398	1,083	0,962	0,847	0,775	0,531
LP3	0,385	0,317	0,076	0,377	0,396	0,322	0,091	0,389	0,414	0,340		0,825	0,627	0,907	20,199	0,796	0,718	11,379	0,598
LP4	0,301	0,301	0,278	-0,002*	0,334	0,323	0,305	-0,008*	0,310	0,334	0,377		1,330	0,849	0,932	1,618	1,197	0,675	1,546
M1	0,252	0,316	0,308	0,241	0,313	0,359	0,348	0,274	0,104	0,350	0,443	0,273		0,915	0,683	2,527	6,696	0,504	0,558
M2	0,445	0,071	0,257	0,344	0,483	0,072	0,251	0,368	0,417	0,102	0,355	0,371	0,353		1,069	0,856	0,789	0,760	0,442
M3	0,384	0,291	0,052	0,350	0,398	0,291	0,050	0,359	0,412	0,316	0,024	0,349	0,423	0,319		0,921	0,745	17,260	0,592
M4	0,294	0,307	0,226	0,220	0,314	0,349	0,312	0,242	0,201	0,342	0,386	0,236	0,165	0,369	0,352		1,716	0,594	0,741
L1	0,117	0,340	0,308	0,289	0,166	0,369	0,354	0,312	0,051	0,371	0,410	0,295	0,069	0,388	0,402	0,226		0,563	0,627
L3	0,456	0,379	0,141	0,430	0,477	0,377	0,108	0,439	0,493	0,392	0,042	0,426	0,498	0,397	0,028	0,457	0,470		0,466
L4	0,410	0,473	0,405	0,284	0,412	0,493	0,472	0,302	0,429	0,485	0,455	0,244	0,472	0,531	0,458	0,403	0,444	0,517	

inf: infinite value

FIGURES


Alcalá del Río 1
Carmona 1
Los Palacios 1
Lepe 1
Moguer 1
Moguer 4
Alcalá del Río 4
Carmona 4
Los Palacios 4
Lepe 4
Carmona 2
Moguer 2
Los Palacios 2
Alcalá del Río 2
Alcalá del Río 3
Carmona 3
Moguer 3
Los Palacios 3
Lepe 3
0.02

Ligand blot and genomic microarray analysis of resistant and susceptible Australian *Helicoverpa armigera* populations.

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ABSTRACT

Insect resistance to *Bacillus thuringiensis* toxins has been deeply studied to avoid their appearance in crop protection programs. A previous work demonstrated that there is a reduction on Cry1Ac binding sites in the Cry1A resistant *Helicoverpa armigera* strain ISOC by comparison with the almost isogenic susceptible strain (ANGR). In the present work, the resistance acquired by ISOC has been further investigated by ligand blot and microarray experiments to determine which molecules could be responsible for the resistance in the Australian resistant strain after being selected with *B. thuringiensis* Cry1Ac toxin under laboratory conditions. Cry1Aa, Cry1Ab and Cry1Ac toxins and *H. armigera* brush border membrane vesicles (BBMV) were used for the ligand blot assays and the results showed that no difference could be observed between the type of toxin and strain used. In all the experiments five bands ranging from 48 to 250 kDa were found to bind Cry1A toxins in the BBMV. For microarray experiments, RNA from midguts obtained from both ANGR and ISOC strains were used to be hybridized in the same array (based on ANGR ESTs midguts) to check their difference in expression. Among all the differences obtained, there were four expressed sequence tags (ESTs) with great reduced expression (higher than 100-fold less) in ISOC compared to ANGR, one of them with predicted transposase function. Another EST was found with more than 100-fold difference of expression in ISOC (EST number 1595), a candidate to be studied in depth for its possible relationship with Cry1Ac resistance in this strain.

All the results taken together and along with those obtained before by Akhurst et al., 2003, the resistance in ISOC might have been achieved as a result of different changes in the strain. This is the first approach using ligand blot and microarray technology to find out differences between two almost isogenic *H. armigera* strains, susceptible and the other one resistant to *B. thuringiensis* toxins.

INTRODUCTION

Helicoverpa armigera is one of the most important pests throughout different countries in Europe, Australia, Asia and Africa. The major threat of this species is the impact it causes on the cotton crop, one of the most economically important crops in the world, by leading to serious damage in different parts of the plants, and also decreasing the final production because of the damage it causes. It has been traditionally controlled with chemical insecticides and has evolved resistance to many of them (Kranthi et al., 2001a; Kranthi et al., 2001b; McCaffery et al., 1998). Alternatives to chemicals such as the use of insecticidal proteins produced by *Bacillus thuringiensis* have been studied for the last years and some of these toxins have been used for the efficient control of this species, although *H. armigera* is not very sensitive to *B. thuringiensis* toxins compared with some relative species (http://www.glfc.forestry.ca/bacillus), and only some toxins have been demonstrated to be effective. Many studies have shown that Cry1Ac is the most active protein against this pest (Chakrabarti et al., 1998; Liao et al., 2002; Padidam, 1992), and this

was one of the reasons why it is the active component of different insecticide formulations and it is the protein expressed in some transgenic cotton events.

The cotton industry and the scientific community are concerned about the possibility of *H. armigera* to develop resistance to Cry1Ac in the field and have put much effort on finding other alternatives for the control of this species. The potential of *H. armigera* and other species to develop resistance to Cry toxins under laboratory conditions have been demonstrated, but only one species, *Plutella xylostella* achieved this resistance in the field (Ferré and Van Rie, 2002). For that reason, understanding the mechanism of resistance in *H. armigera* Cry1Ac resistant strain and in other species can help to prevent evolution of resistance in the field after exposure to *B. thuringiensis* toxins. Among all the mechanisms of resistance developed by insects against *B. thuringiensis* toxins, the best studied one and which has been related with high levels of resistance and cross-resistance is the alteration of binding sites in the insect midgut (Ferré and Van Rie, 2002). Other mechanisms like alteration of proteinase activity in the midgut (Candas et al., Forcada et al., 1996; Li et al., 2004; Oppert et al., 1994; Oppert et al., 1997), and enhanced midgut cell reparation after toxin infection (Martínez-Ramírez et al., 1999) have only been related with low or medium levels of resistance.

