VNIVERSITAT DE VALÈNCIA

Caracterización molecular del mecanismo de patogénesis de las toxinas Cry de *Bacillus thuringiensis* activas contra insectos coleópteros

Tesis doctoral

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INFORMAN: Que Dña. ESTEFANÍA CONTRERAS NAVARRO, licenciada en Bioquímica por la Universitat de València, ha realizado bajo nuestra dirección el trabajo de investigación recogido en esta memoria que lleva por título "Caracterización molecular del mecanismo de patogénesis de las toxinas Cry de *Bacillus thuringiensis* activas contra insectos coleópteros" con el fin de optar al grado de Doctora por la Universitat de València dentro del programa de Doctorado en Biotecnología y,

AUTORIZAN su presentación en el Departamento de Genética de la Facultad de Ciencias Biológicas para tal fin.

Para que así conste, y en cumplimiento de la legislación vigente, firmamos el presente certificado en Burjassot a 20 de septiembre de 2013.

Dra. María Dolores Real García

Dra. Carolina Rausell Segarra

Dra. MARÍA DOLORES REAL GARCÍA, Catedrática de Universidad, y Dra. CAROLINA RAUSELL SEGARRA, profesora titular de Universidad, del Departamento de Genética de la facultad de Ciencias Biológicas de la Universitat de València,

CERTIFICAN: Que las publicaciones presentadas en esta memoria por Dña. ESTEFANÍA CONTRERAS NAVARRO para optar al grado de doctora no han sido utilizadas en otra tesis doctoral y en aquellas publicaciones en las que no figura como primera autora, su trabajo consistió en:

- La producción y purificación de la toxina Cry3Ba y la realización del análisis proteolítico de la misma, tras la interacción con las proteasas de membrana del intestino de *Leptinotarsa decemlineata*, presentados en el artículo: García-Robles *et al*., 2012, Toxicon 60: 1063-1071.

- El silenciamiento de la prohibitina 1 mediante la ingestión de RNA de doble cadena en larvas de *L. decemlineata* combinado con el tratamiento con dos dosis diferentes de la toxina Cry3Aa de *Bacillus thuringiensis*, presentado en el artículo: Ochoa-Campuzano et al., 2013, Pest. Biochem. Physiol. DOI: 10.1016/j.pestbp.2013.09.001.

Para que así conste, y en cumplimiento de la legislación vigente, firmamos el presente certificado en Burjassot a 20 de septiembre de 2013.

Dra. María Dolores Real García Dra. Carolina Rausell Segarra

Para la realización de esta tesis, el autor ha sido beneficiario de una Beca para la Formación de Personal Investigador de carácter predoctoral concedida por la Consellería d'Educació de la Generalitat Valenciana, según la resolución del 25 de agosto de 2008 por el Director General de Política Científica. El trabajo realizado ha sido subvencionado por los proyectos de investigación del Ministerio de Ciencia e Innovación con referencia BIO2007-67860 y AGL2010-22300-C03-03.

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A mis padres, a Josep y a Javi

Índice

Introducción

1. Insectos coleópteros plaga

Los coleópteros comprenden el 40% de las 360.000 especies de insectos conocidas en la actualidad y constituyen el orden más extenso y diverso de los organismos eucariotas. Estos organismos presentan una enorme diversidad morfológica y pueden ocupar cualquier hábitat excepto el marino, donde su presencia es mínima. La mayoría de los coleópteros son fitófagos, y como consecuencia, este orden incluye algunas de las plagas agrícolas, forestales y de grano almacenado más importantes, siendo las larvas las que causan la mayor parte de los daños.

Tribolium castaneum, plaga de grano almacenado, es la especie modelo en este orden cuyo genoma ha sido recientemente secuenciado y constituye una especie ideal para el estudio de nuevos insecticidas. *Leptinotarsa decemlineata* es la plaga más devastadora del cultivo de la planta de patata en el mundo y hasta ahora ha desarrollado resistencia a muchos insecticidas químicos, por lo tanto, para el control de este coleóptero es esencial el desarrollo de nuevos insecticidas con diferentes modos de acción.

1.1 *Tribolium castaneum* (escarabajo rojo de la harina)

Tribolium castaneum, también denominado escarabajo rojo de la harina, es un insecto coleóptero de la familia de los tenebriónidos. Es una de las plagas más importantes de productos alimenticios y grano almacenado que infesta hogares, molinos de harina, tiendas de comestibles y almacenes. Se alimenta generalmente de harina y productos basados en cereales (galletas, pasta, arroz, maíz…), aunque también ingiere frutos secos, legumbres, especias, semillas, chocolate y leche en polvo. Tanto las larvas como los insectos adultos causan daños irreversibles en la calidad de estos productos y pueden producir reacciones alérgicas en los individuos que los ingieren. Esto produce, en muchas ocasiones, grandes pérdidas económicas en los países desarrollados que tienen en este mercado una importante fuente económica (Phillips y Throne, 2010; Prakash *et al.*, 1987).

Durante décadas se han estudiado en el laboratorio diversas especies de *Tribolium*, en especial *T. castaneum* y *Tribolium confusum*, centrándose en aspectos de su genética, nutrición, comportamiento y fisiología. En 1924 se introdujo por primera vez el uso de estas especies en el estudio de genética de poblaciones. Más tarde, se han utilizado como especies modelo para analizar la diferenciación poblacional y especiación. En la actualidad, *T. castaneum* ha resurgido como organismo modelo en biología evolutiva, biología del desarrollo, en investigaciones sobre la metamorfosis y la diversidad del insecto adulto y en control de plagas.

1.1.1 Distribución y ciclo de vida

El escarabajo rojo de la harina está extendido por todo el mundo, sobre todo por áreas templadas. Se sabe poco sobre el nicho ecológico que poseía antes de su introducción en almacenes de alimentos humanos, pero su tolerancia a ambientes secos y cálidos, su reducido sistema visual y la abundancia de genes codificantes de receptores gustatorios y de odorantes que posee sugiere que presentó un estilo de vida subterráneo, en regiones subtropicales áridas, probablemente regiones Indo-australianas (Brown *et al*., 2009).

T. castaneum presenta cuatro fases de desarrollo a lo largo de su vida: huevo, larva, pupa y adulto. La puesta de huevos de los insectos adultos se produce en el mismo grano, harina o productos infestados. Las hembras pueden llegar a poner hasta 20 huevos al día a 30ºC, temperatura a la cual los huevos eclosionan a los 4 días aproximadamente. Las larvas, de una tonalidad entre amarillenta y marrón claro, pueden crecer hasta algo más de 6 mm y tienen normalmente 6 estadios larvarios. Entre 22 y 100 días después de la eclosión empieza el estado de pupa, de unos 8 días de duración y de donde emergen los adultos, de unos 4 mm de longitud, de color rojizo oscuro (Fig. 1).

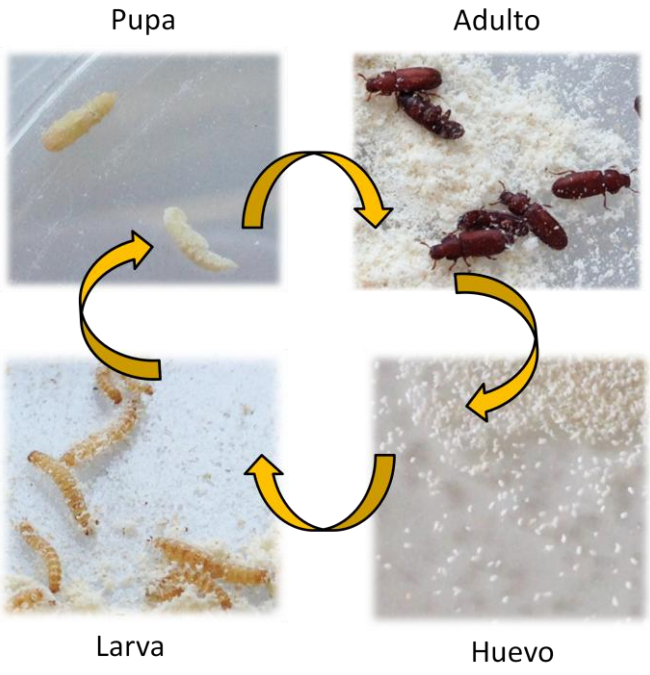

Figura 1. Etapas del ciclo biológico de *T. castaneum*

La elección de *T. castaneum* como organismo modelo en investigación se debe en gran parte a que se reproduce rápidamente, resulta fácil de manejar en el laboratorio y

requiere un mantenimiento mínimo. Su dieta se basa en harina y levadura de cerveza y el desarrollo óptimo se produce a 30-32ºC, temperaturas a las que el tiempo entre generaciones es menor y la supervivencia es más alta, aunque puede reproducirse a partir de 20ºC.

1.1.2 *T. castaneum* como organismo coleóptero modelo en estudios genéticos

Los estudios de genética clásica en *Tribolium* empezaron hace más de 70 años. En 1960 ya se conocían suficientes mutaciones como para construir mapas de ligamiento de siete de los nueve autosomas y del cromosoma X. El desarrollo más importante en el análisis genético con *Tribolium* se produjo con la reversión de determinados fenotipos dominantes, la construcción de cromosomas equilibradores que cubren el 30% del genoma y el desarrollo de sofisticados esquemas de mutagénesis. Los mapas genéticos que existen en la actualidad, basados en marcadores moleculares, han alcanzado como media una resolución de 1 cM, llegando en algunas regiones a 0,007 cM (2 kbp) (Lorenzen *et al.* 2008). Además, también se dispone de regiones mapeadas que son diana de recombinación específica por transposones, que permitirán en un futuro definir la reorganización de cromosomas (Brown *et al*., 2009).

Aunque el análisis genético en *T. castaneum* se ha desarrollado mucho por el uso de genética directa, también es posible el uso de genética reversa, especialmente a partir de la secuenciación de su genoma (Tribolium Genome Sequencing Consortium, 2008). Este organismo, a diferencia de la especie modelo *Drosophila melanogaster*, presenta una eficiente respuesta sistémica al RNA de interferencia (RNAi), que en ocasiones se extiende incluso a la siguiente generación. El mecansimo de RNAi se basa en la degradación de ácidos nucleicos exógenos por parte de los organismos. El suministro de RNA de doble cadena exógeno a un organismo produce la degradación de un RNA mensajero específico de interés y permite el estudio de la función del gen "silenciado" ya que el organismo carece la proteína codificada.

En *T. castaneum* se han usado diferentes métodos de genética reversa mediante RNAi. En el método de RNAi embriónico, el RNA de doble cadena se inyecta directamente dentro del embrión (Brown *et al*., 1999) y en el método de RNAi parental, el RNA de doble cadena se inyecta dentro del hemocele de una pupa hembra (Bucher *et al*., 2002). Estos dos métodos se han utilizado satisfactoriamente en estudios de embriogénesis y desarrollo (Fu *et al*. 2012; Schröder, 2003). Sin embargo, en algunos casos, los efectos del RNAi embriónico y parental no persisten a lo largo del desarrollo de la pupa y adulto y otras veces el silenciamiento de unos genes concretos produce detención en un estadio temprano del desarrollo, haciendo dificultoso el análisis de la

función de estos genes en estadios más tardíos. Por estas causas, se desarrolló posteriormente el método del RNAi larvario, en el que el RNA de doble cadena se inyecta en la cavidad corporal de la larva, induciendo el efecto del silenciamiento en los tejidos de la larva durante diferentes estadios y más tarde en la pupa, llegando incluso a producir efectos morfológicos en el adulto (Tomoyasu y Denell, 2004).

La posibilidad de generar una eficiente respuesta sistémica de RNAi en *Tribolium* permite la identificación de nuevas funciones génicas sin tener información previa en otros organismos modelo, en cualquier momento del ciclo de vida del insecto y aparentemente en cualquier tejido (Posnien *et al*., 2009).

Debido a las características anteriormente mencionadas y como representante del orden de insectos más extenso, *T. castaneum* fue elegido por el Instituto Nacional de la Investigación del Genoma Humano (NHGRI) para ser incluido en la lista de especies para ser secuenciadas, siendo el único escarabajo cuyo genoma ha sido secuenciado hasta la fecha. El genoma de la cepa Ga-2, altamente consanguínea, es de 200 megabases, y fue secuenciado en 2008 (Tribolium Genome Sequencing Consortium, 2008). La base de datos de *T. castaneum*, "Beetlebase" (http://www.beetlebase.org), se desarrolló basándose en el primer ensamblaje del genoma (Tcas_1.0) (Wang *et al*., 2007) y se ha ido actualizando hasta la versión actual, que se basa en el tercer ensamblaje (Tcas_3.0) (Kim *et al*., 2010).

La primera ronda de anotación computacional produjo 16.000 genes aproximadamente, de los cuales 2.000 fueron analizados más a fondo, tomando información de sus homólogos en otras especies y forman el grupo de genes verificados y corregidos manualmente. Por otra parte, el NCBI (Centro Nacional de Información Biotecnológica) realizó un análisis independiente de anotación prediciendo un grupo más reducido de genes, unos 9.000. Además de la secuencia genómica, también se dispone de aproximadamente 70.000 marcadores de expresión (ESTs) de diferentes estadios y específicos de tejido (Brown *et al*., 2009).

1.1.3 Métodos de control

Para evitar los daños ocasionados por *T. castaneum* en los productos almacenados, se ha intentado controlar la proliferación de este insecto a través de la puesta en marcha de prácticas de manejo de plagas (Phillips y Throne, 2010) o a través del uso de productos químicos tóxicos, y entre ellos, los fumigantes han sido los más utilizados (Thoms y Phillips, 2004). Los organofosforados y los piretroides sintéticos son los insecticidas químicos más populares para el control de plagas de productos

almacenados, aunque esta plaga ha desarrollado resistencia a la mayoría de ellos (Mujeeb y Shakoori, 2007).

Como consecuencia del uso intensivo, al final de los años 50, del organofosforado malatión sobre los productos almacenados, en 1961 apareció la primera cepa de *T. castaneum* resistente a este compuesto (Parkin *et al*., 1962). Unos años después, en 1974, esta característica ventajosa era ya bastante común en la especie (Champ y Dyte, 1976) hasta que la cepa resistente ha llegado a sustituir a la cepa susceptible (Arnaud *et al*., 2002). De igual modo, el uso intensivo de otros organofosforados como la fosfina, el diclorvos, el clorpirifós o el fenitrotión ha conducido al desarrollo de resistencia de *T. castaneum* en muchas regiones del mundo (Jagadeesan *et al*., 2012; Halliday *et al*., 1988; Zettler, 1991; Champ y Cambell-Brown, 1970). El uso prolongado de piretroides, desde finales de los años 70, para el control de plagas de producto almacenado también ha llevado a la aparición de cepas de *T. castaneum* con una mayor tolerancia a estos compuestos (Champ y Cambell-Brown, 1970; Champ, 1978; Collins, 1990).

Además de los organofosfatos y piretroides, *T. castaneum* ha desarrollado resistencia a otros compuestos como el lindano (Bhatia y Pradhan, 1972), el bromometano, cianuro de hidrógeno, dibromuro de etileno, carbamatos, etc. (Champ, 1978). Algunos de estos compuestos no están actualmente autorizados para su uso como insecticidas, por ser peligrosos para los seres humanos y otras especies acuáticas y terrestres, y por su persistencia en el medio ambiente. Por tanto, se requieren nuevas alternativas a estos productos, más efectivas y menos dañinas para los usuarios y el medio ambiente.

Algunos productos naturales como los aceites y extractos de plantas como *Juniperus virginiana, Pongamia glabra, Piper nigrum, Cinnamomum aromaticum*, *Evodia rutaecarpa*, *Artemisia vulgaris, Elletaria cardamomum, Allium sativum* o monoterpenos de los cítricos y eucaliptos actúan como repelentes y tóxicos para el escarabajo rojo de la harina (Sighamony *et al*., 1984; Huang y Ho, 1998; Liu y Ho, 1999; Wang *et al*., 2006; Huang *et al*., 2000; Ho *et al*., 1996; Prates *et al*., 1998), aunque por el momento se desconoce el mecanismo de toxicidad de estos productos, los efectos en otros animales y su utilidad en la aplicación comercial (Pathipati, 2012).

1.2 *Leptinotarsa decemlineata* (escarabajo de la patata)

L. decemlineata es un insecto coleóptero de la familia de los crisomélidos y plaga de plantas solanáceas. Comúnmente se denomina escarabajo de la patata, por ser la planta de patata su principal hospedador. Pero además de alimentarse de plantas de patata (*Solanum tuberosum*), puede consumir plantas de berenjena (*Solanum melongena*) y plantas de tomate (*Solanum lycopersicum*) (Jacques, 1988). Se considera el insecto

defoliador de la patata más importante, ya que tanto la larva como el insecto adulto ingieren hojas y tallos de la planta. El ciclo de vida de este insecto, sus hábitos alimenticios destructivos y su facilidad para desarrollar resistencia a insecticidas hace de este insecto un problema relevante en agricultura.

1.2.1 Distribución y ciclo de vida

El escarabajo de la patata es originario de México. Actualmente presenta una amplia distribución por las zonas templadas, incluyendo América del Norte, Europa y Asia. La temperatura óptima para su desarrollo oscila entre 25 y 32ºC. La fecundidad de este insecto es elevada, las hembras pueden poner entre 300 y 800 huevos durante su vida de adulto (Alyokhin, 2009). Los huevos son de color amarillo anaranjado, de forma ovalada y de 1.7-1.8 mm de largo. Las hembras usan un adhesivo amarillento para depositar los huevos en grupos en el envés de las hojas, de los que eclosionan las larvas después de unos 10 días, dependiendo de la temperatura y la humedad. Las larvas, de color rojizo, llegan a medir 1.3 cm una vez maduras y pasan por 4 estadios, que duran unos 21 días en total. Las larvas maduras cavan en el suelo oquedades de 2-5 cm y empiezan a pupar, proceso que dura una media de 5.8 días. Después de este periodo, emergen los adultos, de unos 9.5 mm de largo y de color amarillo claro, con 5 rayas negras por élitro (Capinera, 2001) (Fig. 2).

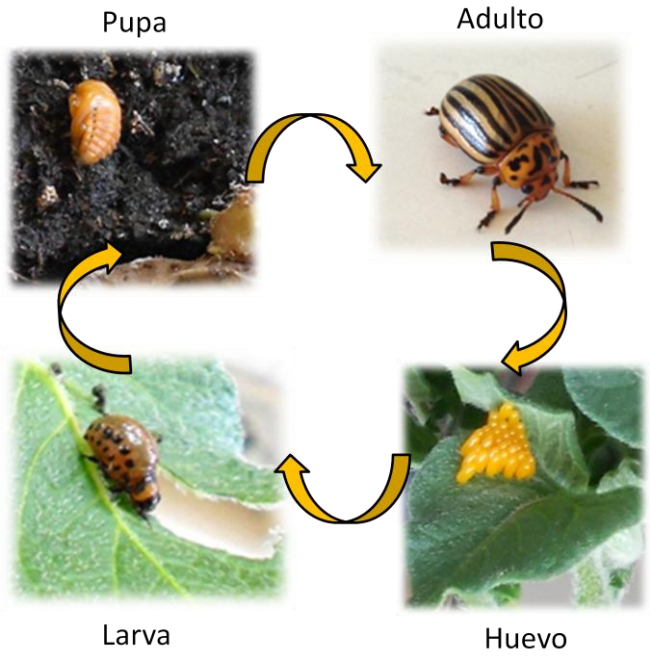

Figura 2. Etapas del ciclo biológico de *L. decemlineata*

1.2.2 Métodos de control

Se ha utilizado una gran diversidad de compuestos químicos con el fin de controlar esta plaga, como por ejemplo DDT (Forgash, 1981), organofosfatos, carbamatos (Stankovic *et al*., 2003; French *et al*., 1992), piretroides (French *et al*., 1992), acetoarsenito de cobre y arseniato de calcio (Alyokhin, 2009). Al igual que *T. castaneum*, diferentes poblaciones de este escarabajo han desarrollado resistencia a gran diversidad de compuestos (Argentine *et al.,* 1995, Argentine y Clark, 1990; Clark *et al.,* 1995), muchos de los cuales además resultan peligrosos para el medio ambiente.

Algunos de los tratamientos naturales que se han ensayado contra el escarabajo de la patata son el hongo entomopatógeno *Beauveria bassiana* (Lipa, 1985), extractos de planta de pimienta (Scott *et al*., 2003), el ácido anacárdico de algunas plantas (Schultz *et al.,* 2006) o aceite de la planta herbácea *Tanacetum vulgare*, que muestra efecto repelente sobre el insecto (Schearer, 1984), además del uso de insectos depredadores de este coleóptero (Brust, 1994). Sin embargo, los tratamientos de origen biológico más efectivos hasta la fecha han sido aquellos basados en la bacteria entomopatógena *Bacillus thuringiensis* (Shelton *et al*., 2002; Lacey *et al*., 1999; Ferro y Gelernter, 1989; Krieg *et al*., 1988).