H. armigera ISOC strain was selected with *B. thuringiensis* toxins and was demonstrated to have lost Cry1Ac major binding to BBMV prepared from insect midguts (Akhurst et al., 2003). This strain was also cross-resistant to Cry1Ab (more

resistant even than to Cry1Ac), and to Dipel and Xentari formulates (with low levels of resistance), but it was not resistant to Cry2Ab (Akhurst et al., 2003). Bioassays performed with ANGR (susceptible strain) and ISOC strain on cotton leaves expressing Bt toxins demonstrated that ISOC could develop completely feeding on transgenic cotton leaves but not the susceptible one (Bird et al., 2005). Furthermore, there was a fitness cost associated with Cry1A resistance in ISOC and the inheritance of resistance was demonstrated to be partially dominant (Bird et al., 2005).

This present work reports a study trying to determine the binding molecules related with resistance in a Cry1Ac resistant strain which has been shown to have lost binding affinity to the insect midgut (Akhurst et al., 2003). Ligand blot experiments were carried out as the first approximation to check differences in binding molecules between ANGR (susceptible) and ISOC *H. armigera* strains. Furthermore, a microarray system with 4984 printed ESTs from *H. armigera* ANGR strain was utilized to detect gene expression differences between two almost isogenic *H. armigera* strains, one susceptible and the other one Cry1Ac resistant. The microarray construction contained different kind of insect molecules which participate in several insect pathways, including some of the candidates molecules that have been related with Cry toxin binding and resistance by other authors, such as aminopeptidases N and cadherin (Banks et al., 2001; Denolf et al., 1997; Gill et al., 1995; Hua et al., 2001; Ingle et al., 2001; Jenkins and Dean, 2001; Knight et al., 1994;

Lee et al., 1996b; Liao et al., 2005; Luo et al., 1997; Masson et al. 1995a; Oltean et al., 1999; Rajagopal et al., 2003; Sangadala et al., 1994; Valaitis et al., 1995)

MATERIALS AND METHODS

B. thuringiensis strains and production

Cry1Ac was obtained from the *B. thuringiensis* HD73 strain, and provided by D. Pinnock (University of Adelaide, South Australia). A clone of the *cry1Ab* gene of the subsp. *kustaki* HD-1 in *Escherichia coli* (ER1648) was used for the production of Cry1Ab. Cry1Aa toxin was produced from a *B. thuringiensis* strain after cloning *cry1Aa* gene which was obtained from the NRD-12 strain of *B. thuringiensis*. Strains producing Cry1Aa and Cry1Ab toxins were kindly provided by L. Masson (National Research Council, Montreal, Canada). All toxins were cultured and obtained as described by Akhurst et al., 2003.

Insect strains

The susceptible laboratory strain ANGR was generated by crossing AN02 with GR, two laboratory *H. armigera* strains, and then maintained under laboratory conditions (Liao et al 2002; Akhurst et al., 2003). The GR strain was provided by Dr. J. Daly (CSIRO Entomology, Canberra). The Cry1Ac resistant strain, ISOC, was a nearly ANGR isogenic line derived after mating insects from BX and ANGR strains followed by selection with Cry1Ac (Bird et al., 2004). The original BX strain from

which ISOC derived was founded by crossing field-collected insects with AN02, and its resistance allele(s) was/were introduced in ANGR by continuous crosses with ANGR (Bird et al., 2004). ISOC, in the fourth generation of backcrosses between BX and AN02, shared least 95% of its genome with ANGR, and the following backcrosses would have increased the similarity between both strains.

Preparation of brush border membrane vesicles (BBMV)

Last instar larvae were dissected form ANGR and ISOC strains to obtain the insect midguts, which were frozen in liquid nitrogen, and kept at -80ºC until required. Brush border membrane vesicles (BBMV) were prepared by the $MgCl_2$ precipitation method (Wolfersberger et al., 1987).

Ligand blot assays

BBMV (100 µg) from each strain were centrifuged at 14000 rpm to eliminate the MET buffer (250 mM mannitol, 17 mM Tris-HCl, 5 mM EGTA, pH 7.5), suspended with 10 µl cold electrophoresis buffer and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then electro-transferred onto a PVDF Hybond-P membrane (Amersham, Piscataway, NJ) and blocked overnight with 3% BSA, 5% skimmed milk in TPBS buffer (1 mM KH_2PO_4; 10 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl, pH 7.4, 0.1 % Tween-20). Pre-stained marker (10 µl) (Amersham) was included in each experiment as a reference. The membranes were cut into different pieces containing the separated BBMV proteins and incubated with 1 µg/ml trypsin activated Cry1Aa, Cry1Ab or Cry1Ac toxins in

TPBS with 0.1 % BSA for 2 h. Non-bound toxin to the BBMV was removed after washing the membrane three times with TPBS, 0.1 % BSA. The membrane was then incubated with the Cry1A antibody (1:4000), washed three times with TPBS with 0.1 % BSA, and incubated with anti-mouse IG from rabbit/mice horseradish peroxidase conjugate (Amersham), for 1h following the manufacturer's recommendations. After washing, bands containing the proteins where the toxin binds to the BBMV were detected with a chromogenic reaction with tetramethylbenzidine (TMB) substrate protected from light.

H. armigera **RNA extraction and quantification**

Ten fourth instar larvae from either ANGR or ISOC *H. armigera* strains were dissected to obtain the midgut used for one RNA extraction in freshly prepared cold MET buffer (250 mM mannitol, 17 mM Tris-HCl, 5 mM EGTA, pH 7.5). After washing the midguts with MET buffer, messenger RNA (mRNA) extraction was performed with the RNeasy kit (Eppendorf, New York, NJ), and further purified using the RNeasy MiniElute Cleanup kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The quantity and quality of mRNA was determined by spectrophotometry (A_{260} nm/A_{280} nm, 1.9-2.1) and by 1% agarose gel electrophoresis under denaturing conditions in formaldehyde.

Reverse transcription (RT) and probe purification

Double strand DNA (cDNA) was prepared from 25 µg mRNA by adding 8 µl of 5x first-strand RT buffer, 3 µl 20 mM dATP, dCTP and dGTP, 3 µl 2mM dTTP, 3 µl

2mM AA-dUTP, and 4 µl of 0.1 mM DTT, to the RNA/primer mix to a final volume of 40 µl. The mix was then incubated at 65°C for 5 minutes followed by the addition of 1 µl of Superscript II reverse transcriptase (Invitrogen, Carisbad, CA) and another 2 h incubation period at 42ºC. Samples were then incubated at 65°C for 30 minutes after the addition of 8 µl of 0.1 M NaOH to hydrolyze and remove any remaining RNA, and neutralized by adding 8 µl of 1 M HCl and 4 µl Tris-HCl, pH 7.5. The neutralized reactions were then purified using a Microcon 30 concentrator (Qiagen), split in two aliquots for the labeling with both fluorescent dyes (Cy3-dCTP and Cy5-dCTP) and dried completely in a speed vacuum.

cDNA labelling reaction and quantification

cDNA (0.5 µg) was dissolved in 20 µl of 0.1 M sodium bicarbonate, pH 9.0, before mixing them with the Cy3-dCTP and Cy5-dCTP dyes (Amersham). After one hour incubation time, the reaction was quenched by adding 5 µl of 4 M hydroxylamine and further incubated 15 minutes. All the steps were carried out protected from light to avoid the degradation of the dyes. The amount of target cDNA and the labeled nucleotides incorporated was obtained by measuring the absorbance of samples with a spectrophotometer at 260, 550 and 650 nm. The amount of target cDNA and the Cy3 and Cy5 dyes incorporated in each sample was calculated with the following formulas: [cDNA (ng) = A_{260} x 37 x total volume of cDNA (µl)]; [cDNA-Cy3 (pmols) = A_{550} x cDNA (µl)/0.15]; [cDNA-Cy5 (pmols) = A_{550} x cDNA (µl)/0.15].

H. armigera library construction

H. armigera microarray constructions used in the experiments were provided by Karl Gordon (CSIRO, Canberra). *H. armigera* cDNA plasmid DNA used for the production of microarrays consisted of 4984 expressed sequence tag (EST) clones obtained from different stages of instar larvae and derived from three different *H. armigera* midgut cDNA libraries, all of them prepared with ANGR strain. Library sequencing was done either at CSIRO Entomology with an automated sequencer or at the AGRF in Brisbane on an ABI 377. Clones were sequenced from the 5' end using CEQ™ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter). Sequencing reactions contained, in 10 µl total volume, 100-300 ng plasmid DNA, 2 µl Quick Start Master Mix, 2 µl Better Buffer (Microzone Ltd, West Sussex, UK), and 10 pmol reverse primer. All sequence traces were read using Phred (Ewing et al. 1998) to yield sequence and quality data in FASTA format. The quality scores were automatically evaluated using Perl scripts and all sequences with a minimum Phred20 score >100 collated. This was done on a semi-automatic basis using the relational database carrying all sequence and clustering data (the Stackpack output). For microarray printing, clones were amplified and their products were purified and dried down in by vacuum centrifugation and resuspended in 6 µl of 50% DMSO. Resuspended clones were printed onto CMT-GAPS II™ coated slides (Corning, Corning NY) using a VersArray ChipWriter Pro microarrayer (BioRad, Philadephia, PA) with Stealth SMP3 pins (Telechem

International, Sunnyvale, CA). Printing was performed at room temperature and 60% humidity.

cDNA array hybridization, detection and analysis

Each slide, containing two printed microarrays, was baked at 80ºC for 3-4 h before the addition of the labeled probes. After that, they were incubated with warm pre-hybridization solution (50 % formamide, 5x SSC, 0.1 % SDS, 0.1 mg/ml BSA) at 42 ºC for 60 minutes, washed twice with 0.1 x SSC for 5 minutes, and with milliQ water for 30 seconds at RT. Labeled cDNA (20-25 pmols) were incubated for 5 minutes at 95 ºC in 25 µl hybridization solution (50 % formamide, 0.1 mg/ml salmon herring sperm DNA, 5x SSC, 0.05 µg/µl polyA blocken, 0.1 % SDS) before the hybridization reaction with the microarray for 16 h at 42 ºC. Before the final detection, the slides were washed several times with different solutions: once with 2 x SSC, 0.1 % SDS for 2 minutes at 42 ºC; 2 x SSC, 0.1 % SDS for 5 minutes at 42 ºC; twice with 0.1 x SSC, 0.1 % SDS for 5 minutes at RT; four times with 0.1 x SSC, 0.1 for 1 minute at RT; and once with 0.01 x SSC for 10s at RT. Once the slides were dried at RT, they were scanned using the GenePix 4000B apparatus (Axon Instruments, Union city, CA) with the GenePix.Pro 6.0 software (Axon Instruments). Further normalization, comparison between different arrays, the dye swap and their analysis were performed with the Acuity 4.0 software (Molecular Devices, Downington, PA). Data were expressed as the $\log_2$ ratio of fluorescence intensities in all samples; the raw data without the background were also analyzed to check the presence of signal in

one of the dyes but not in the other. After that, data were filtered to exclude elements that did not have at least a 1.5-fold intensity/background ratio in at least 5 of the 6 arrays analyzed, as we are looking for great differences between ANGR and ISOC strains, present in all the arrays.

Experimental design and analysis of microarray data

Each cDNA sample from ANGR and ISOC strains was labeled with both dyes (Cy3 and Cy5) and used to be hybridized in the same microarray to check the different expression of each strain in all the printed EST for the experiment. Samples from one strain labeled with one of the dyes and from the other strain labeled with the other dye were combined and hybridized in the same microarray to check the difference in gene expression between them. The opposite experiment was also carried out to minimize the effect of dyes with the other combination strain-dye in the other microarray printed in the same slide. Three samples of both strains were analyzed and this gave six replicates after doing the dye swap with the Acuity 4.0 software (Molecular Devices).