2. Control de plagas con bioinsecticidas

La biotecnología agrícola es una disciplina enfocada a dar solución a problemas de baja producción agrícola y pérdidas económicas de cultivos, intentando lograr así una agricultura sostenible (Menn y Hall, 1999). Una importante causa de pérdida de productividad agrícola es la presencia de insectos plaga de cultivos, por lo que su desarrollo debe ser controlado en los campos a través del uso de insecticidas. Uno de los objetivos de la biotecnología agrícola es reducir la dependencia de insecticidas químicos tradicionales sin afectar o incluso incrementar la productividad del campo, con la consecuente reducción en costes de mantenimiento y disminución de problemas medioambientales. Una alternativa a los insecticidas químicos son los insecticidas biológicos o bioinsecticidas, que resultan inocuos para los usuarios y el medio ambiente.

La industria de los bioinsecticidas incluye organismos entomopatógenos que infectan al insecto plaga, organismos entomófagos que son depredadores o parasitoides del insecto plaga, compuestos con actividad insecticida derivados de plantas o metabolitos de actinomicetos que no resultan tóxicos para el medio ambiente, y organismos y plantas transgénicas (Baum *et al*., 1999).

De forma general, los organismos entomopatógenos son aquellos que infectan al insecto y se reproducen en él hasta causarle la muerte, como hongos, virus, bacterias, protozoarios y nemátodos. Además, tienen la ventaja de que incluso en los casos en que el número de organismos que infectan no es suficiente como para ocasionar la muerte del insecto, el mismo insecto continúa dispersando el agente infectivo y provocando la infección de otros insectos en el área.

Se han detectado bacterias gram-negativas entomopatógenas, como miembros de la familia *Enterobacteriaceae*, algunos de ellos hospedados en el intestino de nemátodos que infectan insectos. Sin embargo, las bacterias gram-positivas entomopatógenas son las más conocidas y estudiadas, y constituyen una de las alternativas a los insecticidas químicos clásicos más eficaces para el control de plagas (de Maagd *et al*., 2003).

2.1 *Bacillus thuringiensis*

La primera bacteria gram-positiva entomopatógena que fue descubierta y con diferencia la más estudiada es *Bacillus thuringiensis (Bt)*. Esta bacteria se puede encontrar en gran diversidad de nichos ecológicos como en el suelo, en la superficie de las plantas, en el polvo que proviene de producto almacenado o en el interior de los insectos. Forma endoesporas en condiciones adversas y, al mismo tiempo, produce inclusiones cristalinas parasporales que contienen una o más proteínas tóxicas por ingestión para insectos (Schnepf *et al*., 1998) (Fig. 3).

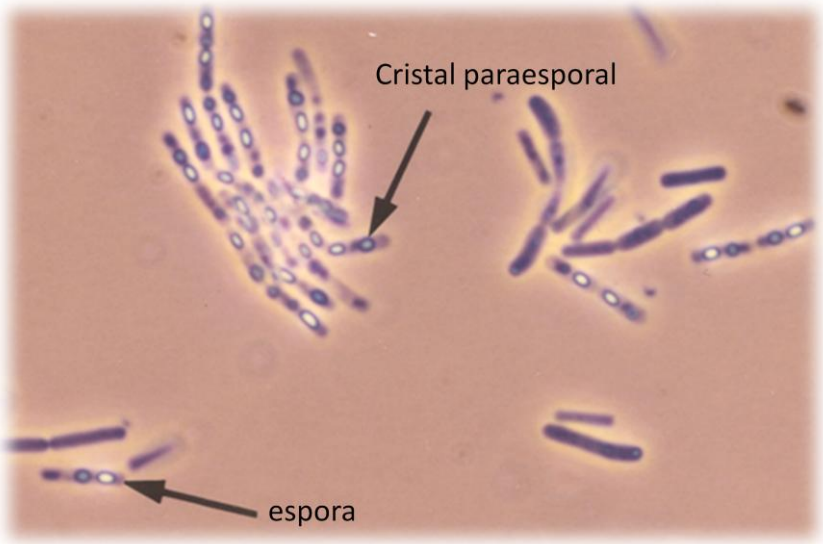

Figura 3. Cultivos de *B. thuringiensis* en estado avanzado de esporulación

Esta bacteria fue descubierta por el biólogo Japonés Shigetane Ishiwata en 1901, trabajando con el gusano de seda, y la denominó *Bacillus sotto*. Diez años después, en la provincia alemana de Turingia, Ernst Berliner aisló la misma bacteria a partir de la polilla mediterránea de la harina y la llamó *Bacillus thuringiensis* (Siegel, 2000), nombre con el que se la conoce en la actualidad. Las propiedades bioinsecticidas de esta bacteria se empezaron a estudiar al descubrir, en el interior de cadáveres de larvas de insectos, esporas e inclusiones cristalinas que ésta produce. Se comprobó que la capacidad insecticida de esta bacteria radicaba en las inclusiones cristalinas, ya que orugas susceptibles que ingerían alimento contaminado con cristales de *Bt* presentaban disrupción de las microvellosidades de las células de membrana del epitelio intestinal, con la consecuente muerte de la larva (Sutter y Raun, 1967).

Desde entonces, se han conseguido aislar centenares de cepas diferentes de *Bt*, presentando cada una de ellas un rango de especificidad limitado a uno o unos pocos órdenes de insectos e incluso a una determinada especie. Se han encontrado cepas tóxicas para insectos lepidópteros, coleópteros, dípteros, himenópteros, homópteros, ortópteros y malófagos y para otros organismos como nemátodos, protozoos y ácaros (Feitelson *et al.*, 1992). Las esporas son capaces de germinar tanto en el interior como en el exterior de los insectos, por lo que se trata de bacterias entomopatógenas facultativas. La gran diversidad de cepas y toxinas de esta bacteria se debe, al menos en parte, a la plasticidad genética, ya que los genes codificantes para sus toxinas residen en plásmidos, muchos de ellos conjugativos por naturaleza y muchas veces como parte de elementos transponibles (Schnepf *et al*., 1998).

Existen formulaciones comerciales basadas en mezclas de esporas y cristales de *Bt* que se usan como bioinsecticidas para proteger, frente a diferentes plagas de insectos, plantas cultivadas, vegetales, frutos, plantas ornamentales y bosques. La subespecie más utilizada es *Bt kurstaki* para el control de distintas especies de lepidópteros. Además, para controlar vectores de diversas enfermedades humanas como la malaria, se usan formulaciones de la subespecie *Bt israeliensis,* activas contra larvas de dípteros (Margalith y Ben-Dov, 2000; Fillinger *et al*., 2003). Algunos coleópteros, como el escarabajo de la patata, pueden ser controlados con las subespecies *Bt tenebrionis, san diego, morrisoni, tolworthi* y *galleriae* (Krieg *et al.,* 1983; Hernstadt *et al.,* 1986, 1987; Sick *et al*., 1990). Actualmente, los aerosoles basados en *Bt* son los bioinsecticidas más usados en el mundo y constituyen aproximadamente el 2% del mercado de los insecticidas (Pardo-López *et al*., 2013).

El estricto rango hospedador de las cepas de *Bt* es útil para combatir plagas de insectos, sin perjudicar otros organismos presentes en un mismo hábitat. Actualmente,

se continúa realizando exhaustivas búsquedas de nuevas cepas de esta bacteria en diferentes ambientes, con el objetivo de ampliar el rango de especificidad a determinadas especies plaga no susceptibles a ninguna cepa de *Bt* conocida. Asimismo, el desarrollo biotecnológico alcanzado en la actualidad ha permitido la modificación de los genes codificantes de las toxinas, con la finalidad de obtener nuevas proteínas más tóxicas y tratar de evitar el posible desarrollo de resistencia que se pudiera generar por parte de insectos susceptibles (Whiteley y Schnepf, 1986).

Por otra parte, ha sido posible la introducción de genes que codifican las proteínas insecticidas de *Bt* en el genoma de las plantas, generando así cultivos transgénicos, los denominados "cultivos *Bt*". Las primeras plantas a las que se les introdujeron estos genes fueron las de tabaco y tomate, y más tarde se desarrollaron otros cultivos como el maíz *Bt*, capaz de producir una proteína insecticida tóxica para diversos lepidópteros como *Helicoverpa punctigera, Helicoverpa zea* y *Pectinophora gossypiella* (Kamle y Ali, 2013). A la primera generación de plantas modificadas genéticamente se les introdujo solamente un gen codificante para una proteína bioinsecticida, pero en las generaciones posteriores se les introdujo dos o tres genes, a veces combinados con genes de resistencia a herbicidas (Kamle y Ali, 2013). En 2011, se plantaron 66 millones de hectáreas en el mundo con maíz *Bt* y algodón *Bt*, los únicos cultivos *Bt* disponibles comercialmente. Entre las ventajas del uso de estas plantas destaca la reducción de la dependencia de insecticidas, la minimización del impacto ambiental y el incremento del beneficio de los agricultores (Tabashnik *et al*., 2013; Shelton, 2012).

2.2 Otras bacterias entomopatógenas gram-positivas

Además de *Bt*, existen otras bacterias entomopatógenas gram-positivas, como *Bacillus cereus*, la cual se considera de la misma especie que *Bt*, con la diferencia de que *B. cereus* no forma cristales pero las esporas pueden producir septicemia en insectos, al igual que las de *Bt* (Helgason *et al*., 2000; Agaisse *et al*., 1999). A *Bacillus sphaericus*, que debe su nombre a la forma esférica de las esporas que produce, también se le ha atribuido actividad insecticida frente a larvas de díptero. Algunas cepas de esta especie producen inclusiones cristalinas, y otras forman proteínas solubles, en lugar de cristales (Charles y Nielsen-LeRoux, 2000). También muestran capacidad insecticida bacterias del género *Penibacillus*, que incluye diversas especies entomopatógenas obligadas ya que no son capaces de esporular en el exterior de los insectos, y por último, se han encontrado también cepas de *Clostridium bifermentans* con actividad insecticida para mosquitos (de Maagd *et al*., 2003).

3. Toxinas producidas por bacterias entomopatógenas gram-positivas

Existe una gran diversidad de toxinas producidas por *Bt* y otras bacterias entomopatógenas gram-positivas. Tradicionalmente las toxinas de *Bt* se han dividido en toxinas Cyt (de citolíticas) y Cry (de cristal) constituyendo estas últimas el más numeroso y conocido grupo de toxinas (Fig. 4), la mayoría de las cuales presentan tres dominios bien diferenciados en su estructura terciaria (Bravo *et al*., 2007) (Fig. 5).

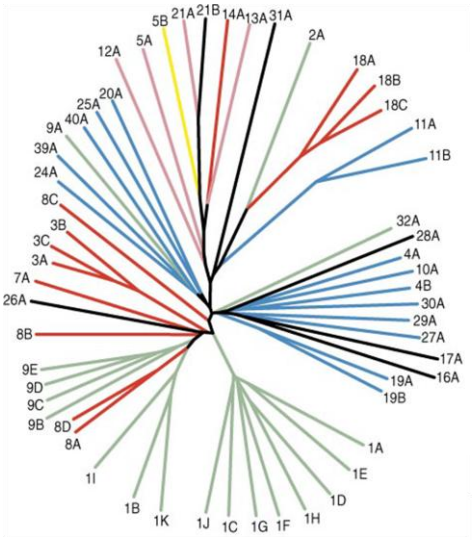

Figura 4. Relaciones filogenéticas de las toxinas Cry de tres dominios. Los colores de las ramas representan la especificidad de las toxinas en los diferentes órdenes de insectos. Las ramas rojas representan toxinas específicas de coleópteros, las verdes específicas de lepidópteros, las azules específicas de de dípteros, las magenta específicas de nematodos y las amarillas específicas de himenópteros. Tomado de Maagd *et al.* (2003).

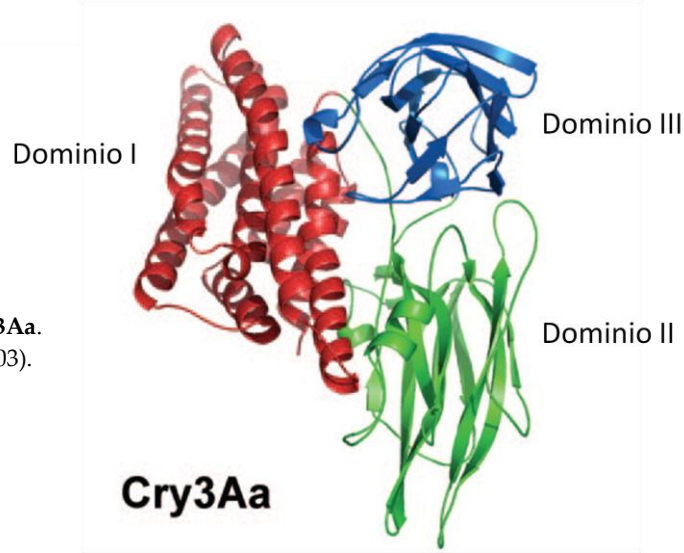

Figura 5. Estructura de Cry3Aa. Tomado de Maagd *et al.* (2003).

Las toxinas Cyt deben su nombre a su actividad citolítica *in vitro*, pero *in vivo* son específicas contra larvas de díptero y se ha observado que tienen capacidad de sinergizar la actividad de otras proteínas insecticidas (de Maagd *et al*., 2003).

El descubrimiento de otras toxinas Cry que no presentan homología con las toxinas en tres dominios y sí con toxinas de otras bacterias entomopatógenas gram-positivas ha incrementado la complejidad de la clasificación. En la actualidad, las toxinas se clasifican atendiendo a la homología de su estructura primaria (Crickmore *et al*., 1998)

en cuatro grupos: las toxinas Cry de tres dominios, las toxinas binarias y relacionadas, las toxinas Mtx2, Mtx3 y relacionadas, y las toxinas Cyt (de Maagd *et al*., 2003).

El grupo de toxinas binarias es un grupo de toxinas que forman cristales, pero no presentan una estructura terciaria en tres dominios. Entre ellas, encontramos las toxinas Bin de *B. sphaericus* y las relacionadas con éstas, como por ejemplo Cry34/35 de *Bt*. Las toxinas Cry34/35 fueron purificadas a partir de una cepa de *Bt* tóxica para el coleóptero *Diabrotica virgifera virgifera*. Ambas proteínas son necesarias para producir el efecto insecticida, de ahí el nombre de toxinas binarias. Cry34 no presenta homología con ninguna proteína de las bases de datos, y Cry35 presenta homología con otras toxinas Bin de *B. sphaericus* (Ellis *et al*., 2002) y probablemente comparte con ellas un plegamiento tridimensional similar (de Maagd *et al*., 2003).

El grupo de toxinas Mtx2 y Mtx3, producidas por *B. sphaericus*, son activas contra mosquitos y presentan homología con diversas toxinas formadoras de poro, como la alfa-toxina de *Clostridium septicum*, la aerolisina de *Aeromonas hydrophila* y la toxina épsilon de *Clostridium perfringens*. *Bt* también produce toxinas relacionadas con las Mtx2 y Mtx3, ejemplos de las cuales son las Cry15Aa, CryC53, Cry38 y Cry23A. La toxina Cry23 (también denominada CryET33) es una proteína de 29 KDa y su toxicidad se manifiesta solo cuando está acompañada de la toxina Cry37 (CryET34), de 14 KDa, que no presenta homología con ninguna proteína de las bases de datos. Cry23 es capaz de formar canales en bicapas lipídicas, y tiene una estructura alargada formada por láminas beta antiparalelas que recuerda a la estructura de los dominios del 2 al 4 de la toxina proaerolisina de *A. hydrophila* (Fig. 6). Cry37 presenta una estructura de beta-sandwich C2, y podría tener una función análoga a la del dominio tipo lectina de la aerolisina (Fig. 6), facilitando la unión a la toxina formadora de canal Cry23 (de Maagd *et al*., 2003). Hasta la fecha, no existen estudios sobre su modo de acción.

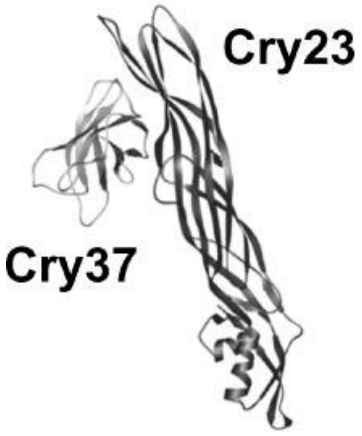

Figura 6. Estructura de las toxinas Cry23/37. Tomado de Pigott y Ellar (2007).

Además de las toxinas mencionadas anteriormente, existen otras producidas durante el crecimiento vegetativo de la bacteria, como las toxinas Mtx1, producidas por *B. sphaericus*, y VIP, producidas por *Bt* y *B. cereus.*

Recientemente, han cobrado importancia las paraesporinas, proteínas producidas por *Bt* que presentan toxicidad para ciertas líneas de células tumorales induciendo apoptosis. Se clasifican en 4 grupos según su espectro citotóxico y su nivel de actividad (Ohba *et al*., 2009).

4. Las toxinas Cry de tres dominios de *Bt*

4.1 Estructura de las toxinas Cry de tres dominios

Las toxinas Cry de tres dominios constituyen el grupo de toxinas producidas por *Bt* más conocido y estudiado. Se encuentran en dos posibles tamaños, 130 ó 70 kDa aproximadamente. Las proteínas de mayor tamaño, como las del tipo Cry1, tienen un dominio carboxi-terminal que parece estar implicado en la formación del cristal, pero este fragmento puede ser proteolizado y es dispensable para la toxicidad, liberando una forma activa en torno a los 60 kDa. Las proteínas de menor tamaño, como es el caso de las toxinas Cry3, no presentan este fragmento C terminal y se encuentran ya en forma activa (Höfte y Whiteley, 1989).

La forma activa presenta en su secuencia primaria hasta cinco bloques conservados (Höfte y Whiteley, 1989) y se propone que las toxinas que poseen estos bloques comparten una estructura terciaria similar (Schnepf *et al*., 1998). Las toxinas Cry de tres dominios se clasifican atendiendo a la homología de su secuencia aminoacídica en tres rangos. El primer rango se designa con un número arábigo y comprende toxinas con una identidad de al menos el 45%, el segundo rango se designa con una letra mayúscula y en él se clasifican proteínas de al menos una identidad del 75%. El tercero se designa con una letra minúscula y lo integran proteínas de al menos un 95% de identidad. Las toxinas con homología mayor al 95% son consideradas variantes alélicas y se designan con un número arábigo (Crickmore *et al*., 1998) (Fig. 7).

Hasta la fecha ha sido determinada, por cristalografía de rayos X, la estructura de seis toxinas Cry diferentes: Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, Cry4Aa y Cry4Ba, siendo la estructura de Cry3Aa la primera en ser obtenida (Fig. 5). Todas ellas presentan una elevada similitud en su estructura, que sugiere un modo de acción similar (Li *et al*., 1991; Grochulski *et al*., 1995; Morse *et al*., 2001; Galitsky *et al*., 2001; Boonserm *et al*., 2005, 2006).