RESULTS AND DISCUSSION

Ligand blotting analysis with Cry1Aa, Cry1Ab and Cry1Ac

We performed ligand blotting experiments with BBMV proteins from the two strains to determine which proteins were associated with Cry1Ac resistance in the *H. armigera* ISOC resistant strain (figure 1). After running the BBMV in an SDS-

PAGE and transferring the proteins onto a PVDF membrane, trypsin-activated Cry1Aa, Cry1Ab and Cry1Ac were found to bind *H. armigera* BBMV proteins in both strains in a similar way. There were five BBMV proteins ranging from 48 to 250 KDa in the ligand blotting results after incubation the membrane with trypsin-activated Cry1Ac in ANGR and ISOC strains (fig. 1), and the proteins were approximately around 48, 78, 100, 210 and 250 KDa by comparison with the molecular marker included as a reference. The results obtained with the other two proteins tested, Cry1Aa and Cry1Ab, shown five bands with similar molecular weights in either both strains, indicating that at this level, there are not differences regarding the source of Cry1A toxin used in the experiment.

A previous study performed with the ISOC strain revealed reduced high affinity Cry1Ac binding to BBMV prepared from insect midguts compared with the ANGR susceptible strain (Akhurst et al., 2003). It was demonstrated that ISOC was cross-resistant to Cry1Ab and to two commercial formulations based on *B. thuringiensis* Cry proteins (XenTari and Dipel), but not to Cry2A toxins. In another work, a Chinese GYBT *H. armigera* Cry1Ac resistant strain was shown to be cross-resistant to Cry1Ab and also to Cry1Aa, but not to Cry2Aa (Xu et al., 2005). All these results and the data obtained from binding experiments carried out with several Spanish susceptible populations (Estela et al., 2004) indicate that Cry1A toxins have common binding sites in *H. armigera* and that these receptors could not be shared by the Cry2 group proteins. The fact that Cry1A and Cry2A toxins do not

share binding sites in this and other species is not surprising since there has been proposed that these toxins have a different mode of action (English et al., 1994; Jurat-Fuentes et al., 2003; Karim et al., 2000). Results reported here show an apparent disagreement with previous data obtained with the same *H. armigera* resistant strain as our results with ligand blot experiments demonstrated Cry1A in vitro binding to BBMV denatured proteins in the resistant strain. These differences are found as methodologies used in each procedure are different. BBMV have to be denatured before their separation in an SDS-PAGE in ligand blotting experiments, and many proteins with the same or similar molecular weights could interact with Cry toxins there, but this phenomenon does not happen when binding experiments are performed in solution. On the other hand, in vitro binding experiments carried out in solution do not need any previous treatment of the vesicles and only some molecules could interact directly with toxins. It is well known that these differences could be found when using binding experiments with BBMV in solution and for ligand blot (Lee et al., 1996a). Furthermore, other three studies performed separately with different techniques with the same strain of a different species. *P. xylostella,* also support the fact that different experimental approaches could result in different results (Escriche et al., 1995; Ferré et al., 1991; Masson et al., 1995b). In the present work, the bands in the membrane, after being compared with a molecular marker as a reference, were approximately around 48, 78, 100, 210 and 250 KDa. Further preliminary analysis of these bands where Cry1A toxins bound to with the MALDI-

TOF system (data not included) showed the coexistence of several molecules in the same band since they could have not been well resolved with one-dimensional SDS-PAGE system. It is known that the vesicles have a complex protein composition consisting in proteins with overlapping molecular sizes that cannot be completely separated with one-dimensional SDS-PAGE. These preliminary results obtained with the UV-laser desorption mass spectrometry in our work revealed the coexistence of several proteins in all the analyzed bands where Cry1A toxins bound to either in ANGR and ISOC, such as aminopeptidase N and alkaline phosphatase, two of the candidate molecules previously related to Cry binding proteins in the literature (Banks et al., 2001; Denolf et al., 1997; Fernández et al. 2006; Gill et al., 1995; Hua et al., 2001; Ingle et al., 2001; Jenkins and Dean, 2001; Jurat-Fuentes and Adang., 2004; Knight et al., 1994; Lee et al., 1996b; Liao et al., 2005; Luo et al., 1997; Masson et al. 1995a; Oltean et al., 1999; Rajagopal et al., 2003; Sangadala et al., 1994; Valaitis et al., 1995). Results found in previous studies along with ours indicate that determining the relationships between toxin binding sites by only one technique might not be conclusive to find out the mechanisms of resistance in a strain.

Different EST expression in ANGR and ISOC strains

The cDNA library used for the experiment has 4984 expression sequence tags (ESTs) obtained from midguts of the *H. armigera* ANGR strain. These ESTs were printed on slides to check the different expression between two almost isogenic *H. armigera* strains (ANGR and ISOC) after hybridizing the labeled cDNAs from both strains

with different dyes on the same slide. We focused our experiments in finding any possible large difference in expression between both strains without exposure to Cry toxins, to check the inherent differences between them. For that purpose, only differences in expression (expressed as the log2 ratio intensity between both strains) higher than 1.5 or smaller than -1,5 were included in our results, which meant at least 4.5 fold upregulation or downregulation, respectively, in the resistant strain. Taken ANGR as a baseline for the EST expression (as it is the strain used for the microarray system construction), some ESTs (numbers given in parenthesis) were found downregulated (table 1) and others were upregulated (table 2) in the resistant strain. Most of EST functions in our results were unknown (Fig. 2 and 3). Furthermore, some different ESTs belonging to proteins with the same or similar function have different level of expression in both strains. Among them, ESTs encoding for proteins related with lipase activity were either downregulated (1984) and upregulated (1330, 1331, and 3648) in ISOC indicating that Cry1Ac resistance found in ISOC might not be related to an alteration in this function. Another downregulated function in the resistant strain was the alcohol dehydrogenase (1440), an important enzyme that participates in detoxifying pathways. A phenol UDP-glucosyltransferase (486), important in glycosilation and also in detoxifying processes, has also a lower level of expression in ISOC. In addition, other enzymes found with greater expression in ISOC than in the susceptible strain were also related with detoxifying processes: some ESTs with predicted glutathione-S-

transferase (GST) activity (295, 957, and 1138), and also another EST with aldehyde dehydrogenase predicted function (3249). GSTs are considered to be important detoxification enzymes as they catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophiles (xenobiotic substrates). Apart from the similar activities either up- and downregulated in both of the strains, many were the differences found between both of them in our experiments. The most important difference between them with known function was the expression of a transposase fragment (3136) that was found very low in ISOC, after being compared with ANGR expression (less than 215 fold). Furthermore, several ESTs with metalloproteinase associated function were also lower in ISOC, four of them with aminopeptidase-N activity (977, 3213, 3214, and 4299) and the other one was a midgut carboxipeptidase (1690). In addition, some other activities that were also found downregulated in the resistant strain such as a carotenoid binding protein, were related with steroid byosinthesis (1232), and a desaturase (1142), which constitutes one of the central lipogenic enzymes that catalyse the insertion of a double bond at the delta position of fatty acids. The EST 3422, with lysozyme activity associated and related with immunity and defense, was found to have also around 5 fold lower expression in ISOC. Other 25 ESTs from the microarray with current unknown predicted function were downregulated in the resistant strain, one of them with an intensity of 5,6 (4061), the second highest difference found in both strains, and many with intensities higher than 2, all of them candidates for further studies as

their difference in expression is important. Furthermore, seven ESTs from the ones detailed before with unknown function, are different parts of the same final predicted molecule, indicating that this could be a good candidate to study in depth that might ascertain the possible differences between both strains as it is consistent along the array.

Among the upregulated EST in the resistant strain, some were found to encode for serine proteinases like trypsin and chymotrypsin (867, 1036, 1594, 2129, and 3670), for digestive metalloproteinases with predicted astacin activity (1837 and 3299), and for another kind of predicted proteinase enzyme but with unknown biological function (4645). The amylase activity (3353, 3497, 3651, and 4779), important for carbohydrate metabolism by cleaving 1,4-glucose linkages, and two different glucosyltransferases, an ecdysteroid UDP-glucosyltransferase (1386), and an UDP-N-acetyl-alpha-D-galactosamine (1181), were also upregulated in the resistant strain. The participation of enzymes related with glycosilation pathways have been previously related to Cry toxin binding and resistance (Griffitts et al., 2001; Griffitts et al., 2003; Griffitts et al., 2005). In addition, the expression of the parvovirus non-structural protein NS1 were also upregulated in ISOC compared with ANGR. The EST number 3676, predicted to be related with armadillo protein function, one component of the multiprotein complex that either maintain or initiate formation of sheets of epithelial cells and form part of the adherent junction structure, had a greater expression in ISOC. The alteration of the cadherin, another

molecule which participates in the adherent junction structure, has also been related with resistance to Cry toxins in this and other some insect species (Gahan et al., 2001; Hua et al., 2001; Ihara et al., 1998; Jenkins and Dean, 2001; Jurat-Fuentes et al., 2004; Keeton and Bulla, 1997; Morin et al., 2003; Nagamatsu et al., 1998; Vadlamudi et al., 1993; Wang et al., 2005). Another interesting EST upregulated in the resistant strain was a predicted high mobility group protein D (HMG-D) (4667), known to have high affinity for bent or distorted DNA and bent linear DNA. Other 18 ESTs, including one with predicted lipocalin activity (4665), with unknown o uncertain function had also higher expression in the resistant strain. Among all of the EST with unknown function upregulated in the resistant strain, there were four with intensities higher than 2, one of them with a intensity around 6.5 (the highest between both strains), very interesting for further analysis that might help to explain the appearance of Cry1Ac resistance in ISOC. Other two EST from the unknown activity list (555, 987) and belonging to the same final molecule were also upregulated in the ISOC.

After clustering the data with similar or related functions from the microarray experiments in a cake-shaped graph, it was found that 69 % of the downregulated (fig. 2) and 42 % of the upregulated (fig. 3) ESTs have an unknown predicted function. Another important group of molecules found either downregulated (fig. 2) or upregulated (fig. 3) in ISOC in our results was related with proteolysis and peptidolysis. There was a reduction in the aminopeptidase N expression in the

resistant that could not be seen in the ligand blot experiments as the amount of mRNA and their translation to proteins are not always totally related. This kind of transmembrane ectopeptidase molecules has previously been associated as Cry receptor molecules (Banks et al., 2001; Denolf et al., 1997; Gill et al., 1995; Hua et al., 2001; Ingle et al., 2001; Jenkins and Dean, 2001; Knight et al., 1994; Lee et al., 1996; Liao et al., 2005; Lorente et al., 1997; Luo et al., 1997; Masson et al. 1995a; Oltean et al., 1999; Rajagopal et al., 2003; Sangadala et al., 1994; Simpson and Newcomb, 2000; Valaitis et al., 1995; Yaoi et al., 1997) but not with the appearance of resistance to *B. thuringiensis*. ISOC has also an increased expression of trypsin and chymotrypsin proteins, two important digestive proteinase enzymes known to participate in Cry toxin activation in the insect midgut. Since ISOC has been selected with non-trypsin activated toxins, possible changes in the resistant strain could include not also alteration of binding in the insect midgut but also other mechanisms previous to binding, as the alteration of proteinases in the insect midgut. This is not the first work where a resistant strain has been associated with variations in the composition or activity of the midgut proteolytic enzymes, a phenomenon normally related with low or medium level of resistance to *B. thuringiensis* toxins. In previous works with other resistant strains, it was found either a reduction or an increase in the proteinase activity in the resistant strain after being compared with a susceptible one from the same species (Candas et al., 2003; Forcada et al., 1996; Li el al, 2004; oppert et al., 1994; Oppert et al., 1997).