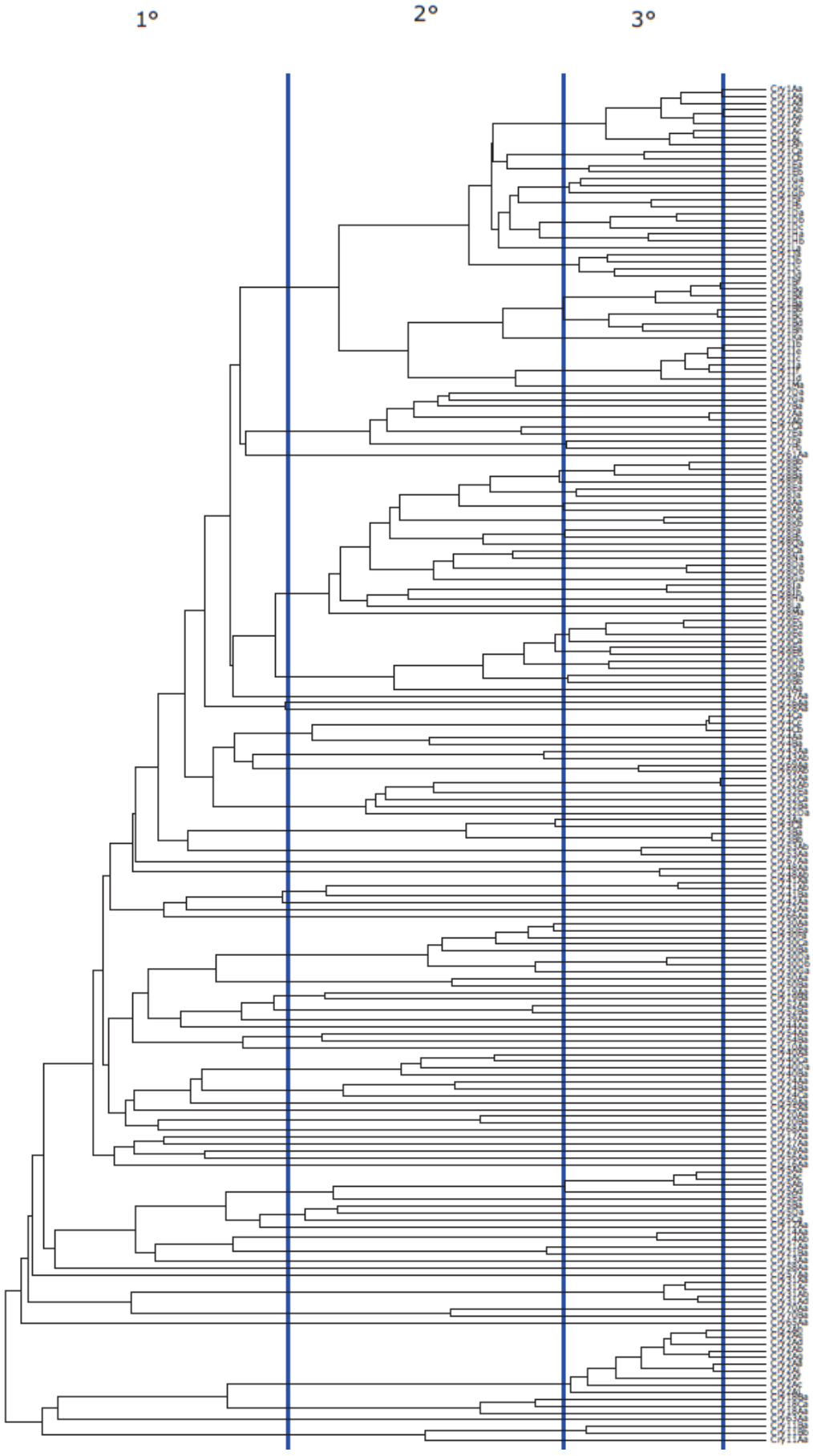

Figura 7. Dendograma de las proteínas Cry de tres dominios. Las líneas verticales indican los límites de identidad que marcan los tres primeros rangos de nomenclatura.
Tomado de http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/. Última actualización.

El dominio I de estas proteínas está formado por siete hélices alfa, donde la hélice central se rodea de las otras seis hélices anfipáticas. La mayoría de las hélices tienen una longitud superior a 30 Å, por lo que son capaces de atravesar la membrana lipídica. Este dominio presenta una estructura similar al de otras toxinas bacterianas formadoras de poro, como la colicina A y la toxina diftérica. Debido a estas características, el dominio I ha sido implicado en la formación de poros líticos en el epitelio intestinal del organismo diana (Pigott y Ellar, 2007; de Maagd *et al*., 2003)

El dominio II consiste en tres láminas beta antiparalelas que forman un prisma beta. Es el dominio más variable entre toxinas, y su estructura ha sido comparada con la de algunas lectinas. Experimentos de mutagénesis dirigida y de intercambio de segmentos indican que este dominio está implicado en el reconocimiento de moléculas receptoras (Li *et al*., 1991; Schnepf *et al*., 1998).

El dominio III es un beta-sandwich de dos láminas beta antiparalelas que presenta cierta similitud a módulos de unión a carbohidratos. Es menos variable entre toxinas que el dominio II y, al igual que éste, parece estar implicado en el reconocimiento de moléculas receptoras (Schnepf *et al*., 1998; Pigott y Ellar, 2007).

4.2 Modo de acción de las toxinas Cry de tres dominios

El modo de acción de las toxinas Cry de tres dominios se ha caracterizado principalmente en lepidópteros, y consta de distintos pasos. En primer lugar, las inclusiones cristalinas formadas por la bacteria son ingeridas por la larva susceptible. Las condiciones del intestino favorecen la solubilización y liberación de las toxinas Cry de las inclusiones cristalinas. En lepidópteros, se sabe que para la solubilización es especialmente importante el pH alcalino del intestino de la larva (Schnepf *et al*., 1998). Durante la solubilización, las toxinas Cry de aproximadamente 130 kDa son liberadas de los cristales en forma de protoxinas y es necesario el procesamiento de su extremo C terminal para la liberación de la forma activa. El procesamiento es llevado a cabo por proteasas presentes en el intestino de los insectos, siendo las proteasas de tipo tripsina y quimotripsina las más abundantes en el intestino medio de lepidópteros (de Maagd *et al*., 2001). En coleópteros, el pH del intestino oscila entre neutro y ligeramente ácido y entre las proteasas más abundantes se encuentran cisteín y serín proteasas (Murdock *et al*., 1987; Oppert *et al*., 2002; Leplé *et al*., 1995; Vinokurov *et al*., 2006).

El siguiente paso es la unión de la forma activa a receptores específicos presentes en la membrana apical de las microvellosidades del intestino del insecto. Está generalmente aceptado que la unión a estos receptores es clave para el desencadenamiento del proceso tóxico (Pigott y Ellar, 2007).

4.2.1 Receptores implicados en el modo de acción de las toxinas Cry de tres dominios

La relevancia de la interacción de las proteínas Cry con algunas de las proteínas presentes en la membrana del intestino de los insectos susceptibles en el proceso tóxico ha despertado el interés de los investigadores en identificar y clonar dichos receptores. Son muchas las proteínas identificadas potencialmente capaces de unir toxinas Cry, pero las mejor caracterizadas y validadas como receptores funcionales en diferentes insectos son las aminopeptidasas N, las cadherinas, las fosfatasas alcalinas, los glicolípidos y recientemente también un transportador ABC.

Cadherina

Las cadherinas constituyen una superfamilia diversa de proteínas que presentan repeticiones de dominios de unión a calcio, también llamadas repeticiones tipo cadherina. Se trata de proteínas glicosiladas y ancladas a la membrana, generalmente situadas en los puntos de adhesión intercelulares (Carthew, 2005), aunque también se ha confirmado que están presentes en el epitelio intestinal de diversos lepidópteros susceptibles a diferentes toxinas del grupo Cry1A, como en *Manduca sexta, Bombyx mori, Heliothis virescens, Ostrinia nubilalis, P. gossypiella, Helicoverpa armigera o Lymantria dispar*, y que unen toxinas de dicho grupo (Pigott y Ellar, 2007).

Se ha demostrado que las cadherinas actúan como receptores funcionales en diferentes lepidópteros, relacionándose la expresión de las cadherinas de *M. sexta*, *B. mori* y *H. virescens* en células de mamífero con la aparición de susceptibilidad a toxinas del grupo Cry1A (Dorsch *et al*., 2002; Hua *et al*., 2004b; Nagamatsu *et al*., 1999; Jurat-Fuentes y Adang, 2006b). Actualmente, se siguen depositando en las bases de datos proteínas homólogas a estos receptores tipo cadherina en otros lepidópteros.

En otros órdenes de insectos, se han identificado también cadherinas que funcionan como receptores para toxinas Cry. En el díptero *Aedes aegypti* se ha encontrado una cadherina capaz de unir las toxinas Cry11Aa y Cry11Ba (Chen *et al*., 2009a; Likitvivatanavong *et al*., 2011) y se ha comprobado que actúa como mediador de la toxicidad para Cry11Aa mediante su silenciamiento con RNAi (Rodríguez-Almazán *et al*., 2012). En el díptero *Anopheles gambiae* también se ha detectado una cadherina que une Cry4Ba (Hua *et al*., 2008).

En coleópteros como *D. virgifera virgifera,* se ha clonado una cadherina similar a aquellas que tienen la función de receptor en lepidópteros (Sayed *et al*., 2007) y en el coleóptero *Henosepilachna vigintioctomaculata* se ha identificado una cadherina que une

Cry7Ab3 (Song *et al*., 2012). En *Tenebrio molitor*, otro insecto de este orden, se ha identificado y clonado una cadherina capaz de unir Cry3Aa y se ha demostrado su papel como mediador de la toxicidad mediante silenciamiento con RNAi (Fabrick *et al*., 2009).

Se han identificado diferentes regiones de la cadherina de *M. sexta* por las que puede unir la toxina Cry1Ab, entre ellas la repetición de cadherina 7 (CR7) (Gómez *et al*., 2001), CR11 (Dorsch *et al*., 2002) y la región proximal a la membrana incluyendo la repetición de cadherina más próxima a ésta, aunque sólo esta última zona ha sido confirmada como región funcional de unión y desencadenante de la toxicidad (Hua *et al*., 2004a). Con el objetivo de establecer relación entre las regiones de unión de las toxinas Cry en las cadherinas y la toxicidad, se han intoxicado larvas de insectos acompañados de fragmentos de cadherinas que contienen los epítopos de unión a las toxinas. Las larvas de *M. sexta* que ingerían toxina Cry1Ab acompañada de un fragmento de la cadherina que contenía la región de unión funcional resultó en un aumento de toxicidad, lo que llevó a sugerir que el fragmento ejerce un papel similar al del receptor de Cry1Ab (Chen *et al*., 2007). En cambio, en otras especies como *H. armigera*, se ha demostrado que fragmentos de cadherina que contenían la región de unión acompañados de la toxina Cry1Ac actúan como competidores, disminuyendo la unión de la toxina con su receptor y como consecuencia la mortalidad (Liu *et al*., 2009).

En el díptero *A. gambiae* también se ha observado sinergismo en el efecto de la toxina Cry4Ba cuando ésta se acompaña de un fragmento de la cadherina (Hua *et al*., 2008), pero en este mismo insecto, a su vez, se ha identificado una segunda cadherina capaz de unir Cry11Ba y se ha descrito que un fragmento de ésta es capaz de disminuir la toxicidad en el insecto (Hua *et al*., 2013). También se ha observado que fragmentos de la cadherina de *T. molitor* que contiene la región de unión a Cry3Aa o de *D. virgifera virgifera* son capaces de incrementar la toxicidad de Cry3Aa o Cry3Bb (Fabrick *et al*., 2009; Park *et al*., 2009) en éstos y en otros coleópteros crisomélidos (Gao *et al*., 2011; Park *et al*., 2009). La influencia ejercida por estos péptidos en la mortalidad de las toxinas Cry a las que unen representa otro tipo de evidencia de la importancia del receptor cadherina en diferentes órdenes de insectos.

Aminopeptidasa N (APN)

La APN es una peptidasa dependiente de zinc abundante en el intestino medio de lepidópteros, anclada a la parte exterior de la membrana plasmática a través de glicosilfosfatidilinositol (GPI) (Takesue *et al*., 1992). Esta proteasa corta entre aminoácidos preferentemente neutros en el extremo amino terminal de las proteínas, y

juega un papel importante en la digestión de las proteínas de la dieta junto con otros enzimas digestivos (Sjöström *et al.*, 2000; Wang *et al*., 2005). Existen muchos estudios que confirman, en diferentes lepidópteros (*M. sexta, H. virescens, B. mori, H. armigera, Plutella xylostella, L. dispar, Epiphyas postvittana, Spodoptera litura…),* la unión de APN a toxinas Cry1A, Cry1B, Cry1C y Cry1F a las que son susceptibles, tanto en condiciones nativas como desnaturalizantes (Pigott y Ellar, 2007). Sin embargo, son pocos los estudios que validan proteínas APN como receptores funcionales. En *S. litura* se ha implicado una APN como mediador de la toxicidad de Cry1Ca *in vivo* mediante silenciamiento con RNAi (Rajagopal *et al*., 2002). Mediante la misma técnica, en *H. armigera* se ha caracterizado una APN como receptor funcional de Cry1Ac, cuya expresión confiere susceptibilidad a células de insecto (Sivakumar *et al*., 2007). Finalmente, la expresión de una APN de *M. sexta* en una cepa transgénica de *D. melanogaster* también le confirió al insecto susceptibilidad a la toxina Cry1Ac (Gill y Ellar, 2002).

En otros órdenes de insectos se han identificado también APN que pueden actuar potencialmente como receptores de las toxinas de *Bt*. En el díptero *Anopheles quadrimaculatus* se ha encontrado una APN que une la toxina Cry11Ba, tóxica para mosquitos (Abdullah *et al*., 2006), y en *A. aegypti* APN también mostraron capacidad de unión a la toxina Cry11A (Chen *et al*., 2009b) y Cry4A (Bayyareddy *et al*., 2009). En *A. gambiae* se ha encontrado una APN que une Cry11Ba (Zhang *et al.,* 2008b), y además un fragmento de esta APN es capaz de inhibir la toxicidad en el insecto al ser ingerido junto con la toxina, mientras que otro fragmento de ésta es capaz de aumentar la toxicidad. En este caso, la inhibición por parte de uno de los péptidos y el sinergismo de otro se ha relacionado con la inhibición o el favorecimiento de la unión de la toxina al epitelio intestinal del mosquito, respectivamente (Zhang *et al.,* 2010). En *A. aegypti* se han validado tres APN como receptores funcionales de Cry4Ba mediante silenciamiento (Saengwiman *et al*., 2011). En el orden de los coleópteros, sólo se ha encontrado un transcrito codificante para un posible receptor APN en *Asymmathetes vulcanorum* (Gómez *et al*., 2012) pero no se ha demostrado su función como receptor de toxinas Cry.

Fosfatasa Alcalina (ALP)

La ALP es un metaloenzima que ejerce el papel de eliminar los grupos fosfato de muchos tipos de moléculas (Kim y Wyckoff, 1991) y algunas formas de esta proteína que están ancladas a la parte exterior de la membrana plasmática a través de GPI, al igual que APN, han mostrado capacidad de unión a toxinas Cry. Se demostró la unión

de las toxinas Cry1Ac y Cry1Ab a ALP de *M. sexta* (McNall y Adang, 2003; Arenas *et al*., 2010) y de Cry1Ac a ALP de *H. virescens* (Jurat-Fuentes y Adang, 2004). En este último trabajo se demostró la implicación de este receptor como mediador de la toxicidad confiriendo susceptibilidad a células de *Drosophila* que expresaban ALP.

En dípteros, se ha confirmado la unión de Cry11Aa a una ALP de *A. aegypti* y además se ha demostrado la importancia de esta interacción mediante la atenuación *in vivo* de la toxicidad, exponiendo epítopos de esta ALP mediante "phage display" (Fernández *et al*., 2006). En este insecto, también se ha demostrado la unión de Cry4Ba a ALP expresada en células y la importancia de este receptor en la aparición de susceptibilidad a la toxina (Dechklar *et al*., 2011). Además, se ha comprobado su papel como receptor funcional de Cry4Ba y Cry11Aa mediante RNAi (Jiménez *et al*., 2012). En *A. gambiae* se ha confirmado también la unión de Cry11Ba a ALP del insecto, y además un fragmento del receptor es capaz de disminuir la toxicidad *in vivo* (Hua *et al*., 2009).

En el orden de los coleópteros también se han encontrado ALP capaces de unir toxinas Cry, como la ALP de *Anthonomus grandis*, que une Cry1Ba6 (Martins *et al*., 2010) o la ALP de *T. molitor* que une Cry3Aa (Zúñiga-Navarrete *et al*., 2013).

Glicolípidos

La información sobre este tipo de receptor proviene en mayor medida del aislamiento de organismos mutantes del nematodo *Caenorhabditis elegans* resistentes a Cry5B y Cry14A (Griffitts *et al*., 2003). En estos estudios, se confirmó que un glicoesfingolípido confería susceptibilidad al nematodo y también que glicolípidos extraídos del intestino de *M. sexta* presentaron unión a diferentes toxinas Cry1A, aunque no se ha establecido aún su papel en el mecanismo de toxicidad (Griffitts *et al*., 2005).

Transportador ABC

Los transportadores con casete de unión a ATP (transportador ABC) son proteínas ancladas a la membrana a través de 12 hélices alfa transmembrana. Utilizan la energía de la hidrólisis del ATP para exportar compuestos desde el citoplasma hacia el exterior celular o dentro de compartimentos intracelulares (Heckel, 2012).

McNall y Adang (2003) identificaron un transportador ABC en *M. sexta* como posible molécula de unión a la toxina Cry1Ac, y recientemente, el transportador ABC de la subfamilia C2 (transportador ABCC2) de *B. mori* ha sido validado como receptor

funcional de Cry1Ab y Cry1Ac, ya que la expresión de este receptor en células confirió susceptibilidad a las toxinas. La expresión del transportador junto con la cadherina incrementó la susceptibilidad de las larvas del insecto a las toxinas Cry1A, Cry1F y Cry8Ca (Tanaka *et al*., 2013).

Otros receptores

Se han identificado otras proteínas de insectos susceptibles capaces de unir toxinas Cry que podrían actuar como receptores, además de los receptores descritos anteriormente, que son los mejor caracterizados. Entre ellas, encontramos actina, transportadores de péptidos y canales de sodio, las subunidades B y E de la V-ATPasa, las subunidades alfa y beta de la ATP sintasa, una metaloproteasa de zinc de la familia M1, serín proteasas, prohibitinas, desmocolinas, una proteína relacionada con el choque térmico (McNall y Adang, 2003; Bayyareddy *et al*., 2009; Krishnamoorthy *et al*., 2007; Chen *et al*., 2010a), un glicoconjugado de 270 KDa (Valaitis *et al*., 2001), una proteína no identificada de 252 KDa (Hossain *et al*., 2004), una metaloproteasa ADAM (Ochoa-Campuzano *et al*., 2007), una glucosidasa (Yamaguchi *et al*., 2013) y una alfa amilasa (Fernández-Luna *et al*., 2010). La posible implicación de estas proteínas en el mecanismo de acción de las toxinas Cry pone de manifiesto la gran complejidad del mismo.

4.2.2 Modelos que explican el modo de acción de las toxinas Cry en tres dominios

Dado el elevado número de toxinas Cry, los diferentes receptores candidatos para las toxinas y la diversidad de insectos que actualmente se estudian, no resulta sorprendente que el modo de acción de las toxinas Cry sea controvertido. Existen diversos modelos basados mayoritariamente en estudios con insectos lepidópteros que difieren en cómo se produce el efecto tóxico y la importancia que se le da a los distintos receptores involucrados (Fig. 8).

Modelo de Bravo (formación de poro)

Este modelo está basado en el estudio del modo de acción de la toxina Cry1Ab en el lepidóptero *M. sexta* y propone que tanto el receptor tipo cadherina como APN se requieren para la toxicidad. Postula que la unión de alta afinidad de la forma activa de la toxina al receptor tipo cadherina promueve un cambio de conformación que facilita la proteolisis por parte de proteasas asociadas a membrana del intestino, del extremo amino terminal de la toxina Cry1Ab, que incluye la hélice 1 alfa (Gómez *et al*., 2002).

Este corte proteolítico provoca la oligomerización tetramérica de la toxina, que presenta una afinidad más elevada por el receptor tipo APN que la toxina monomérica. Este segundo receptor dirige la estructura oligomérica a las regiones ricas en colesterol (Gómez *et al*., 2007; Bravo *et al*., 2004), donde se facilita su inserción en la membrana formando un poro, con la consecuente circulación libre de iones a su través, influjo de agua y lisis osmótica de la célula (Knowles y Ellar, 1987) (Fig. 8). Más recientemente, el modelo se ha completado con estudios de mutagénesis dirigida, que indican que posiblemente la toxina monomérica se une primero con baja afinidad a APN, receptor muy abundante en el intestino del lepidóptero. Después se une con alta afinidad a la cadherina, que es poco abundante y tras producirse la proteólisis del extremo amino terminal y oligomerización, el tetrámero de toxinas se unen de nuevo a APN, mecanismo conocido como "ping-pong". (Pacheco *et al*., 2009).

Modelo de Zhang (transducción de la señal)

Este modelo se fundamenta en los resultados obtenidos en células de insecto donde se expresó el receptor cadherina de *M. sexta* (Zhang *et al.,* 2005) y más recientemente también el de *A. gambiae* (Ibrahim *et al.,* 2013) e implica solo a éste receptor en el modo de acción. Propone que la toxina Cry monomérica se une a este receptor, iniciando cascadas de señalización dependientes de Mg^{2+}. La unión de la toxina al receptor promueve la activación de la adenilato ciclasa a través de la proteína G. El aumento de AMP cíclico intracelular produce la activación de la proteína quinasa A, que conlleva la desestructuración de canales iónicos y el citoesqueleto, con la consecuente muerte celular por oncosis. Este modelo no cuestiona la existencia de la forma oligomérica de la toxina, pero postula que ésta se inserta en la membrana de forma inespecífica y no es la causante de la aparición de poros líticos (Zhang *et al*., 2005; Zhang *et al*., 2006).

Modelo de Jurat-Fuentes

A partir de los estudios realizados con la toxina Cry1Ac en el lepidóptero *H. virescens* y cepas resistentes de este insecto, Jurat-Fuentes y Adang (2004, 2006a) propusieron la integración de los modelos anteriores. Postulan que la unión de la toxina monomérica al receptor tipo cadherina promueve la oligomerización de la misma e incrementa su afinidad por receptores anclados a la membrana a través de GPI, como APN, al igual que se explica en el modelo de Bravo y también a ALP. Estos dos últimos receptores dirigen el oligómero a estructuras ricas en colesterol de la membrana donde se forma un poro, y además, esta unión también puede promover la activación de cascadas de señalización intracelular reguladas por fosfatasas. De esta

forma, la citotoxicidad se debe tanto a la lisis osmótica generada por la formación del poro como por la activación de cascadas de señalización intracelulares, dos eventos que no son excluyentes entre sí.

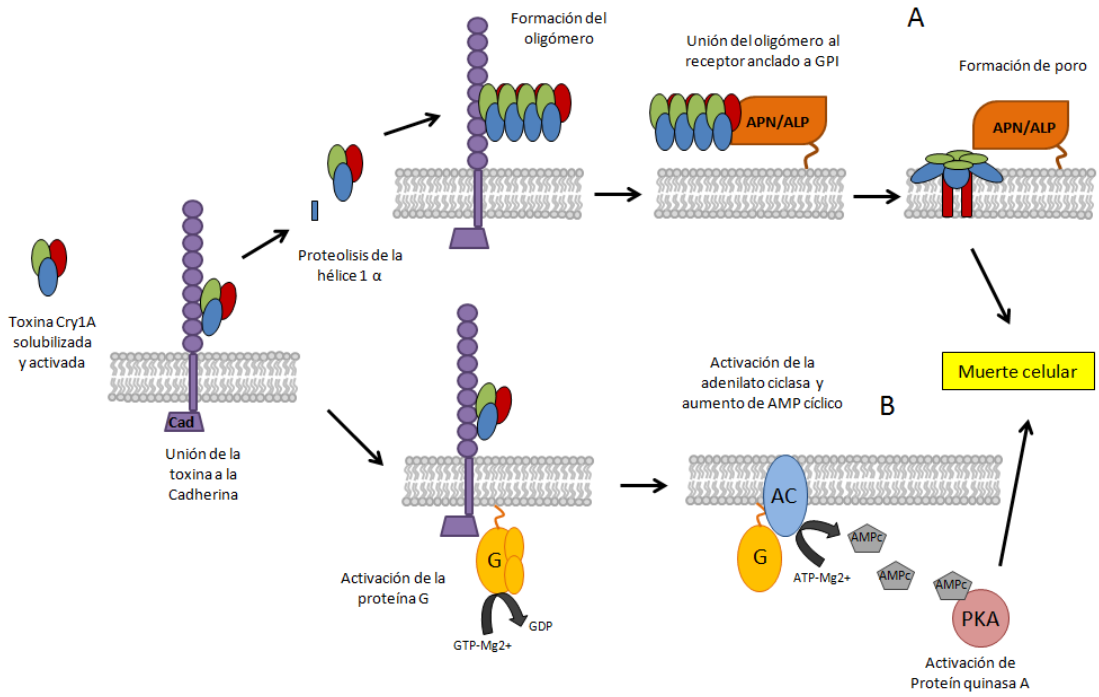

Figura 8. Mecanismo de acción de las toxinas Cry. A) Modelo de Bravo (formación de poro). B) Modelo de Zhang (transducción de la señal). Adaptado de Soberón *et al.* (2009).

5. Mecanismos de resistencia a la acción de las toxinas Cry

El uso intensivo de *Bt* y sus genes *cry* incrementa la probabilidad de desarrollar resistencia por parte de los insectos a los bioinsecticidas basados en *Bt* y especialmente en plantas transgénicas, en las que la alta presión de selección generada en los insectos puede desencadenar mecanismos de defensa con el fin de eludir la acción tóxica, como el desarrollo de una elevada respuesta inmune del insecto (Rahman *et al.*, 2004; Hernández-Martínez *et al.*, 2010).

Sin embargo, los casos de resistencia más estudiados son aquellos en los que se produce el bloqueo de alguno de los pasos en el mecanismo de acción. Para estudiar las bases moleculares de la resistencia se han seleccionado en los laboratorios cepas resistentes y se ha observado que la ausencia de unión de la toxina a sus receptores es la causa de aparición de resistencia más frecuente. Esta pérdida de unión puede deberse a distintos factores como la degradación o falta de activación de la toxina por proteólisis o a la ausencia del receptor.

5.1 Resistencia por proteolisis de la toxina

En diferentes estudios, se ha comprobado que una de las posibles causas de resistencia implica a las proteasas que interaccionan con las toxinas en el intestino del insecto. Estas investigaciones sugieren que los cambios en la actividad o composición de dichas proteasas están relacionados con la alteración de la susceptibilidad a las toxinas Cry. Así, se ha comprobado que las proteasas de una cepa resistente de *H. virescens* eran capaces de degradar más rápidamente la toxina que las proteasas de la cepa susceptible (Forcada *et al*., 1996) y en *Spodoptera littoralis*, se ha demostrado que la actividad de las proteasas del intestino se incrementa a lo largo del desarrollo, incremento asociado a una mayor degradación de la toxina y a la pérdida de sensibilidad a ésta (Keller *et al*., 1996). También se han encontrado dos cepas resistentes de *Plodia interpunctella* que han perdido una proteasa del intestino involucrada en la activación de las toxinas Cry por proteólisis (Oppert *et al*., 1997) y además, se ha relacionado la presencia de diferentes formas de proteasas digestivas con resistencia de *L. decemlineata* a Cry3Aa (Loseva *et al*., 2002).

5.2 Resistencia por ausencia de receptor

La ausencia de unión de las toxinas Cry al epitelio intestinal del insecto susceptible ha sido relacionada, en muchos casos, con mutaciones que afectan a sus receptores. Se ha demostrado que la disrupción en el gen de la cadherina conduce a resistencia del insecto a las toxinas Cry (Gahan *et al*., 2001; Xu *et al*., 2005; Fabrick *et al*., 2011), así como de APN (Zhang *et al*., 2009; Chang *et al*., 2012). Se han identificado casos en los que se ha detectado unos niveles reducidos de ALP en las cepas resistentes (Jurat-Fuentes *et al*., 2011) y también se ha implicado la modificación de carbohidratos con resistencia a las toxinas Cry en *C. elegans* (Griffitts *et al*., 2001). Cambios en los niveles de V-ATPasa y F-ATPasa también se han relacionado con cepas resistentes de *P. interpunctella* (Candas *et al*., 2003). Además, recientemente, se ha detectado una mutación en el gen ABCC2 codificante de un transportador ABC responsable de la resistencia de *H. virescens* a Cy1Ac y de *B. mori* a Cry1Ab (Gahan *et al*., 2010; Atsumi *et al*., 2012). La presencia de mutaciones que afectan a receptores de las toxinas Cry en cepas resistentes refuerza la importancia de la interacción entre la toxina y su receptor, demostrando la función de los mismos como mediadores de la acción tóxica *in vivo*.

5.3 Resistencia a *Bt* en el campo

A pesar del uso intensivo de formulaciones basadas en *Bt* durante los últimos 15 años, en el campo han aparecido de momento pocos casos de resistencia. Uno de los insectos que ha demostrado una mayor capacidad para desarrollar resistencia a toxinas de *Bt* es *P. xylostella*. Los primeros casos de resistencia a *Bt* en el campo se detectaron en este lepidóptero en Filipinas (Tabashnik *et al*., 1990; Ferré *et al*., 1991), en Hawai, y en el centro de Estados Unidos (Tabashnik, 1994). Sin embargo, la elevada presión de selección a la que son sometidos los insectos alimentados con cultivos *Bt* ha generado resistencia en numerosas especies. Se detectó tolerancia de *P. gossypiella* en plantaciones transgénicas de algodón que expresaban Cry1Ac, en India y en China (Bagla, 2010; Wan *et al*., 2012) y también de larvas de *H. armigera* en algodón *Bt* que expresa Cry1Ac en China (Liu *et al*., 2010) o Cry2A en Australia (Downes *et al*., 2010). También se han encontrado larvas de *Spodoptera frugiperda* resistentes a maíz *Bt* que expresa Cry1F, en Puerto Rico (Storer *et al*., 2010) y larvas de *Busseola fusca* resistentes a maíz Bt que expresa Cry1Ab en Sudáfrica (Kruger *et al*., 2011).

6. Respuesta de los organismos susceptibles a las toxinas Cry

Con el objetivo de entender cómo se desarrolla el proceso de intoxicación e intentar así diseñar estrategias más efectivas para el control de plagas con productos basados en *Bt*, se han realizado estudios de la respuesta generada en diferentes organismos a las toxinas Cry.

En *C. elegans*, la toxina Cry5B indujo genes relacionados con la ruta de señalización de MAPK (proteín quinasa acivada por mitógenos) (Huffman *et al*., 2004), con la respuesta de estrés de la llamada "proteína desplegada" (UPR) del retículo endoplasmático, regulada a su vez por la primera (Bischof *et al*., 2008) y con la ruta DAF-2 insulina/IGF-1 (Chen *et al*., 2010b). En este nematodo, la ruta de señalización de hipoxia, además, resultó protectora frente a la acción patogénica de la toxina Cry21A (Bellier *et al*., 2009). En los insectos *M. sexta* y *A. aegypti* se confirmó igualmente la importancia de la ruta de las MAPK en la respuesta a las toxinas Cry (Cancino-Rodezno *et al*., 2010) y la respuesta a estrés UPR en este último insecto (Bedoya-Pérez *et al*., 2013).

A. aegypti y *H. armigera* respondieron a distintas toxinas Cry aumentando la demanda energética e incrementando los niveles de componentes del citoesqueleto (Cancino-Rodezno *et al*., 2012; Yuan *et al*., 2011). Los lepidópteros *Choristoneura fumiferana* y *H. armigera*, a su vez, indujeron la expresión de genes codificantes para proteínas involucradas en la detoxificación (van Munster *et al*., 2007; Yuan *et al*., 2011) y

en *C. fumiferana*, además, proteínas relacionadas con la defensa inmune o asociadas a estrés (van Munster *et al*., 2007).

Entre los genes reprimidos tras la intoxicación de toxinas Cry se encontraron, en *A. aegypti,* algunos que codifican proteínas implicadas en el plegamiento y reciclaje de proteínas (Cancino-Rodezno *et al*., 2012), y en *C. fumiferana* y *H. armigera*, genes que codifican enzimas intestinales (van Munster *et al*., 2007; Yuan *et al*., 2011).

En los coleópteros *D. virgifera virgifera* y *T. molitor*, tras el tratamiento con toxinas Cry también se encontraron sobreexpresados genes codificantes de proteínas estructurales y relacionados con el citoesqueleto, así como proteínas implicadas en patogénesis y respuesta inmune (Sayed *et al*., 2010; Oppert *et al*., 2012). En *T. molitor*, además, se indujo la expresión de genes relacionados con el transporte de iones y funciones mitocondriales para generar energía vía respiración celular y se reprimieron aquellos que codificaban enzimas metabólicos asociados con glicolisis, el ciclo del ácido cítrico, catabolismo de lípidos, actividad antioxidante y enzimas intestinales (Oppert *et al*., 2012).

En líneas generales, parece que las toxinas Cry reprimen en los insectos la expresión de enzimas intestinales que pueden estar involucrados en la adaptación del insecto a la exposición de toxinas de *Bt* y de enzimas metabólicos, posiblemente como resultado de la parálisis característica tras la intoxicación (Oppert *et al*., 2012). Por otra parte, con el objetivo de combatir mediante la respuesta innata la invasión bacteriana, se induce la producción de energía para contrarrestar el estrés ocasionado por la toxina, la expresión de proteínas relacionadas con el citoesqueleto para reforzar el epitelio intestinal dañado y proteínas implicadas en la detoxificación y sistema inmune (Yuan *et al*., 2011; van Munster *et al*., 2007).

7. Mecanismos de respuesta innata en los insectos

Los insectos han colonizado prácticamente todos los nichos ecológicos, y por tanto deben afrontar gran diversidad de parásitos y microorganismos patógenos. Han diseñado eficientes y complejas estrategias de defensa innata o no adaptativa contra estos invasores, ya que carecen de defensa adquirida, que desarrolla memoria inmunológica. Así, algunos patrones moleculares presentes en los patógenos como el lipopolisacárido y peptidoglicano bacteriano y β-1,3 glucanos de la pared celular de los hongos son reconocidos por proteasas y proteínas implicadas en el reconocimiento de patógenos del hospedador, poniendo en marcha esta respuesta innata (Hoffmann, 1995).

La respuesta innata codifica factores para el reconocimiento y eliminación de los invasores y comprende la respuesta celular y la respuesta humoral. La respuesta celular está mediada por los hemocitos, e incluye los mecanismos de fagocitosis y formación de nódulos (unión de hemocitos a agregados de bacteria y material extracelular) que pueden llevar también a la encapsulación (formación de capas de hemocitos sobre organismos diana más grandes como parasitoides, nematodos, etc. que no pueden ser fagocitados) (Lavine y Strand, 2002).

La respuesta humoral comprende la producción de lisozima y péptidos antibacterianos y antifúngicos por parte del cuerpo graso (homólogo funcional del hígado de los mamíferos) que se liberan a la hemolinfa para destruir los microorganismos invasores. También contribuyen a la síntesis de estos péptidos aunque en menor medida los hemocitos, túbulos de Malphigi, cutícula, células pericárdicas e intestino medio. En coleópteros, se han identificado y caracterizado péptidos antibacterianos como defensinas, coleoptericinas, cecropinas y otros péptidos antifúngicos (Gillespie *et al.*, 1997).

Además, en la respuesta humoral se inducen dos cascadas proteolíticas que llevan a la formación del coágulo y la melanización. Ésta última es el resultado de una cascada de serín proteasas dependiente de calcio, donde la proteína clave es el enzima fenoloxidasa, que genera melanina e intermediarios reactivos de oxígeno y nitrógeno (Hoffmann, 1995).

7.1 Fenoloxidasa

Es un enzima con actividad oxidorreductasa, presente en la hemolinfa en su forma precursora profenoloxidasa (proPO), en hemocitos y/o plasma y también en la cutícula y en células epiteliales del intestino. Además de ser un enzima implicado en la respuesta inmune, es también importante en la esclerotización y pigmentación de muchos tejidos (Söderhäll y Cerenius, 1998).

La presencia de compuestos de origen microbiológico promueve la proteólisis de la forma precursora por medio de serín proteasas, liberando la forma activa fenoloxidasa (PO). La PO forma parte de un sistema complejo, constituido por proteínas de reconocimiento y unión a elementos presentes en los microorganismos y parásitos invasores, la cascada de serín proteasas activadoras y otros factores reguladores del sistema como inhibidores de proteasas (por ejemplo, serpinas).

Después de la activación, la PO forma quinonas a partir de la oxidación de fenoles, lo que conduce a la formación de melanina por polimerización no enzimática (Fig. 9).

Los compuestos reactivos del oxígeno generados durante el proceso son tóxicos para los microorganismos y la melanina formada actúa como escudo protector contra los invasores retardando o inhibiendo su crecimiento y/o para sellar la herida (Cerenius y Söderhäll, 2004).

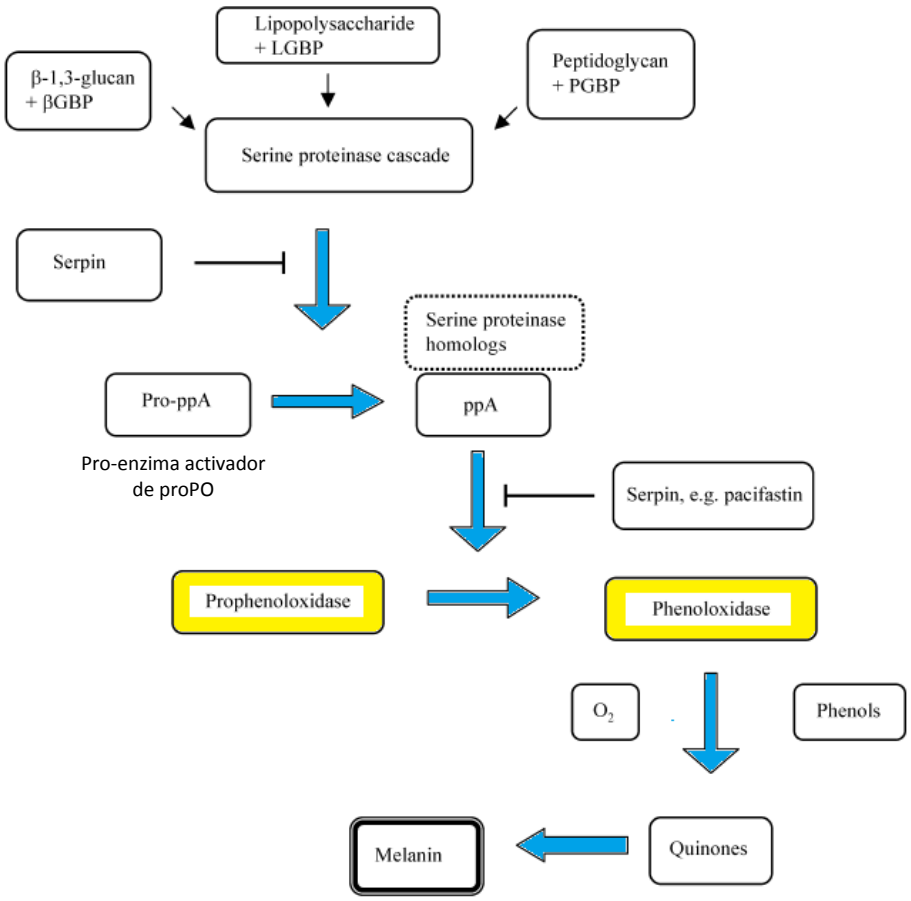

Figura 9. Cascada de activación del enzima profenoloxidasa. Tomado de Cerenius y Söderhäll (2004).

Se ha caracterizado el enzima proPO de diferentes artrópodos y todos ellos presentan una estructura dimérica constituida por subunidades de 80 kDa (Söderhäll y Cerenius, 1998). Forman parte de una superfamilia génica que incluye hemocianinas y proteínas hexaméricas de hemolinfa.

En el genoma de *T. castaneum* se han identificado 3 genes codificantes de proPO que presentan una identidad de secuencia aminoacídica del 99,6%, y por homología de secuencia, 103 genes codificadores de serín proteasas de entre los cuales se han propuesto candidatos a activar a PO. También se han identificado proteínas que podrían actuar como cofactores, y además, 65 homólogos no catalíticos de serín proteasas y hasta 31 serpinas inhibidoras de serín proteasas que se encargan de regular

que la activación proteolítica de proPO ocurra de forma local y transitoria en respuesta a la infección (Zou *et al*., 2007).

En la hemolinfa de los insectos, además de la fenoloxidasa, se encuentran otras proteínas involucradas en la inmunidad y otros procesos fisiológicos y del desarrollo, siendo una de las más destacables la apolipoforina III.

7.2 Apolipoforina III

La apolipoforina III (apoLpIII) es una proteína implicada en el metabolismo de lípidos en los insectos que adicionalmente participa en reacciones inmunológicas, actuando como proteína de unión al lipopolisacárido bacteriano o como potenciadora de la actividad bacteriolítica de la hemolinfa.

Se ha caracterizado la apoLpIII en diferentes especies de insectos, la mayoría de ellos ortópteros y lepidópteros, y constituye una apolipoproteína modelo para el estudio de las apolipoproteínas denominadas intercambiables, que se asocian de forma reversible con la lipoforina estructural o no intercambiable.

7.2.1 Implicación de apoLpIII en el transporte de lípidos

En los insectos, el transporte de lípidos en circulación se lleva a cabo mediante la formación de complejos de lípidos y proteínas que dan lugar a apolipoproteínas que estabilizan los componentes lipídicos y median el metabolismo de la partícula (Weers y Ryan, 2006).

La apolipoproteína más importante en la hemolinfa de los insectos es la lipoforina que está compuesta por dos subunidades, la apolipoforina I (apoLp-I) de aproximadamente 240 kDa, y la apolipoforina II (apoLpII) de unos 80 kDa. Estas dos apolipoforinas provienen de un mismo precursor que sufre un procesado post-traduccional por parte de una furina del insecto y forman parte de la partícula lipoproteica estructural o no intercambiable madura en una ratio 1:1 (Weers y Ryan, 2006).