Here, we found higher expression of different ESTs, encoding for serine proteinases such as trypsin and chymotrypsin, in ISOC that could change the way the toxin is activated in the insect midgut and might vary the final activity of the enzyme. Furthermore, another group of different ESTs but all of them participating in lipid metabolism pathways were also found downregulated (fig. 2) or upregulated (fig. 3) in ISOC. The group belonging to glycosilation and detoxification molecules upregulated in the resistant strain was higher than in ANGR, but some of them (5 %) were downregulated in the resistant strain (fig. 2 and 3). The other minor groups of molecules clustered together that were downregulated in ISOC were transposition, steroid byosintesis metabolism, and immunity and defense (fig. 2). On the other hand, the groups found only upregulated in ISOC were related with carbohydrate methabolism, viral infection, DNA reparation, and participating in the adherent junction structure (fig. 3).

The fact that ISOC has an increased expression of molecules related with proteolysis and peptidolysis, lipid and carbohydrate metabolism, DNA reparation and detoxification might indicate that selection pressure could have forced the insect to adapt to the new situation by modifying their own metabolism. Taken all the results showed in the present work along with the ones obtained by Akhurst et al. (2003) might indicate that resistance in the ISOC strain could be explained by a reduction in Cry1Ac binding affinity in the insect midgut, but additional factors

may be participating as other genes have changed their expression after *H. armigera* selection with Cry1Ac.

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TABLES

TABLE 1. Downregulated EST in the ISOC strain by comparison with ANGR. Intensities are expressed as the mean of log2 ratio between both strains. Negative signs have been removed from the intensity values.

EST NUMBER	GIVEN NAME	PREDICTED FUNCTION	BIOLOGICAL PROCESS	INTENSITY (mean)
4061	no_match	unknown	unknown	5,607
3136	Transposase (Fragment)	Transposase	Transposition	5,389
3048	no_match	unknown	unknown	4,817
2459	no_match	unknown	unknown	3,444
137	no_match	unknown	unknown	3,395
4925	no_match	unknown	unknown	2,653
486	Phenol UDP-glucosyltransferase	Phenol UDP-glucosyltransferase	Glycosilation/ Detoxification	2,604
2244	no_match	unknown	unknown	2,435
1440	CG7675-PB, isoform B	Alcohol deshidrogenase	Detoxification	2,295
4462	CG30413-PA	unknown	unknown	2,103
3426	CG30413-PA	unknown	unknown	2,055
3427	CG30413-PA	unknown	unknown	2,015
2686	CG30413-PA	unknown	unknown	1,837
3733	CG30413-PA	unknown	unknown	1,812
3734	CG30413-PA	unknown	unknown	1,787
4280	CG30413-PA	unknown	unknown	1,509
1385	no_match	unknown	unknown	2,009
232	no_match	unknown	unknown	1,968
2239	no_match	unknown	unknown	1,931
4649	no_match	unknown	unknown	1,897
3604	no_match	unknown	unknown	1,862
3024	no_match	unknown	unknown	1,808
727	CG9954 protein	unknown	unknown	1,773
1984	Lipase-1	Lipase	Lipid metabolism	1,702
3213	HaAPN-3	Aminopeptidase-N	Proteolysis and peptidolysis	1,694
977	HaAPN-3	Aminopeptidase-N	Proteolysis and peptidolysis	1,652
3214	HaAPN-3	Aminopeptidase-N	Proteolysis and peptidolysis	1,644
4299	HaAPN-3.	Aminopeptidase-N	Proteolysis and peptidolysis	1,643
1232	Carotenoid-binding protein.	Steroid byosinthesis	Steroid byosinthesis	1,681
1690	Midgut	Midgut	Proteolysis and	1,651

	carboxypeptidase 1 (Fragment)	carboxypeptidase	peptidolysis	
1183	no_match	unknown	unknown	1,646
2787	no_match	unknown	unknown	1,643
1142	Desaturase	Desaturase	Fatty acid anabolism	1,634
3587	Bmb037792	unknown	unknown	1,624
3422	Lysozyme	Lysozyme	Cell wall catabolism	1,613
2810	no_match	unknown	unknown	1,585
983	Bmb020895	unknown	unknown	1,563

TABLE 2. Upregulated EST in the ISOC strain by comparison with ANGR. Intensities are expressed as the mean of log2 ratio between both strains.

EST NUMBER	GIVEN NAME	PREDICTED FUNCTION	BIOLOGICAL PROCESS	INTEN-SITY (mean)
1595	no_match	unknown	unknown	6,524
1036	Trypsin	Trypsin	Proteolysis and peptidolysis	3,826
2129	Trypsin	Trypsin	Proteolysis and peptidolysis	3,397
867	Serine proteinase	Serine proteinase	Proteolysis and peptidolysis	2,754
1594	HzC20 chymotrypsinogen	Chymotrypsin	Proteolysis and peptidolysis	2,118
3670	Chymotrypsin	Chymotrypsin	Proteolysis and peptidolysis	1,904
957	Glutathione S-transferase	Transferase activity	Detoxification	2,775
295	Glutathione S-transferase	Transferase activity	Detoxification	2,278
1138	Glutathione S-transferase	Transferase activity	Detoxification	1,701
4665	Gallerin	Lipocalin protein	unknown	2,199
796	Viral protein Parvovirus non-structural protein NS1	Parvovirus non-structural protein NS1	Viral infection	2,187
673	DnV Ns-1	Parvovirus non-structural protein NS1	Viral infection	1,667
1480	Bmb006598	unknown	unknown	2,129
953	no_match	unknown	unknown	2,126
1386	Ecdysteroid UDP-glucosyltransferase	Transferase activity	Glycosiation/ Detoxification	2,084
3353	Alpha-amylase-2	Alpha-amylase	Carbohydrate metabolism	2,005
3651	Alpha-amylase	Alpha-amylase	Carbohydrate metabolism	1,748
4779	CG14935-PB, isoform B	Alpha-amylase	Carbohydrate metabolism	1,523
3497	Amylase	Amylase	Carbohydrate metabolism	1,513
1201	CG6129-PB, isoform B	unknown	unknown	2,003
3594	no_match	unknown	unknown	1,977
4937	no_match	unknown	unknown	1,969
1331	Lipase-1	Lipase	Lipid metabolism	1,966
3648	Lipase	lipase	Lipid metabolism	1,96