Durante periodos largos de movilización de lípidos, como en el vuelo de los insectos, la hormona adipoquinética estimula, en el cuerpo graso, la conversión de las reservas de tracilglicéridos en diacilglicéridos (DAG) y éstos son liberados a la hemolinfa. La lipoforina, en forma de partícula lipoproteica de alta densidad (HDLp) que se sintetiza también en el cuerpo graso, se libera a la hemolinfa donde ejerce la función de recogida de estos DAG, pasando a ser una lipoforina de baja densidad

(LDLp). Al mismo tiempo, el aumento del nivel de lípidos en la hemolinfa induce la síntesis de una partícula lipoproteica de la familia de las apolipoproteínas intercambiables, llamada apolipoforina III (apoLpIII), que se asocia a la partícula LDLp. Todo el complejo viaja por la hemolinfa hasta el músculo de vuelo u otro tejido diana, donde una lipasa hidroliza los DAG que serán utilizados para la generación de energía. La apoLpIII se disocia del complejo y se regenera la partícula HDLp original que vuelve al cuerpo graso para recoger y transportar otra ronda de lípidos (Van der Horst *et al.*, 2002) (Fig. 10).

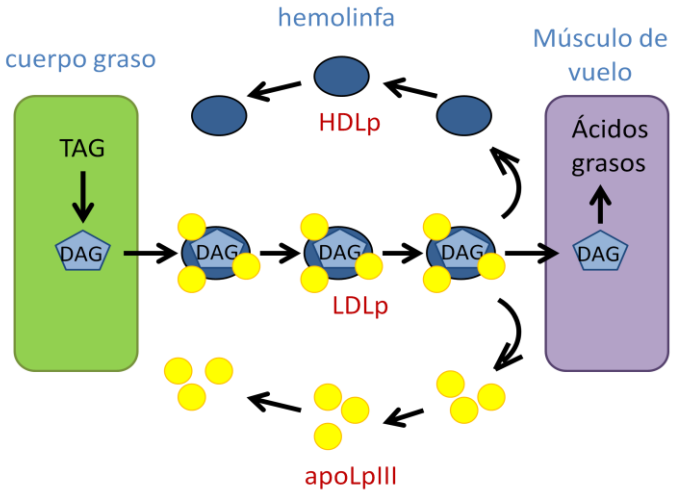

Figura 10. Ciclo de transporte de lípidos en la hemolinfa de los insectos. En respuesta a la hormona adipoquinética, los DAG del cuerpo graso son recogidos por la partícula HDLp, que al mismo tiempo recluta a apoLpIII presente en la hemolinfa. La partícula pasa a ser LDLp y viaja por la hemolinfa hasta el músculo de vuelo, donde se liberan los DAG y HDLp y apoLpIII se regeneran. Adaptado de Weers y Ryan (2006).

Las moléculas de apoLpIII, de bajo peso molecular (entre 161 y 166 aminoácidos), están compuestas por cinco hélices anfipáticas conectadas por lazos cortos, estructuradas de forma cilíndrica con los residuos hidrofóbicos hacia el interior y los residuos hidrofílicos hacia el exterior (Breiter *et al.*, 1991; Wang *et al.*, 1997) (Fig. 11). En la conformación en que no se une a lípidos, apoLpIII se comporta como una proteína globular, pero al asociarse a lípidos se produce un cambio conformacional importante en el que se exponen los residuos hidrofóbicos para interactuar con los lípidos (Niere *et al.*, 2001).

7.2.2 Implicación de apoLpIII en la inmunidad

Estudios de expresión de apoLpIII en *M. sexta* revelaron que esta proteína aumentaba en tejido muscular durante la muerte celular programada, lo cual sugiere

que esta molécula podría tener un papel alternativo al del transporte de lípidos (Sun *et al*., 1995). En este sentido, se ha descrito que la apoLpIII de *Hyphantria cunea* y *P. xylostella* aumentan después de la infección con *E. coli* (Kang *et al*., 2003; Son y Kim, 2011) y en *A. gambiae* tras la infección con *Plasmodium berghei* (Gupta *et al*., 2010). En *Galleria mellonella* también se observó un aumento de apoLpIII después de la inyección con bacterias gram negativas como *E. coli*, *Klebsiella pneumoniae* y *Pseudomonas aeruginosa* y gram positivas como *Micrococcus luteus*, y *Bt kurstaki* y *alesti* (Zdybicka-Barabas y Cytryńska, 2011; Andrejko y Mizerska-Dudka, 2011; Andrejko *et al*., 2008) al igual que en *T. molitor*, donde se observaron transcritos codificantes de apoLpIII sólo en larvas tratadas con la toxina de *Bt* Cry3Aa y no en larvas sin tratar (Oppert *et al*., 2012). Estas observaciones relacionaron el aumento de apoLpIII con la respuesta a diversos patógenos, entre ellos *Bt*, de modo que, además de su función en el transporte de lípidos, fue implicada en el sistema inmune de los insectos.

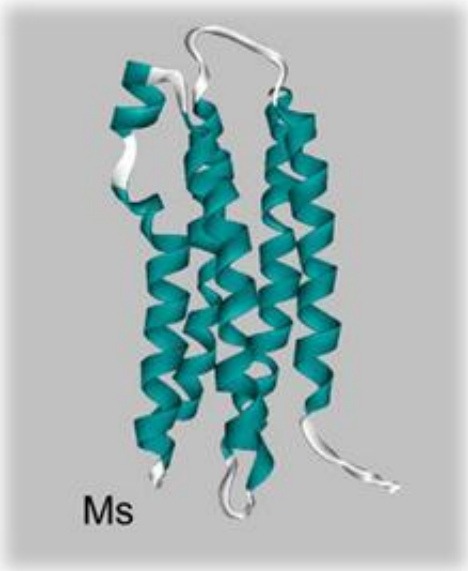

Figura 11. Estructura de la apolipoforina III de *M. sexta*. Tomado de Weers y Ryan (2006).

ApoLpIII como proteína de reconocimiento de patógenos

El papel de apoLpIII como proteína relacionada con el sistema inmune se confirmó al observarse una interacción entre la apoLpIII de diversos insectos con el ácido lipoteicoico de la superficie de las bacterias gram positivas (Halwani *et al*., 2000), con lipopolisacárido de las bacterias gram negativas (Pratt y Weers, 2004) y con β-1,3 glucanos de la pared de los hongos (Whitten *et al*., 2004). Esta propiedad le otorga la posibilidad de unirse a la superficie celular de los invasores y formar parte del proceso de reconocimiento de patógenos por parte del sistema inmne del insecto. La capacidad

innata de detectar patógenos es esencial para la existencia de los organismos multicelulares, y se ha conseguido a través de la evolución de moléculas que reconocen estructuras que no sean propias (Janeway, 1992). ApoLpIII es una proteína abundante en la hemolinfa, implicada en el transporte de lípidos sólo cuando se transportan grandes cantidades de éstos. Por ello, se hipotetiza que siempre se dispone de una cantidad de apoLpIII libre en la hemolinfa que puede actuar como proteína de reconocimiento de patógenos (Weers y Ryan, 2006).

Se han observado cambios e interconversiones entre lipoforinas después del inicio de la respuesta inmune, como por ejemplo un descenso en HDLp y un aumento de LDLp (Dettloff *et al*., 2001b), por lo que se ha sugerido que apoLpIII, en su conformación de unión a lípidos, actúa como un activador de la respuesta inmune, mientras que la conformación libre no tiene esta función (Dettloff *et al*., 2001a). Es posible que el cambio conformacional de la proteína permita crear alteraciones en su superficie que generen una señal para una respuesta inmune secundaria (Weers y Ryan, 2006).

ApoLpIII como proteína estimuladora del sistema inmune

Se ha descrito que apoLpIII está implicada tanto en la regulación de la respuesta inmune humoral como celular. En *G. mellonella*, la inyección de esta proteína produjo un aumento de la actividad antibacteriana y lisozímica, además de incrementar la fagocitosis de células de levadura por los plasmatocitos del insecto, lo que llevó a catalogar a apoLpIII como un factor proteico estimulador del sistema inmune (Wiesner *et al*., 1997). Esta proteína es capaz de actuar como potenciador de la actividad lítica contra bacterias (Halwani y Dunphy, 1999) y como agente hemoaglutinante y causante de la agregación de eritrocitos (Iimura *et al*., 1998) y por ello, estimula la formación de nódulos melanizados y encapsulación de cuerpos exógenos por parte de los hemocitos (Whitten *et al*., 2004; Son y Kim, 2011). La apoLpIII de *G. mellonella* fue capaz de estimular la producción de péptidos antibacterianos y lisozima así como la cascada del enzima proPO (Park *et al*., 2005, Niere *et al*., 1999). En este mismo insecto, se observó que la activación de la cascada de la proPO en la hemolinfa en respuesta a la inyección de glucanos dependía de la edad de las larvas, y se correlacionó con la cantidad de apoLpIII en la hemolinfa (Mullen y Goldsworthy, 2003). Más recientemente, apoLpIII ha sido implicada en la regulación de enzimas antioxidantes especialmente de manganeso superóxido dismutasa, y regula indirectamente especies reactivas de oxígeno en *H. cunea* (Kim *et al*., 2011).

Se han identificado varias moléculas de reconocimiento de patógenos en los insectos, pero antes de la implicación de apoLpIII en el sistema inmune, poco se conocía sobre el mecanismo posterior que lleva a la inducción de la respuesta innata. Los esfuerzos dedicados en encontrar un activador endógeno de la inmunidad llevaron a identificar a apoLpIII como molécula estimuladora del sistema inmune, lo que resultó sorprendente por la conocida participación de esta proteína en el metabolismo de lípidos. Desde entonces, se han llevado a cabo muchos estudios relacionando esta molécula en la respuesta inmune innata, tanto como molécula de reconocimiento de patógenos, como activadora de la actividad antimicrobiana en la hemolinfa o como activadora de la respuesta celular y humoral entre la que destaca en ésta última la cascada de proPO.

Objetivos

Los bioinsecticidas basados en *Bacillus thuringiensis (Bt)* constituyen una herramienta de gran valor para el control de plagas de insectos, ya que presentan una forma de aplicación muy específica e inocua para los usuarios y el medio ambiente. Entender, tanto el mecanismo de toxicidad de estos bioinsecticidas como la respuesta desencadenada en los insectos diana, tiene como objetivo proporcionar una base útil para el desarrollo de insecticidas mejorados dirigidos contra estas importantes plagas agrícolas, permitiendo ampliar el rango y los niveles de toxicidad de las proteínas insecticidas y abordar problemas como la aparición de resistencia.

En el presente trabajo nos hemos centrado en el estudio del modo de acción de las toxinas Cry en insectos coleópteros. Para ello hemos escogido dos especies de insecto relevantes en este orden: la especie modelo *Tribolium castaneum* por su condición de plaga, la disponibilidad de la secuencia de su genoma y las numerosas herramientas genéticas y genómicas desarrolladas en dicho organismo que facilitan enormemente el análisis funcional y *Leptinotarsa decemlineata*, una de las plagas más importantes a nivel mundial en este orden. Nos planteamos los siguientes objetivos específicos:

1. **Evaluación de la actividad insecticida de diferentes cepas de *Bt* en larvas de *T. castaneum*.**

2. **Análisis de la respuesta de *T. castaneum* a cepas de *Bt* tóxicas para el insecto.**
   - Identificación de proteínas de *T. castaneum* diferencialmente reguladas tras el tratamiento con *Bt*.
   - Estudios funcionales de las proteínas implicadas en la respuesta de *T. castaneum* a *Bt.*

3. **Estudio del modo de acción de las toxinas Cry activas contra *T. castaneum*.**
   - Identificación de receptores implicados en el modo de acción de las toxinas Cry tóxicas para *T. castaneum.*

4. **Estudio del modo de acción de las toxinas Cry3 en *L. decemlineata*.**
   - Identificación de receptores implicados en el modo de acción de Cry3Aa en *L. decemlineata.*
   - Análisis de los patrones de proteólisis de las toxinas Cry3 y su interacción con la metaloproteasa ADAM.

Artículos

Artículo 1: Proteome response of *Tribolium castaneum* larvae to *Bacillus thuringiensis* toxin producing strains

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Proteome Response of *Tribolium castaneum* Larvae to *Bacillus thuringiensis* Toxin Producing Strains

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Abstract

Susceptibility of *Tribolium castaneum* (Tc) larvae was determined against spore-crystal mixtures of five coleopteran specific and one lepidopteran specific *Bacillus thuringiensis* Cry toxin producing strains and those containing the structurally unrelated Cry3Ba and Cry23Aa/Cry37Aa proteins were found toxic (LC_{50} values 13.53 and 6.30 μg spore-crystal mixture/μL flour disc, respectively). Using iTRAQ combined with LC-MS/MS allowed the discovery of seven novel differentially expressed proteins in early response of Tc larvae to the two active spore-crystal mixtures. Proteins showing a statistically significant change in treated larvae compared to non-intoxicated larvae fell into two major categories; up-regulated proteins were involved in host defense (odorant binding protein C12, apolipophorin-III and chemosensory protein 18) and down-regulated proteins were linked to metabolic pathways affecting larval metabolism and development (pyruvate dehydrogenase Eα subunit, cuticular protein, ribosomal protein L13a and apolipoprotein LI-II). Among increased proteins, Odorant binding protein C12 showed the highest change, 4-fold increase in both toxin treatments. The protein displayed amino acid sequence and structural homology to *Tenebrio molitor* 12 kDa hemolymph protein b precursor, a non-olfactory odorant binding protein. Analysis of mRNA expression and mortality assays in Odorant binding protein C12 silenced larvae were consistent with a general immune defense function of non-olfactory odorant binding proteins. Regarding down-regulated proteins, at the transcriptional level, pyruvate dehydrogenase and cuticular genes were decreased in Tc larvae exposed to the Cry3Ba producing strain compared to the Cry23Aa/Cry37Aa producing strain, which may contribute to the developmental arrest that we observed with larvae fed the Cry3Ba producing strain. Results demonstrated a distinct host transcriptional regulation depending upon the Cry toxin treatment. Knowledge on how insects respond to Bt intoxication will allow designing more effective management strategies for pest control.

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Introduction

The entomophatogenic bacterium *Bacillus thuringiensis* (Bt) represents an environmentally safe alternative for pest control producing parasporal inclusions, which contain one or several insecticidal proteins. The greatest variety of toxins found in the crystals of Bt are proteins of the Cry (for Crystal) or Cyt (for Cytotoxic) type [1]. The largest group of Cry toxins corresponds to the 3-domain Cry proteins (Cry-3D), including at least 40 different groups with more than 200 different gene sequences [2]. Other Cry proteins display no homology to the Cry-3D proteins, such as Cry35Ab and Cry36Aa proteins, Cry34Ab and Cry35Ab proteins, and the coleopteran active Cry23Aa and Cry37Aa proteins, homologous to BinA and BinB binary toxins or Mtx toxins from *Bacillus sphaericus* [3].

The red flour beetle, *Tribolium castaneum* (Tc), is a major global pest of stored grain, cereal products, and peanuts for human consumption [4]. This coleopteran insect, readily adaptable to all classes of insecticides, is an ideal subject for the identification of new pesticide targets for which many genetic and genomics tools have been developed and it has become the genetic model for agriculturally important coleopteran species [5]. Tc bioassays with Bt toxins, Cry3Aa, Cry8Ea, Cry8Fa, Cry8Ga, Cry23Aa/Cry37Aa, Cyt2Ca, Cry34Ab/Cry35Ab and Cry1F, have been carried out showing that Cry23Aa/Cry37Aa and Cyt2Ca were active, Cry3Aa intermediate-active and Cry8Ea, Cry8Fa, Cry8Ga, Cry34Ab/Cry35Ab and Cry1F did not have insecticidal activity against this insect [6,7].

Nowadays much research is being carried out to elucidate the molecular basis of Bt Cry toxins entomopathogenic action. The most extensively studied insecticidal Bt proteins are Cry-3D toxins and although their mode of action is not completely understood, it is generally accepted that involves toxin solubilization in the midgut of the susceptible larvae, membrane receptor binding and oligomerization of the toxin followed by pore formation in the brush border membrane [8]. Potential evidence for cell-death signalling pathways in insects as a result of Bt toxins activity has also been reported [9,10]. However, the present knowledge about toxin-induced cellular phenomena lags behind our understanding of the physiological process of Bt intoxication. In nematodes, various signaling pathways have been involved in Cry toxicity and defensive host responses, which include p38 mitogen-activated protein kinase [11], unfolded protein response [12], DAF-2 insulin/IGFR signaling pathways [13] and hypoxia response

pathways [14]. Regarding insects, several reports have characterized some of the defensive response of insects to Cry toxins by means of substraction hybridization libraries in *Choristoneura fumiferana* and *Manduca sexta* larvae treated with sublethal concentrations of Cry1Ab toxin [15,16], transcriptional analysis in *Diabrotica virgifera* challenged with Cry3Bb toxin [17], gene silencing in *M. sexta* challenged with Cry1Ab toxin and *Aedes aegypti* intoxicated with Cry11Aa spore-crystal preparations [18], transcriptome profiling in *Tenebrio molitor* larvae after ingestion of Cry3Aa toxin [19], and proteome analyses in *Helicoverpa armigera* intoxicated with Cry1Ac [20] and *A. aegypti* exposed to Cry11Aa toxin [21], using 2D-electrophoresis and mass spectrometry.

An alternative for the analysis of proteins in a global manner is the iTRAQ technique, a powerful proteomics method that provides higher coverage than other strategies, which has been scarcely used to evaluate the physiological importance of proteins related with the Bt mode of action since only two reports have used iTRAQ to analyze differential protein alterations associated with Bt resistance [22,23].

Traditional insect pest control methods used for stored-grain products are based on synthetic chemical pesticides that are not IPM compatible and contribute to contamination of food products constituting a risk for workers and consumers. Therefore, chemical free or biologically based approaches to control stored-product insects that have proven efficacy need to be developed [4]. Bt represents a useful alternative to conventional insecticides, formulated in bioinsecticides or delivered in transgenic plants. Bt based strategies for pest control mostly relies on Bt granular or spray surface applied products that contain mixtures of bacterial spores and insecticidal toxins. Unfortunately, currently commercialized coleopteran active Bt formulations have not proven effective against Tc and new preparations based on Bt strains more active against this insect are needed. The efficacy of the treatments might be influenced by the insect response to all components of the insecticidal formulation.

In this work, we implemented an iTRAQ proteomic analysis combined with LC-MS/MS to study the differential response of Tc larvae after intoxication with two spore-crystal mixtures of Bt strains active against this insect that produce structurally unrelated Cry toxins. Proteins differentially expressed in Bt treated larvae fell into two major categories, proteins involved in host defense and proteins linked to metabolic pathways affecting larval development, which account for both a general Bt defensive response and toxin-specific physiological regulation.

Results and Discussion

Bt Cry toxin producing strains display toxicity against Tc

To determine the susceptibility of Tc larvae to five coleopteran specific Bt toxin producing strains (Cry3Aa, Cry3Ba, Cry3Ca, Cry23Aa/Cry37Aa and Cry34Ab/Cry35Ab) and one lepidopteran specific Bt toxin producing strain (Cry1Ac) a single dose of 3 µg of spore-crystal mixture of each Bt toxin expressing strain per microliter of flour disc were initially assayed. In the experimental conditions, after seven days of treatment, Cry1Ac, Cry3Aa, Cry3Ca and Cry34Ab/Cry35Ab spore-crystal mixtures were not significantly active compared to non-treated larvae, whereas spore-crystal mixtures of Cry3Ba and the Tc active Cry23Aa/Cry37Aa strain [24] yielded 20±4% and 35±5% mortality, respectively (Table 1). As expected, the lepidopteran specific Cry1Ac toxin producing strain, used as a negative control, and Cry34Ab/Cry35Ab spore-crystal mixtures, already reported inactive against Tc [7], did not show toxicity. Although Cry3Aa spore-crystal mixture did not produce significant Tc larvae mortality, larval weight reduction and increased developmental time was observed in Cry3Aa treated larvae, as previously described [25].

Table 1. Susceptibility of Tc to Bt spore-crystal mixtures.

Treatment (3 µg of spore-crystal mixture/µL flour disc)	% Mortality (Day 7)
Cry3Aa	3±3
Cry3Ba	20±4*
Cry3Ca	0±0
Cry23Aa/Cry37Aa	35±5*
Cry34Ab/Cry35Ab	0±0
Cry1Ac	0±0
none	0±0

*Significantly different with respect to non-treated larvae (Student's *t* –test, $P<0.05$).
doi:10.1371/journal.pone.0055330.t001

Different spore-crystal mixture concentrations of Cry3Ba or Cry23Aa/Cry37Aa were assayed against Tc larvae and results are shown in Figure 1. As a negative control, a Cry1Ac spore-crystal mixture was also assayed. The profiles of Tc dose-response curves to both Bt active spore-toxin treatments were similar with a 70% maximum mortality percentage achieved after seven days of exposure, supporting that in the assay conditions, surviving larvae might be able to mount a defensive response that counteracted the toxic action. Alternatively, this maximum mortality percentage might represent the efficacy limit of the assayed treatments in this insect due to other factors, such as the insect genetic variability underlying Bt susceptibility. The LC_{50} and 95% fiducial limits calculated by Probit analysis [26] were 6.30 (4.89–8.37) µg spore-crystal mixture/µL flour disc for Cry23Aa/Cry37Aa and 13.53 (10.24–17.72) µg spore-crystal mixture/µL flour disc for Cry3Ba spore-crystal preparations. LC_{50} differences between both treatments might be due either to a differential toxic effect or to variation in the toxin content of the spore-crystal culture mixture used in the toxicity assay. Regardless of the reason, in both treatments, insects were sensing the strain active components since after two days of treatment with an LC_{50} of Cry3Ba or Cry23Aa/Cry37Aa spore-crystal mixtures, no appreciable flour disc consumption was observed evidencing cessation of feeding (Figure 1). In contrast, significant disc consumption was seen in assays with untreated larvae and larvae treated with an amount as high as 25 µg Cry1Ac spore-crystal mixture/µL flour disc (Figure 1).

Differential protein response of Tc larvae to Bt toxin producing strains

Toxins contained in the two spore-crystal mixtures that were shown active against Tc, Cry3Ba and Cry23Aa/Cry37Aa, belong to two different classes of Cry proteins. Cry3Ba is a Cry-3D toxin of approximately 70 kDa that lacks the C-terminal extension found in the 130 kDa Cry-3D toxins, which is dispensable for toxicity [1]. The Cry23Aa/Cry37Aa binary toxin consists of two proteins both required for toxicity, Cry23Aa toxin homologous to the dipteran active Mtx 2/3 proteins of *B. sphaericus*, and Cry37Aa toxin not related to other Bt crystal proteins [3]. Since Cry3Ba and Cry23Aa/Cry37Aa proteins are structurally unrelated and might have a distinct mode of action, we decided to analyze whether there is a differential insect response to each treatment and we undertook a proteomic approach using an iTRAQ technique combined with liquid chromatography-tandem mass spectrometry

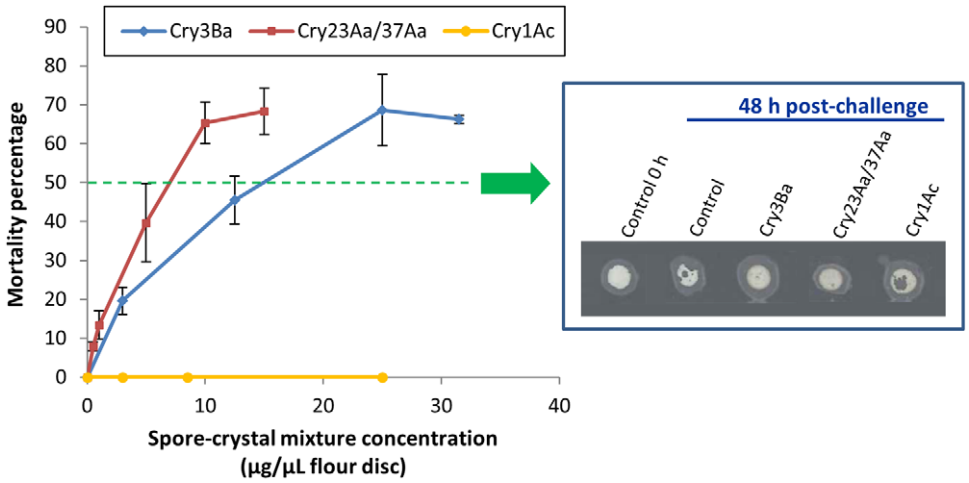


Figure 1. Dose-mortality assays with Cry3Ba, Cry23Aa/Cry37Aa and Cry1Ac spore-crystal mixtures in Tc larvae. Bioassays were performed on eight to ten day-old larvae fed on flour discs containing spore-crystal mixtures of Cry3Ba, Cry23Aa/Cry37Aa and Cry1Ac toxin producing Bt strains. Arrow points to images of 10 µL flour discs on which Tc larvae were fed for two days containing water (control), an approximately LC_{50} of Cry3Ba and Cry23Aa/37Aa spore-crystal preparations (12.5 µg/µL flour disc and 5.0 µg/µL flour disc, respectively), and 25 µg Cry1Ac spore-crystal mixture/µL flour disc (negative control).
doi:10.1371/journal.pone.0055330.g001

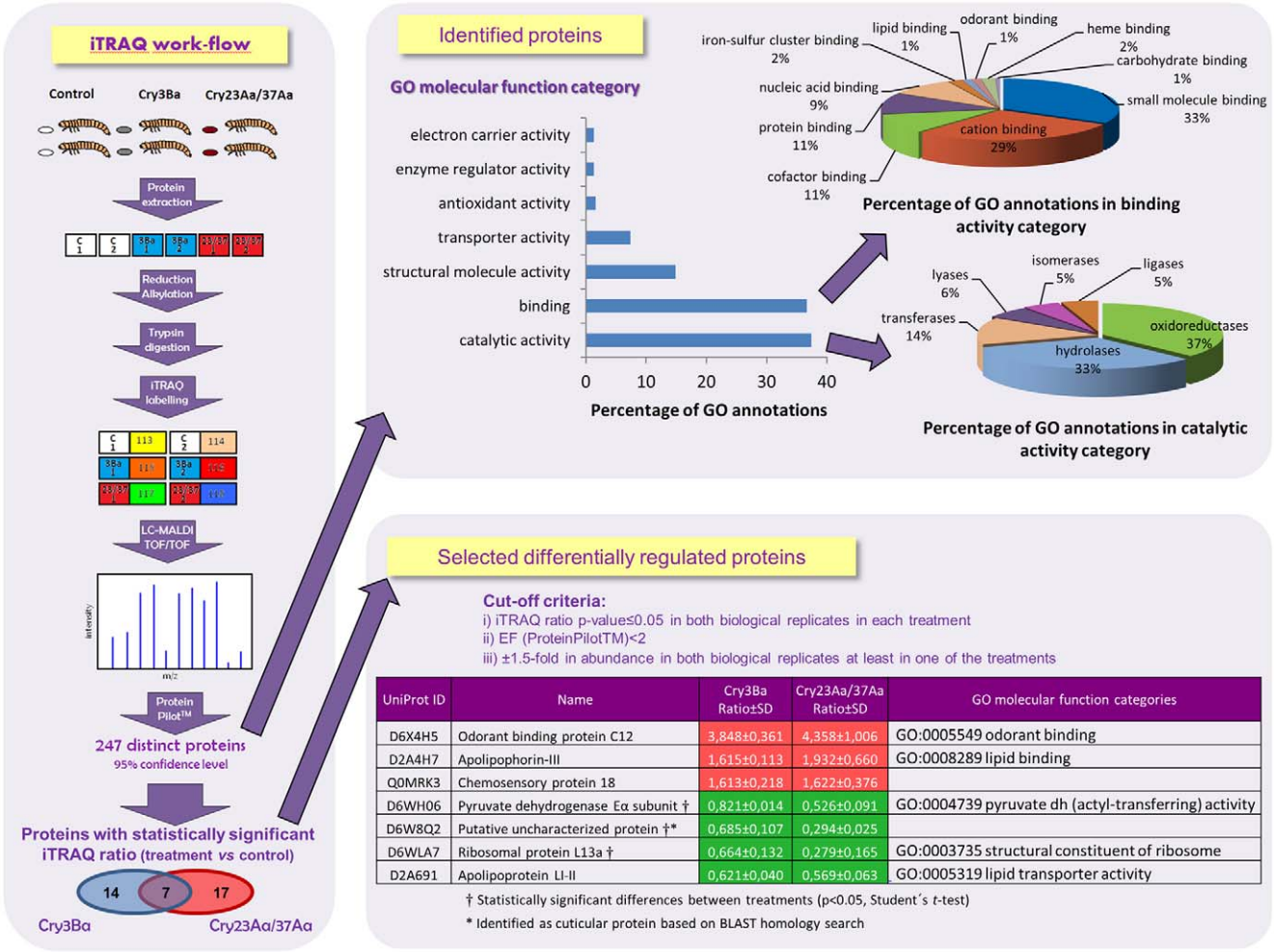
on Tc Bt treated larvae. We chose to intoxicate Tc larvae with approximately a LC_{50} dose of Cry3Ba or Cry23Aa/Cry37Aa spore-crystal mixtures to assure an active response of the insect but, in an attempt to prevent damaged gut epithelium recovery from occurring, treatments were limited to two days, when less cell damage has occurred and therefore the number of cells undergoing repair is likely reduced. Larvae were harvested right after the two days of treatment, when significant cessation of feeding was observed, well in advance of the onset of mortality, and detection of early expressed response proteins was expected. As control, larvae fed with flour discs mixed with the corresponding volume of water were used. A workflow of the iTRAQ experiment is depicted in Figure 2.

A total number of 1,669 non-redundant Tc peptide MS/MS spectra were generated, and 335 proteins were identified at a false discovery rate of 1% with PSPEP (ProteinPilot^{TM} software, ABSciex). The number of assembled proteins accurately quantified was 247 and 239, in response to Cry3Ba or Cry23Aa/Cry37Aa spore-crystal treatments, respectively (Table S1), among which 239 proteins were overlapped between the two treatments. Of the total number of 247 non-redundant identified proteins, 19.4% were identified with more than 5 peptides (Figure S1). Eighty three per cent of total identified proteins were classified according to GO molecular function categories (Figure 2). The largest GO molecular function category was catalytic activity represented mostly by oxidorreductases and hydrolases, followed by binding category, where small molecule and cation binding proteins were mainly found.

To analyze the differentially expressed proteins in response to spore-crystal treatments, the i-TRAQ ratios (treatment versus control) of the quantified proteins with a p-value$\leq$0.05 were selected. Twenty-one proteins were found with statistically significant ratios at least in one replicate in Cry3Ba treated samples, of which 11 proteins exhibited a relative change in protein ratio below $-$1.5-fold or above 1.5-fold (4 out of the 11 proteins showed $\pm$1.5-fold change in both replicate samples). In Cry23Aa/Cry37Aa treated samples, 24 proteins showed statistically significant ratios at least in one replicate, of which 20 proteins exhibited a relative change in protein ratio of $\pm$1.5-fold (7 out of the 20 proteins showed $\pm$1.5-fold change in both replicate samples). The 4 proteins found in Cry3Ba treated samples that showed a 1.5-fold change in both replicate samples were common to 4 of the 7 proteins found for Cry23Aa/Cry37Aa treatment that showed 1.5-fold differential expression for both of the two replicate samples. In the comparative analysis between treatments, differentially expressed proteins were selected based on the following cut-off criteria: (i) p-value$\leq$0.05 in both biological replicates in each treatment, (ii) error factor $<$2 (generated by ProteinPilot^{TM}), (iii) an average of at least $\pm$1.5-fold in abundance in response to Bt intoxication in both replicates at least in one of the treatments. Seven Tc proteins (15.6% of total number of selected proteins with i-TRAQ ratios with a p-value$\leq$0.05) showed a statistically significant change; the specific proteins and their expression alterations (fold change compared to control mass tag levels) are listed for the Cry3Ba and Cry23Aa/Cry37Aa treatments, respectively (Figure 2). Three out of these seven proteins (odorant binding protein C12, apolipophorin-III and chemosensory protein 18) were increased and showed +1.5-fold change in both replicate samples for both treatments, and four out of the seven proteins (pyruvate dehydrogenase Eα subunit, cuticular protein, ribosomal protein L13a and apolipoprotein LI-II) were decreased, when larvae treated with either of the two Bt spore-crystal mixtures were compared with non-treated control larvae. Apolipoprotein LI-II showed $-$1.5-fold change in both replicate samples for both treatments whereas pyruvate dehydrogenase Eα subunit, cuticular protein and ribosomal protein L13a showed a fold change smaller than 1.5 in Cry3Ba spore-crystal treated larvae that was significantly different to the 1.5-fold change reduction observed in Cry23Aa/Cry37Aa spore-crystal treated larvae ($p<0.05$, Student's *t*-test). In the following sections, we contend that Tc might respond to challenge by two Cry-3D toxin-spore mixtures by altering expression of proteins involved in host defense and or developmental pathways.

Tc differentially increased proteins are likely to be implicated in host defense

Among the iTRAQ differentially increased proteins, the odorant binding protein (OBP) showed a remarkable 4-fold



UniProt ID	Name	Cry3Ba Ratio±SD	Cry23Aa/37Aa Ratio±SD	GO molecular function categories
D6X4H5	Odorant binding protein C12	3,848±0,361	4,358±1,006	GO:0005549 odorant binding
D2A4H7	Apolipophorin-III	1,615±0,113	1,932±0,660	GO:0008289 lipid binding
Q0MRK3	Chemosensory protein 18	1,613±0,218	1,622±0,376	
D6WH06	Pyruvate dehydrogenase Eα subunit †	0,821±0,014	0,526±0,091	GO:0004739 pyruvate dh (actyl-transferring) activity
D6W8Q2	Putative uncharacterized protein †*	0,685±0,107	0,294±0,025	
D6WLA7	Ribosomal protein L13a †	0,664±0,132	0,279±0,165	GO:0003735 structural constituent of ribosome
D2A691	Apolipoprotein LI-II	0,621±0,040	0,569±0,063	GO:0005319 lipid transporter activity

† Statistically significant differences between treatments (p<0.05, Student´s *t*-test)

* Identified as cuticular protein based on BLAST homology search

Figure 2. Quantitative iTRAQ proteomic analysis. A work-flow indicating the steps followed in the iTRAQ analysis performed on two biological replicates of each toxin treatment and the classification of the iTRAQ identified proteins according to GO molecular function categories are shown. Proteins showing an i-TRAQ ratio (treatment *vs* control) with a Protein Pilot p-value≤0.05 at least in one replicate sample were 21 in Cry3Ba spore-crystal treated larvae and 24 in Cry23Aa/Cry37Aa spore-crystal treated larvae. Seven differentially regulated proteins are listed for Cry3Ba and Cry23Aa/Cry37Aa treatments and were selected according to the following cut-off criteria: (i) i-TRAQ ratio p-value≤0.05 in both biological replicates in each treatment, (ii) error factor <2 (generated by ProteinPilot™), (iii) an average of ±1.5-fold in abundance in response to Bt intoxication in both biological replicates at least in one of the treatments. Using Student's *t*-test, statistically significant differences between toxin treatments were detected for pyruvate dehydrogenase Eα subunit, cuticular protein and ribosomal protein L13a (p<0.05).
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increase in both treatments, whereas a moderate increase of around 1.6-fold was seen for apolipophorin III and chemosensory protein 18, when comparing to untreated controls (Figure 2).

Besides its function in lipid transport, apolipophorin III has also been reported to mediate insect immune responses in several species such as *Galeria mellonella*, *Hyphantria cunea*, *Heliothis virescens*, *Locusta migratoria* and *Anopheles gambiae* [27]. In the case of coleopteran insects, apolipophorin-III gene was described to be up-regulated after larval exposure to Cry3Aa toxin in *T. molitor* [19]. Chemosensory proteins and OBP participate in sensing odors and/or pheromones [28] and have also been shown to be induced by microbial infections leading some authors to suggest a link between the olfactory system and the immune system in invertebrates [29,30,31].

Interestingly, in the increased protein group no significant differences between Cry3Ba and Cry23Aa/Cry37Aa Tc larvae treatments were observed (p>0.05, Student's *t*-test), indicating that induction of the corresponding genes might be a common mechanism after Bt infection that might constitute the main early defensive response of the insect, regardless of the actual process by which these two different Bt spore-crystal mixtures target cells. As OBP showed the highest change in protein levels after treatments and its role in immunity is not well established we selected this protein to further analyze its involvement in Tc host defense.

To validate at the transcription level the increased OBP protein change detected in the iTRAQ analysis, we used quantitative real time PCR (qRT-PCR) to compare mRNA expression in untreated control larvae and larvae treated with the Cry3Ba and Cry23Aa/Cry37Aa active spore-crystal mixtures or the non-toxic spore-crystal mixtures of the Cry1Ac strain (Figure 3). Results show that the OBP gene was differentially expressed after the three treatments relative to control larvae (12.4-fold, 8.0-fold and 4.5-fold up-regulation corresponding to Cry3Ba, Cry23Aa/Cry37Aa and Cry1Ac treatments, respectively). Non-significant differences between the amount of OBP transcript induced by Cry3Ba and Cry23Aa/Cry37Aa treatments were observed (p>0.05, Student's

t-test). However, statistically significant differences between OBP transcript levels induced by any of these treatments and those induced by Cry1Ac spore-crystal treatment were found ($p<0.05$, Student's *t*-test). Results suggest that although OBP gene might be induced as a result of a general host defense response in Tc, its induction may be even more enhanced in response to Tc active Cry spore-crystal mixtures and therefore, this OBP protein might play a role in Tc response to Bt intoxication.

A search of NCBI protein database was carried out to find sequences similar to Tc OBP (NCBI accession no. EEZ97740). *T. molitor* 12 kDa hemolymph protein b precursor (Tm THP12), characterized as an odorant binding protein in this insect [32], displayed the highest similarity (64% amino acid sequence identity). The amino acid sequence alignment of both proteins is shown in Figure 4A. Both proteins are similar to other members of the insect OBP family, particularly in structurally important regions, carrying an N-terminal signal sequence (predicted cleavage site between positions 18 and 19 using SignalP 4.0 [33], and containing four out of the six aligned cysteine residues that are diagnostic of insect OBPs [34], as described for other hemolymph OBPs [32].

We have predicted the three dimensional homology model of Tc OBP using the automated comparative protein modeling server SWISS MODEL Workspace [35], based on the solved structure of the Tm THP12 OBP (PDB accession no. 1C3Y). Consistent with the idea that both proteins share the same fold (Figure 4B), the model exhibits the same overall structure as Tm THP12 (QMEAN4 score of 0.569, Z-score of −2.26) (Figure S2).

A phylogenetic tree was inferred by the Neighbor joining method with a Gonnet matrix-based model by using Mega software [36] (Figure 5). The Tc OBP protein identified to be increased in this work clustered together with *T. molitor* 12 kDa hemolymph OBPs, indicating that Tc OBP is more similar to Tm THP12 OBP than to any of the 265 apparently functional OBPs annotated in the Tc genome [37]. It has been hypothesized that Tm THP12 and putative orthologs might be carriers of a number of small hydrophobic compounds that would normally be transported through the hemolymph [38]. Expression of OBP genes in non-olfactory tissues might indicate that the encoded proteins are likely to have non-olfactory physiological functions [39].

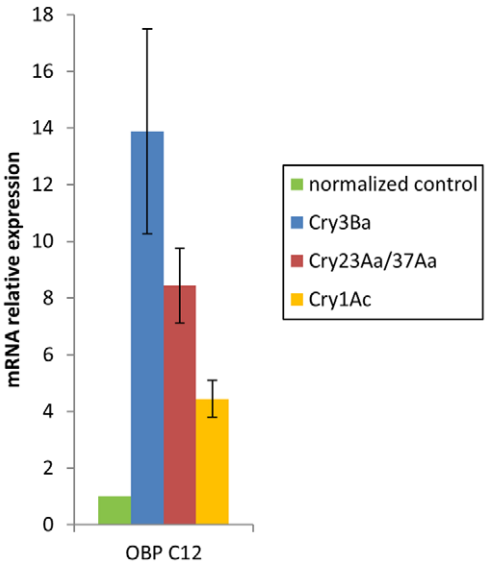

Figure 3. Tc OBP transcriptional analysis. qRT-PCR analysis of mRNA expression levels of Tc OBP in larvae exposed to Cry3Ba, Cry23Aa/Cry37Aa and Cry1Ac spore-crystal mixtures relative control larvae, normalized to the RPS18 mRNA. Error bars indicate standard errors of the means from two biological replicates of twenty-four individuals per replicate.
doi:10.1371/journal.pone.0055330.g003

In order to assess whether Tc OBP is involved in insect defense against Bt, its expression was knocked down by means of RNAi. To choose the appropriate larval size for silencing, we obtained the transcription profile of the OBP gene in different Tc larval developmental stages using qRT-PCR (Figure 6A), with RPS18 mRNA as internal control. The abundance of OBP transcripts did not significantly change in larvae of a weight range of 0.25 to 1.15 mg, except for a reduced transcript expression observed in 1.0 mg weight larvae. Since the OBP expression profile remained stable in larvae less than 1.0 mg weight, we selected larvae of 0.2 to 0.4 mg weight for silencing assays. We employed qRT-PCR to examine mRNA levels of Tc OBP in dsRNA-injected larvae and control (buffer-injected) larvae and the analysis showed a 93% reduction of Tc OBP transcript compared to control larvae (Figure 6B). Knockdown of the Tc OBP genes transcripts did not induce larval mortality, suggesting that the genes normal expression must not be essential for Tc larvae viability.