1330	Alpha/beta-hydrolipase	Alpha/beta-hydrolipase	Lipid metabolism	1,649
987	CG3934 CG3934	unknown	unknown	1,902
555	CG3934 CG3934	unknown	unknown	1,676
4773	CG17259-PA (LP20978p)	unknown	unknown	1,88
3299	CG15255-PA Hypothetical protein BG:BACR44L22.1	Astacin activity	Proteolysis and peptidolysis	1,844
1837	CG15255-PA Hypothetical protein BG:BACR44L22.1	Astacin activity	Proteolysis and peptidolysis	1,75
1181	UDP-N-acetyl-alpha-D-galactosamine	Transferase activity	Glycosylation/ Detoxification	1,723
2637	CG9009-PA (BcDNA GH02901)	unknown	unknown	1,722
1335	no_match	unknown	unknown	1,681
3676	Hypothetical protein Armc7 (Mus musculus 3 days ne)	Related to Armadillo	Adherent junction	1.67
4492	no_match	unknown	unknown	1,663
3249	CG14120 protein	Aldehyde dehydrogenase	Detoxification	1,644
4767	Hypothetical LOC496658	unknown	unknown	1,633
4667	High mobility group protein D (HMG-D).	Regulation of transcription.	DNA reparation	1,617
1606	Bmb034160	unknown	unknown	1.612
3850	no_match	unknown	unknown	1,581
4645	CG9849 protein (RE13814p)	Peptidase activity	Proteolysis and peptidolysis	1,551
1635	no_match	unknown	unknown	1,54
386	Bmb031223	unknown	unknown	1,53

FIGURES

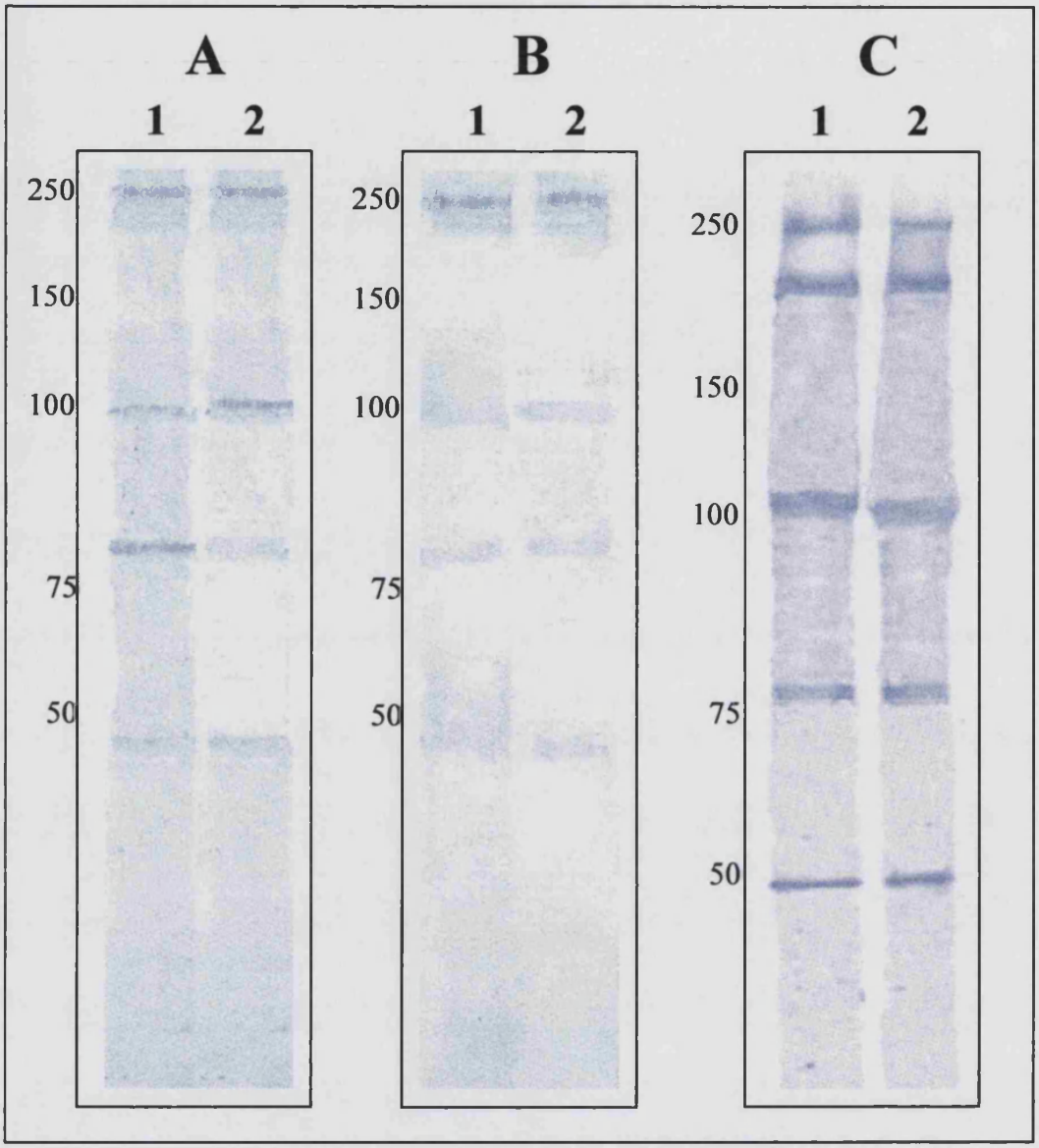

Fig.1. Ligand blotting with Cry1Aa (A), Cry1Ab (B), and Cry1Ac (C) and *H. atmigera* BBMV. Lanes 1. ANGR BBMV, and lanes 2. ISOC BBMV. Molecular marker given on the left.