We next carried out toxicity assays with Cry3Ba, Cry23Aa/Cry37Aa or Cry1Ac spore-crystal mixtures on OBP dsRNA-injected and buffer-injected control larvae (Figure 6C). Results showed a significant increase in mortality of dsRNA-injected larvae treated either with Cry3Ba or Cry23Aa/Cry37Aa ($p<0.05$, Student's *t*-test), consistent with the proposed immune defense function of non-olfactory OBPs induced upon pathogen challenge [29,30,31]. The non-toxic Cry1Ac spore-crystal mixture treatment did not produce larval mortality neither in dsRNA-injected larvae nor in buffer-injected control larvae (Figure 6C). Although OBP gene induction was higher in Cry3Ba than in Cry23Aa/Cry37Aa treated larvae, differences observed in mRNA abundance were not statistically significant (Figure 3) and accordingly, in OBP silenced larvae mortality increase compared to buffer-injected control larvae was the same (around 10% increase) in both Cry3Ba and Cry23Aa/Cry37Aa spore-crystal treatments (Figure 6C). Results support OBP as a Tc responsive protein to Bt challenge that together with other gene products might hold a primary defense function. Understanding the effect of other host defense genes involved in Tc susceptibility to Bt will allow estimating the relative contribution of this specific OBP gene to the overall insect host defense.

Tc differentially reduced proteins are related to metabolism and development

iTRAQ down-regulated proteins were found linked to metabolic pathways that might affect larval development. In a comparison of the protein decrease observed in Cry3Ba and Cry23Aa/Cry37Aa treatments, three out of the four down-regulated proteins showed significant differences between treatments ($p<0.05$, Student's *t*-test), pyruvate dehydrogenase Eα subunit, cuticular protein and ribosomal protein L13a (Figure 2). In contrast, apolipoprotein LI-II precursor protein, the common precursor of apolipoprotein I and apolipoprotein II that constitutes the basic structure of the major insect hemolymph lipophorin, showed a similar 0.6 ratio decrease in Tc larvae for both treatments. Insect lipophorin plays important roles in transporting dietary lipids from the gut to the storage depot, the fat body, while also distributing stored or biosynthesized lipids to peripheral tissues [40]. It has been reported that there is a trade-off between immune stimulation and expression of storage protein genes upon bacterial challenge [41,42]. Interestingly, in contrast to the

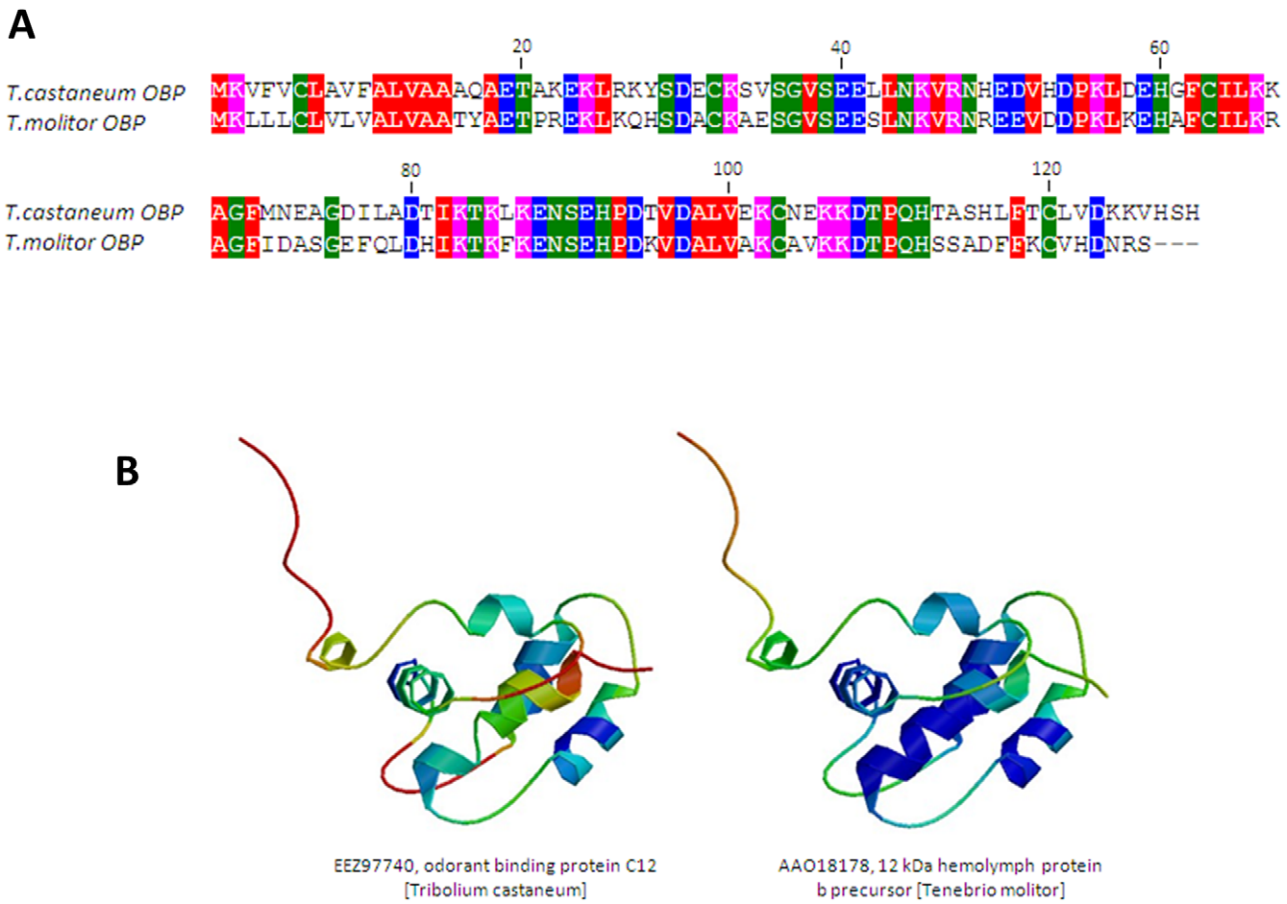

Figure 4. Tc OBP protein structural features. (**A**) Sequence alignment of the OBP amino acid sequences of Tc and Tm using Clustal omega [56]. (**B**) Predicted three-dimensional homology model of the Tc OBP protein using SWISS-MODEL Workspace server [32] based on Tm THP12 (1C3Y.pdb). The Tm THP12 fold (1C3Y.pdb) is shown for comparison.
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apolipoprotein LI-LII precursor reduction, we have observed that the immune-related apolipoprotein III protein increased, which is consistent with the reported down-regulation of accumulation of storage proteins as a consequence of activation of the immune system, considered a general strategy to redirect resources to combat injury or infection [42].

The three down-regulated proteins that showed significant differences between Cry3Ba and Cry23Aa/Cry37Aa treatments might be relevant to understand whether there is a differential insect response to each spore-crystal treatment.

Ribosomal protein L13a is a noncanonical ribosomal protein that carries out tasks often unrelated to the protein synthesis of the ribosome [43]. It has been described that regulated release of L13a from the 60S ribosomal subunit is a mechanism of transcript-specific translational control of genes involved in inflammatory processes in mammals [44]. In yeast it has been demonstrated that inactivation of the two yeast L13a homologous resulted in severe growth retardation and cell death [45]. Although the function of ribosomal protein L13a in insects is unknown, the reduced protein levels detected in the iTRAQ analysis would support a role in the midgut paralysis and cessation of feeding that characterizes Bt intoxication.

Pyruvate dehydrogenase (Eα) is the first component of the pyruvate dehydrogenase complex (PDC), responsible for the decarboxylation of pyruvate to acetyl-CoA in the mitochondria matrix, after which the acetyl-CoA enters the citric acid cycle [46]. The ability to arrest development and metabolism to cope with environmental challenges improves the survival of many species and it has been described that a hypometabolic state is characterized by suppression of oxidative pathways of energy production, involving PDC down-regulation [47]. In *Caernohabditis elegans* it has been reported that resistance to Cry pore-forming toxins can be achieved by mutations that up-regulate the hypoxia response mediated by the hypoxia inducible factor 1 (HIF-1) [14], which induces the expression of pyruvate dehydrogenase kinase that in turn inhibits PDC, leading to a suppression of mitochondrial oxidative phosphorylation. Accordingly, in Tc, pyruvate dehydrogenase Eα subunit reduction after challenging with Cry3Ba and Cry23Aa/Cry37Aa preparations would not be unexpected if it were part of the insect response to protect itself from pore-forming toxins.

The cuticle is a dynamic structure that responds to external factors such as insecticides and desiccation [48]. It is conceivable that Bt intoxication might have consequences for the expression of genes underlying cuticular functions as a part of the metabolic arrest response, affecting timing of moulting and distribution of developmental stages. In Colorado potato beetle the transition from a state of high metabolic rates and active feeding to very low metabolic rates, no feeding, and very little movement when the beetle enters diapause is characterized by a differential regulation of cuticular protein transcripts [49,50].

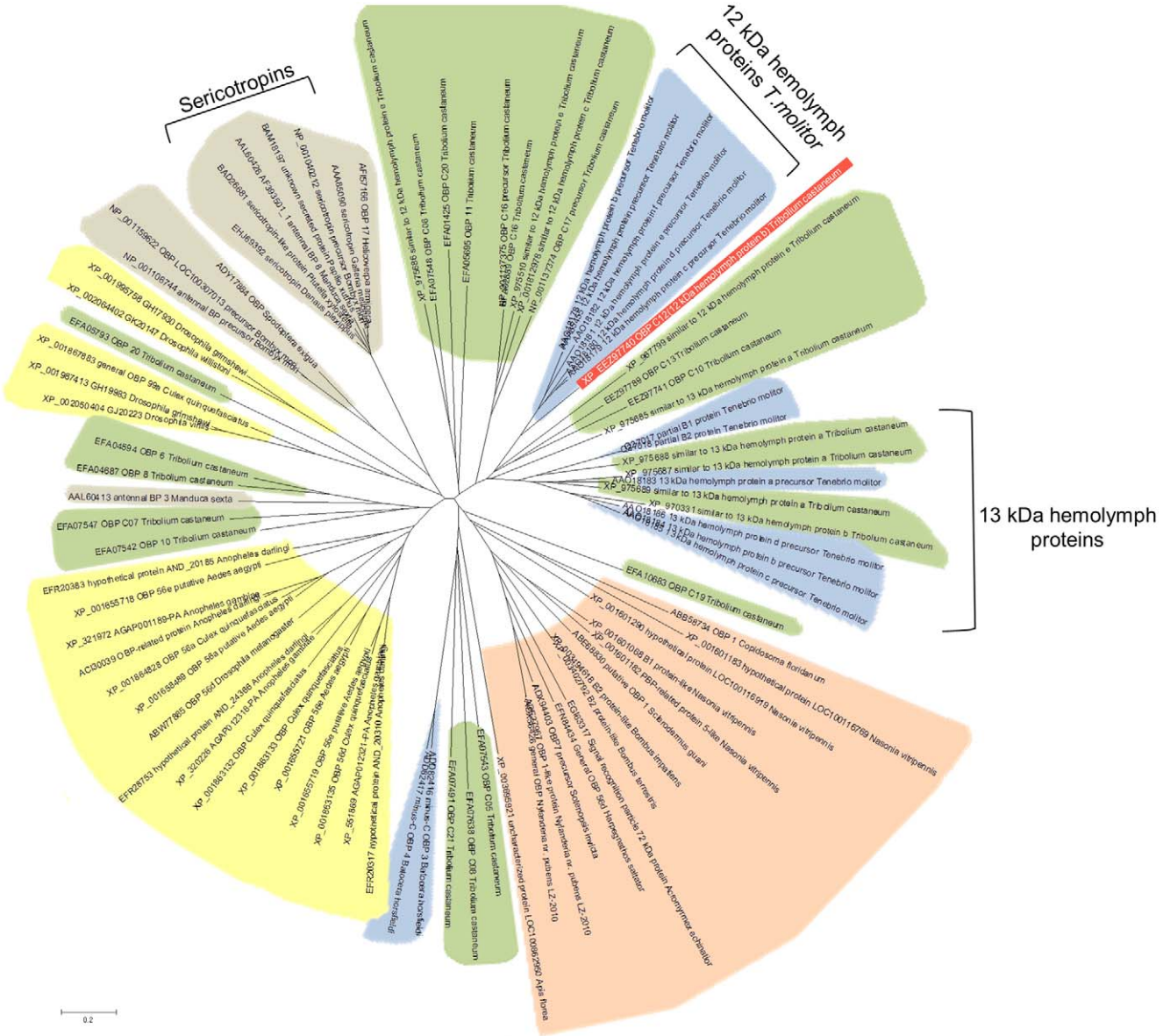


Figure 5. Unrooted phylogenetic tree of insect OBP proteins. The phylogenetic reconstruction was generated with MEGA version 4.0.2 software [35]. In red is shown the Tc OBP up-regulated upon Cry3Ba and Cry23Aa/Cry37Aa treatment challenge and in green, homologous Tc OBPs identified searching the BeetleBase protein database. Depicted in blue are other coleopteran OBPs, in grey lepidopteran OBPs, in yellow dipteran OBPs and in pink hymenopteran OBPs, which were identified to show homology to Tc OBP by searching the NCBI protein database.
doi:10.1371/journal.pone.0055330.g005

To gain more insight into the functional significance of the differential reduction of pyruvate dehydrogenase Eα subunit, cuticular protein and ribosomal protein L13a in CryBa and Cry23Aa/Cry37Aa treatments, expression of the corresponding genes was further assessed at the transcription level using qRT-PCR in untreated control larvae and intoxicated larvae (Figure 7A). For ribosomal protein L13a, no significant differences in gene expression were observed in any of the toxin treatments with respect to non-treated control larvae. For pyruvate dehydrogenase Eα subunit and cuticular protein significant differences between mRNA abundance in treated and control larvae were only found in Cry3Ba treatment. Intriguingly, at protein level both spore-crystal treatments led to down-regulation of the two proteins and a significant higher reduction was observed in Cry23Aa/Cry37Aa intoxicated larvae. These results suggest a distinct transcriptional regulation depending upon the type of Bt toxin used in larval treatment. As these two proteins might be involved in metabolic and developmental processes, differential transcriptional regulation might influence how larvae recover from Bt challenge. Therefore, we decided to intoxicate Tc larvae with a dose of Cry3Ba or Cry23Aa/Cry37Aa spore-crystal mixtures causing 15–20% mortality after 7 days of toxin treatment to assure low insect mortality and then surviving larvae were fed flour discs without toxin to let the gut epithelium recover from toxin damage. Figure 7B shows that the pupation profile of surviving larvae in Cry23Aa/Cry37Aa treatment was similar to that of non-treated control larvae although the pupation rate was reduced for Cry23Aa/Cry37Aa treated larvae, reaching a maximum of 58% pupation (relative to pupation percentage in control larvae) on day 35th after the initial treatment. In contrast, on that day, only 7% of

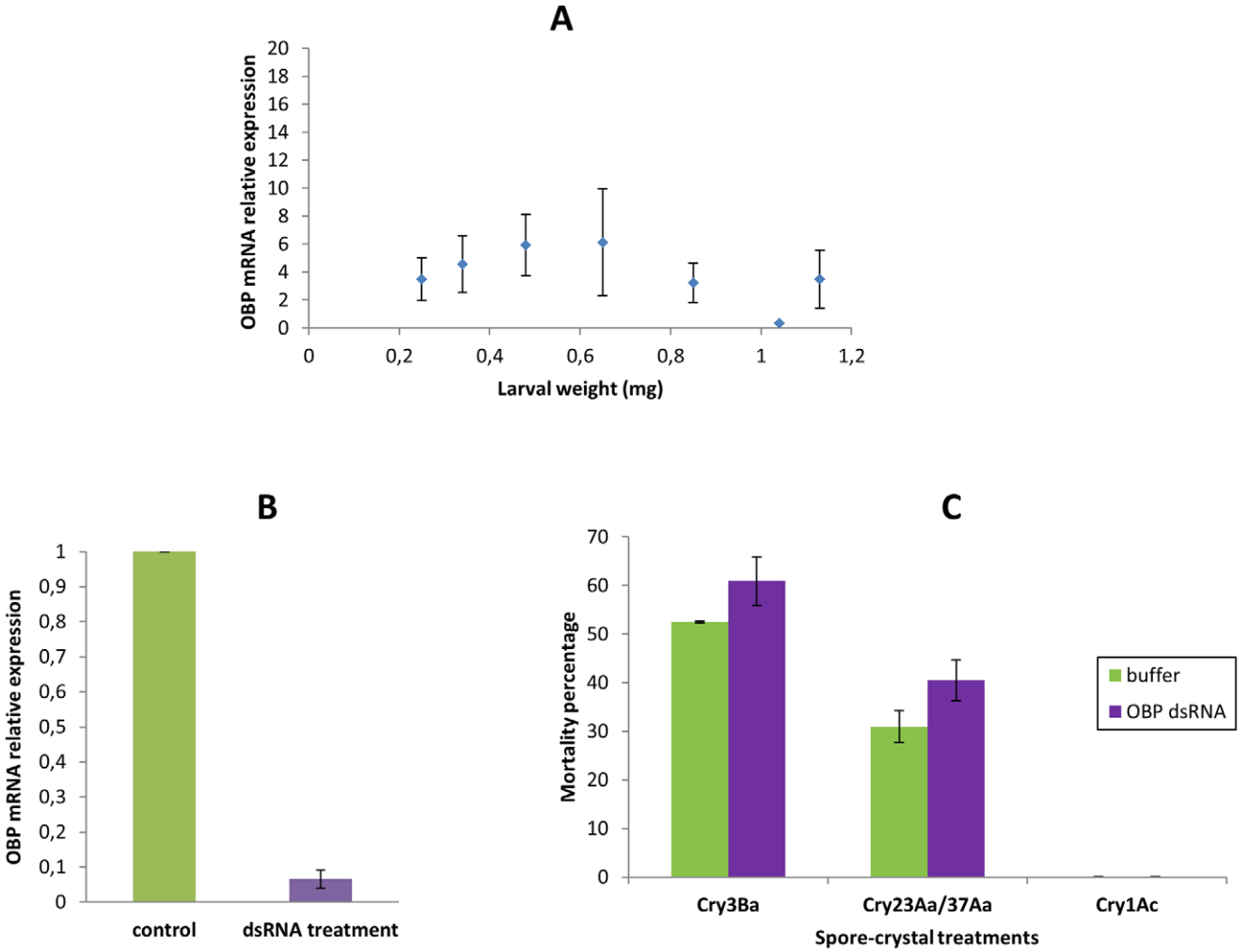
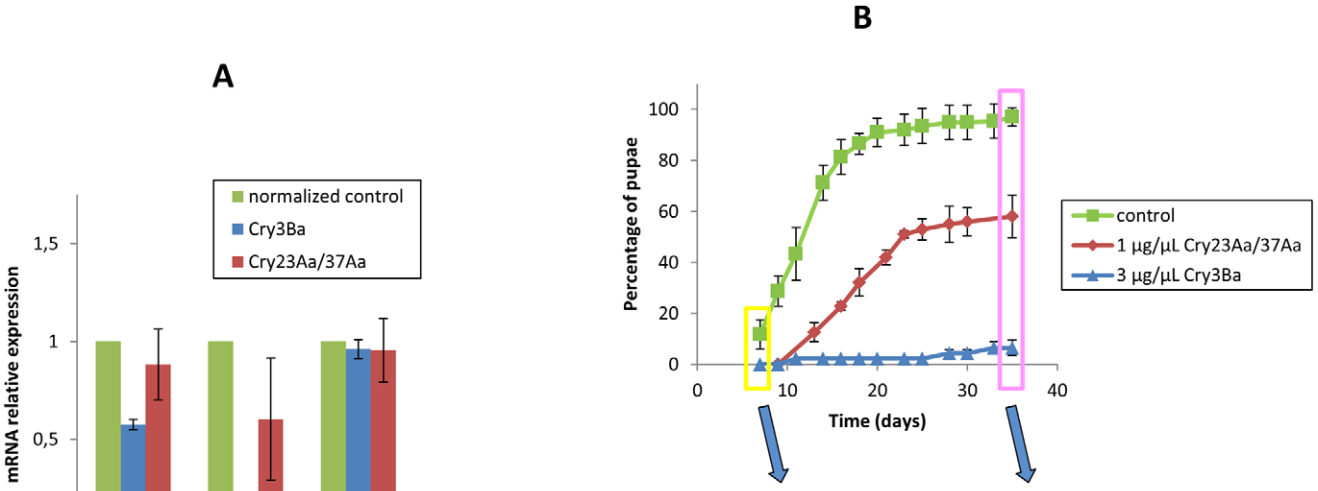


Figure 6. Tc OBP functional characterization. (**A**) qRT-PCR analysis of the OBP mRNA expression levels in Tc larvae of different weight, using RPS18 mRNA as reference. (**B**) OBP silencing in Tc larvae in response to injection of dsRNA against Tc OBP. The relative amount of Tc OBP transcript estimated by qRT-PCR in control and silenced larvae was compared normalized to the expression of *RPS18* gene. The statistical significance of the gene expression between the two samples was evaluated using Student's *t*-test and significant knockdown was observed ($p<0.05$). Error bars represent standard error of the mean of two biological replicates. (**C**) Mortality percentage following Cry3Ba, Cry23Aa/Cry37Aa or Cry1Ac spore-crystal treatments on either buffer-injected (control) or OBP dsRNA-injected larvae. Mortality experiments were performed using fifty Tc larvae in each spore-crystal treatment and the corresponding controls. Error bars represent standard error of the mean of at least two biological replicates. Mortality increase observed upon Bt treatments in OBP silenced larvae respect to buffer-injected larvae was statistically significant using Student's *t*-test ($p<0.05$).
doi:10.1371/journal.pone.0055330.g006

Cry3Ba surviving larvae reached pupation. Additionally, differences between Cry3Ba and Cry23Aa/Cry37Aa treatments were observed regarding larval mortality on day 35th after the initial treatment, being three times higher for Cry3Ba than for Cry23Aa/Cry37Aa intoxicated larvae. The larval developmental arrest observed in Cry3Ba surviving larvae as opposed to the 60% of Cry23Aa/Cry37Aa surviving larvae that recovered from toxin treatment and reached pupation is in agreement with the transcriptional repression of pyruvate dehydrogenase and the cuticular protein genes observed only after Cry3Ba treatment (Figure 7A). Other coleopteran active toxins structurally related to Cry3Ba, also altered transcription of genes linked to metabolic processes. Cry3Bb toxin has been reported to impact larval metabolism and development in *D. virgifera* [17], and Cry3Aa toxin exposure resulted in a repression of genes encoding metabolic enzymes associated with proteolysis, glycolysis, TCA, and fatty acid metabolism in *T. molitor* [19].