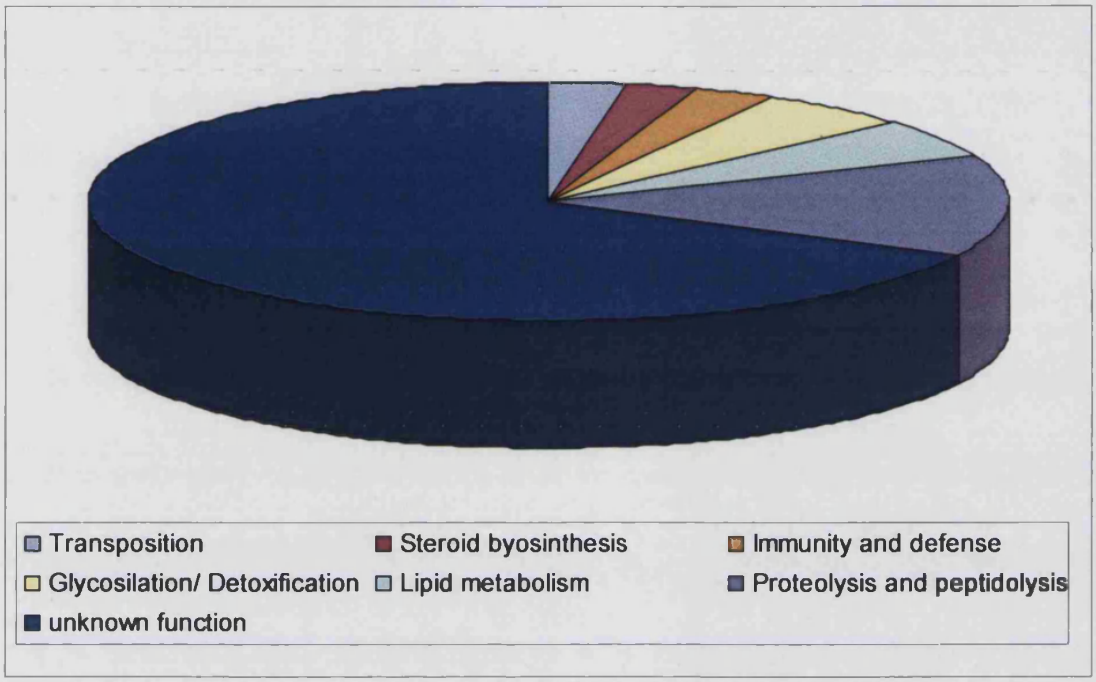

Fig. 2. Downregulated functions in the ISOC strain by comparison with ANGR. Each group represents several ESTs with related function from table 1.

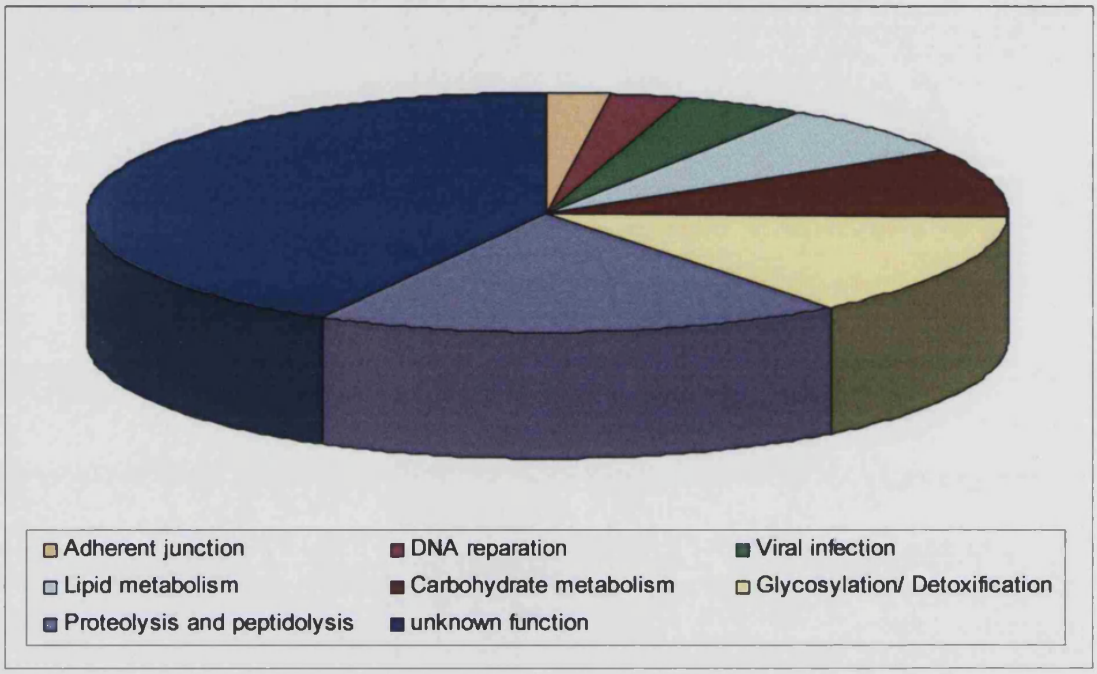

Fig. 3. Upregulated functions in the ISOC strain by comparison with ANGR. Each group represents several ESTs with related function from table 2.

UNIVERSITAT DE VALÈNCIA FACULTAT DE CIÈNCIES BIOLÒGIQUES

Reunido el Tribunal que suscribe, en el día de la fecha acordó otorgar a esta Tesis Doctoral de

D./Dª. **ANNA ESTELA BOLTA**

la calificación de Excellent cum laude

Valencia, a 12. de enero de 2007

EL/LA SECRETARIO/A,

EL/LA PRESIDENTE/A

Drª. Dª. Ana González Garrido

Dr. D. Pedro Castañera Domínguez