Conclusions

The use of iTRAQ combined with LC-MS/MS has led to the discovery of several novel differentially expressed proteins in Tc larvae in response to spore-crystal mixtures of Bt strains producing two structurally unrelated Cry toxins, Cry3Ba and Cry23Aa/Cry37Aa. Tc larvae early response involved up-regulation of three host defense related proteins (odorant binding protein C12, apolipophorin-III and chemosensory protein 18) and down-regulation of four proteins that play a role in larval metabolism and development (pyruvate dehydrogenase Eα subunit, cuticular protein, ribosomal protein L13a and apolipoprotein LI-II). At the transcriptional level, pyruvate dehydrogenase and cuticular genes were decreased in Tc larvae exposed to the Cry3Ba producing strain compared to the Cry23Aa/Cry37Aa producing strain, which may contribute to the developmental arrest that we observed with larvae fed the Cry3Ba producing strain.



	Day 7th after treatment			Day 35th after treatment		
	% larvae	% pupae	% dead larvae	% larvae	% pupae	% dead larvae
Control	88	12	0	3	97	0
Cry23Aa/37Aa	84	0	16	22	58	20
Cry3Ba	80	0	20	35	7	58

Figure 7. Tc differential response to Cry3Ba and Cry23Aa/Cry37Aa spore-crystal treatments. (**A**) Relative levels of Tc pyruvate dehydrogenase (Pdh), cuticular protein and ribosomal protein L13a (RPL13a) mRNAs normalized to the corresponding untreated control, determined by qRT-PCR analysis in control larvae and larvae exposed to Cry3Ba and Cry23Aa/Cry37Aa spore-crystal mixtures, using RPS18 mRNA as reference. Error bars indicate standard errors of the means from two biological replicates of twenty-four individuals per replicate. (**B**) Monitoring of Tc larvae development following Cry3Ba and Cry23Aa/Cry37Aa spore-crystal treatments that produced 15–20% mortality. Percentage of pupae was recorded after seven days of spore-crystal treatment and up to day 35, once surviving treated larvae were transferred to spore-crystal-free diet.
doi:10.1371/journal.pone.0055330.g007

Better understanding how target insects respond to Bt intoxication will allow improving Bt strategies for pest control and counteracting resistance development.

Materials and Methods

Toxin production

Cry3Aa, Cry3Ba, Cry3Ca and Cry1Ac crystals were produced in Bt strains BTS1, BTS00125L, BTS02109P and HD73, respectively. The binary toxins Cry23Aa/Cry37Aa and Cry34Ab/Cry35Ab were produced in Bt strains EG10327 (Ref. No. NRRL B-21365) and PS149B1 (Ref. No. NRRL B-21553), respectively, obtained from the Agricultural Research Culture Collection, Northern Regional Research Laboratory (NRRL), USA.

All bacterial strains were grown in solid sporulation medium [51] at 30±1°C until complete autolysis. Lysed bacteria were resuspended in 2x PBS pH 7.4 and washed twice with 0.02% Triton X-100 in 2x PBS pH 7.4 and twice with water. Following centrifugation at 6,000x *g* for 10 min at 4°C, spore-crystal mixtures were resuspended in water and stored at −20°C until use.

Insects

A laboratory colony of Tc founded from Ga-2 strain adults kindly provided by Dr. Beeman (USDA) was used. Insects were reared on whole-grain flour with 5% brewer yeast powder at 30±1°C in darkness.

Toxicity assays

Toxicity assays were performed on Tc larvae eight to ten days old after egg eclosion, fed for seven days on 20 µL flour discs (20% flour, w/v), prepared as in [52], containing 3 µg Cry3Aa, Cry3Ba, Cry3Ca, Cry23Aa/Cry37Aa, Cry34Ab/Cry35Ab and Cry1Ac spore-crystal mixtures per microliter of flour disc for treatments or water, in control assays. Assays were performed in 96-well polystyrene plates (Sterilin, Thermofisher) with one flour disc and one larva per well. Twenty-four larvae were assayed for each toxin and at least three replicates were carried out. Mortality was recorded after 7 days under laboratory rearing conditions. Cry3Ba and Cry23Aa/Cry37Aa LC_{50} were estimated from several spore-crystal mixture doses using Probit analysis [26].

For toxicity assays on silenced larvae, four days after dsRNA injection, larvae were exposed to 12.5 µg/µL Cry3Ba or 5.0 µg/µL Cry23Aa/Cry37Aa spore-crystal mixtures in flour discs. Flour discs prepared with water were used as controls. At least two replicates of fifty larvae were assayed for each toxin and mortality was recorded after 7 days under laboratory rearing conditions.

iTRAQ Analysis

Forty Tc eight-day-old larvae (0.38±0.01 mg) treated with 12.5 µg Cry3Ba spore-crystal mixture/µL flour disc or 5.0 µg Cry23Aa/Cry37Aa spore-crystal mixture/µL flour disc for two days, and untreated control larvae were used to prepare protein extracts from whole larvae in 4 M urea pH 7.4, 0.05% Protease Max surfactant (Promega), 48 µM pepstatin A, 20 µM E-64. Following centrifugation at 10,000x *g* for 10 min at 4°C, the supernatant was separated, protein concentration measured by the protein-dye method of Bradford [53] using BSA as a standard and stored at −80°C.

Two independent biological replicates of toxin treated or control larvae were labeled with i113, i114, i115, i116, i117, i118 iTRAQ reagents according to the manufacturer's protocol (Applied Biosystems). The tag labeling order was untreated control

replicate-1 113; untreated control replicate-2 114; Cry3Ba spore-crystal mixture treated replicate-1 115; Cry3Ba spore-crystal mixture treated replicate-2 116; Cry23Aa/Cry37Aa spore-crystal mixture treated replicate-1 117; Cry23Aa/Cry37Aa spore-crystal mixture treated replicate-2 118. Labelled protein samples, reduced and alkylated, were digested using trypsin. The resulting labelled peptides were then pooled for further processing and analyzed by integrated LC and MALDI-TOF/TOF analyzer QSTAR ESI. MS/MS spectra were analyzed using the Paragon algorithm in ProteinPilot^{TM} software (ABSciex) with the default search program with digestion enzyme trypsin and methyl methanethiosulfonate as cysteine modification. Data was normalized for loading error by bias correction calculated with ProGroup algorithm and to reduce false positive identification results, a minimum unused ProtScore of 1.3 equivalent to 95% confidence and false discovery rate (FDR) less than 1% were required for all reported proteins. A protein was considered significantly identified when one or more high-confidence (>95%) unique peptides were assigned and the iTRAQ quantification fold difference *p*-value was <0.05. The protein search was performed against NCBI protein Tc database. Gene Ontology (GO) terms were retrieved from UniProt database (http://www.uniprot.org).

RNA isolation and cDNA synthesis

Total RNA was isolated from Tc larvae using Trizol LS Reagent (Invitrogen), following the manufacturer's protocol, and the purified RNA was treated with DNase I (DNA-free, Ambion, Inc.). The ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280} ratio) was used to assess the purity of RNA samples and RNA quality was evaluated by 1% agarose gel electrophoresis and quantified spectrophotometrically (NanoDrop 2000, Thermo Scientific). AMV reverse transcriptase (2 U/µL, final concentration) (Roche) was employed for first strand cDNA synthesis using 1 µg RNA, 50 ng/µL oligo $(dT)_{15}$ (Promega) and 2.5 µM random hexamers (Applied Biosystems).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on each template in a final volume of 25 µL using 100 ng on a StepOnePlus Real-Time PCR system (Applied Biosystems) thermocycler, following the manufacturer's recommendations, using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene specific forward and reverse primers (Table S2) were designed with Primer Express software (Applied Biosystems) using sequences retrieved from BeetleBase website (http://www.Beetlebase.org) [54] under the following accession numbers TC011411-RA (OBP C12 protein), TC002948-RA (pyruvate dehydrogenase protein), TC013477-RA (ribosomal protein L13a) and TC000726-RA (cuticular protein). Primers were obtained from IDT Technologies and optimization of primer concentration was performed using 10 ng cDNA per reaction. Single, sharply defined melting curves confirmed the specificity of the chosen primers, as well as minimization of primer-dimers formation. The primer concentrations used are included in Table S2. Primer effieciencies were calculated using different cDNA concentrations in the range 80–8000 pg/µL (Table S2). Amplifications were carried out using two biological replicates of cDNA, each one from RNA obtained from twenty larvae, and the mean values of three technical replicates were analyzed. RPS18 (ribosomal protein S18) gene (Accession number TC014405-RA), reported as a stable reference gene for qRT-PCR in TC [55], was used to normalize gene expression (forward and reverse primer sequences included in Table S2). Gene expression relative-fold was calculated with the comparative C_t ($\Delta\Delta C_t$) method, using the StepOne software (Applied Biosystems). Data were analyzed by Student's *t*-test for statistically significant differences ($p<0.05$).

To analyze the expression of Tc odorant binding protein gene during larval development, quantitative real-time PCR was performed as described above on RNA obtained from larvae of different weight within a range of 0.25 to 1.2 mg.

RNAi

RNA isolation and cDNA synthesis was performed as described above, from eight to ten day-old larvae after egg eclosion. cDNA was used as template for PCR amplification using Prime Star polymerase (Takara) and specific primers generated from Tc OBP gene sequence (NCBI accession no. EEZ97740), containing a T7 polymerase promoter sequence at their 5′ end (Table S2). PCR product (1 µg) was used for *in vitro* transcription to prepare dsRNA using Ambion MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. Purified dsRNA was stored at −80°C until injected into Tc larvae.

Eight day old larvae were anaesthetized for 5 min on ice before ventral injection of approximately 0.2 µL of 1 µg/µL dsRNA in injection buffer (1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl), using thin wall capillars (World Precision Instruments) in a microinjection system (Narishige). Control larvae were injected with buffer. Following injection, larvae were grown under standard rearing conditions.

In OBP silenced larvae, OBP transcript levels were evaluated 8 days after dsRNA injection by qRT-PCR from total RNA, using the OBP forward and reverse primers described in Table S2.

Supporting Information

Figure S1 Number of peptide identifications per protein in the iTRAQ analysis.
(TIF)

Figure S2 Tc OBP SWISS MODEL Workspace automated model.
(PDF)

Table S1 Proteins identified in the iTRAQ analysis.
(XLSX)

Table S2 Primers used in qRT-PCR to analyze the expression of genes corresponding to iTRAQ differentially expressed proteins upon toxin treatments and to generate dsRNA in RNAi experiments.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CR MDR. Performed the experiments: EC. Analyzed the data: EC CR MDR. Wrote the paper: CR MDR.

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Artículo 2: *Tribolium castaneum* apolipophorin III acts as an immune response protein against *Bacillus thuringiensis* Cry3Ba toxic activity

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Short Communication

Tribolium castaneum Apolipophorin-III acts as an immune response protein against *Bacillus thuringiensis* Cry3Ba toxic activity

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ABSTRACT

In this study, a 2.1-fold Apolipophorin-III mRNA up-regulation was found in *Tribolium castaneum* larvae challenged with *Bacillus thuringiensis* Cry3Ba spore–crystal mixture. Knockdown of Apolipophorin-III by RNAi resulted in increased *T. castaneum* larvae susceptibility following Cry3Ba spore–crystal treatment, demonstrating Apolipophorin-III involvement in insect defense against *B. thuringiensis*. We showed that Apolipophorin-III participates in *T. castaneum* immune response to *B. thuringiensis* activating the prophenoloxidase cascade since: (i) phenoloxidase activity significantly increased after Cry3Ba spore–crystal treatment compared to untreated or Cry1Ac spore–crystal treated larvae and (ii) phenoloxidase activity in Cry3Ba spore–crystal treated Apolipophorin-III silenced larvae was 71 ± 14% lower than that of non-silenced intoxicated larvae.

1. Introduction

Apolipophorin-III (ApoLp-III) is a hemolymph protein that associates with lipoproteins to facilitate lipid transport (Weers and Ryan, 2006). Moreover, ApoLp-III has been also identified as an immune-stimulating protein in several insect species such as *Galleria mellonella* (Zdybicka-Barabas and Cytryńska, 2011), *Hypanthia cunea* (Kim et al., 2004), *Heliothis virescens* (Chung and Ourth, 2002) and *Locusta migratoria* (Mullen et al., 2004). Insects lack adaptive immune systems but develop an innate immune response that comprises a hemocyte-mediated cellular component and a humoral component including antimicrobial peptides and the prophenoloxidase (ProPO) cascade (Marmaras and Lampropoulou, 2009). ApoLp-III has been involved in humoral immune reactions against invading pathogens acting as a pattern recognition protein (Whitten et al., 2004), inducing antibacterial activity (Zdybicka-Barabas and Cytryńska, 2011) and activating ProPO cascades (Mullen and Goldsworthy, 2003; Park et al., 2005), and participates in cellular immune reactions stimulating phagocytosis and encapsulation (Whitten et al., 2004; Wiesner et al., 1997).

ApoLp-III protein was shown to be increased in *Tribolium castaneum* (Tc) larvae exposed to spore–crystal mixtures of *Bacillus thuringiensis* (Bt) coleopteran active strains producing Cry3Ba or Cry23Aa/Cry37Aa toxins (Contreras et al., 2013), suggesting that induction of the corresponding gene might be part of the defensive response of the insect.

It is generally accepted that Cry toxins mode of action involves toxin solubilization and proteolytic activation in the midgut of the susceptible larvae, membrane receptor binding and oligomerization of the toxin followed by pore formation in the brush border membrane although, alternatively, activation of cell-death signaling pathways has been also reported that might play an active role in pathogenesis as a result of Bt toxins activity (Pigott and Ellar, 2007). Several mechanisms of resistance to Bt toxins have been identified involving the loss or modification of receptors, altered proteolysis of protoxin and/or toxin, repair and/or replacement of damaged cells and, more recently, Bt resistance in some insects has been also associated to an elevated immune status (Pardo-López et al., 2013).

The response of insect midgut cells to Cry toxin action is complex and involves the modulation of many proteins required in cellular respiration, metabolic processes, signaling cascades, membrane restructuring and immune response (Cancino-Rodezno et al., 2012; Oppert et al., 2012). In this study, we used RNAi to investigate the immune function of ApoLp-III in Tc larvae and showed that ApoLp-III induction caused by Cry3Ba spore–crystal mixture treatment of Tc larvae is involved in regulation of PO enzyme activity, which plays crucial roles in the innate immune system of the insects and has been demonstrated to be associated with pathogen resistance in several insect systems (Barnes and Siva-Jothy, 2000; Eleftherianos et al., 2006).

2. Materials and methods

2.1. Insects

A laboratory colony of Tc Ga-2 line insects were reared on whole-grain flour with 5% brewer yeast powder at 30 ± 1 °C in darkness.

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2.2. Quantitative real-time PCR (qRT-PCR)

RNA isolation, cDNA synthesis and qRT-PCR was performed as in Contreras et al. (2013) using 50 ng cDNA and 900 nM of forward (5′CCAAAACGCCGCTCAAAC3′) and 300 mM reverse (5′TTGC AAATTGTTGCTGACTTCA3′) primers, designed with Primer Express software (Applied Biosystems) using ApoLp-III sequence (accession number TC015373). RPS18 (ribosomal protein S18) expression was used as an endogenous control as described before (Contreras et al., 2013) and gene expression relative-fold was calculated with the comparative C_t ($\Delta\Delta C_t$) method, using the StepOne software (Applied Biosystems).

2.3. RNAi

cDNA was used as template for PCR amplification using Prime Star polymerase (Takara) and specific primers (5′GAATTGTA ATACGACTC-ACTATAGGCACTCGTCAGTCACACACTTC3′ and 5′GAATTG TAATAC-GACTCACTATAGGGCAAGAAAGGCCGTTCAATAC3′) generated from Tc ApoLp-III Beetlebase gene sequence (TC015373) following conditions described in Contreras et al. (2013). In ApoLp-III silenced larvae, ApoLp-III transcript levels were evaluated 8 days after dsRNA injection by qRT-PCR from total RNA, using the ApoLp-III forward and reverse primers described in Section 2.2.

2.4. Toxicity assays

Cry3Ba and Cry1Ac spore–crystal mixtures were prepared from Bt strains BTS00125L and HD73, respectively, as in Contreras et al. (2013). In toxicity assays on ApoLp-III silenced larvae, 4 days after dsRNA injection, larvae (0.4–0.5 mg weight) were exposed to 12.5 μg Cry3Ba or Cry1Ac spore–crystal mixtures per microliter of flour disk for treatments or water in control assays, in 96-well polystyrene plates (Sterilin, Thermofisher) for 7 days under laboratory rearing conditions, and mortality was recorded after this period.

2.5. Phenoloxidase (PO) activity

Hemolymph from 15 Tc larvae untreated or treated for 2 days with12.5 μg Cry3Ba or Cry1Ac spore–crystal mixtures per microliter of flour disk were collected in 0.1 M Bis–Tris pH 6.5 buffer on ice to minimize spontaneous activation of ProPO cascade in the hemolymph *in vitro*, using thin wall capillaries (World Precision Instruments). Hemolymph protein concentration was determined according to Bradford (1976) using BSA as a standard. Hemolymph samples (1.5 μg total protein) were added to 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA) (Thermo Fisher Scientific) in 0.1 M Bis-Tris pH 6.5 and absorbance at 490 nm was measured in a Spectronic Genesys 5 spectrophotometer. PO activity was expressed as concentration of dopachrome formed per mg total hemolymph protein. Negative controls were performed by adding 0.01% PO inhibitors, 1-phenyl-2-thiourea (PTU) or tropolone.

In buffer-injected or ApoLpIII dsRNA-injected larvae, PO activity was measured 4 days after treatment with 12.5 μg Cry3Ba spore–crystal mixture per microliter of flour disk.

2.6. ProPO detection in western blot and larval tissue sections

Protein extracts (20 μg total protein) were prepared from forty whole Tc larvae of 0.4–0.5 mg weight in 4 M urea pH 7.4, 0.05% Protease Max surfactant (Promega), 48 μM pepstatin A, 20 μM E-64, and immune western blot was performed using *Manduca sexta* ProPO antibody (1:500), kindly provided from Dr. Kanost, as described in Jiang et al. (1997), alkaline phosphatase-conjugated anti-rabbit secondary antibody and ECL detection.

To visualize the distribution pattern of ProPO on Tc larval tissues, 20 μm thick sections of whole larvae untreated or treated for 2 days with 12.5 μg Cry3Ba or Cry1Ac spore–crystal mixtures per microliter of flour disk, were prepared. Tissue sections were obtained with a cryostat, fixed with 1% paraformaldehyde and incubated with the anti-ProPO antibody donated by Dr. Kanost (Jiang et al., 1997) as primary antibody and chicken anti-rabbit IG antibody coupled to Alexa Fluor 488 as secondary antibody. Nuclei were stained with DAPI solution (0.2 μg/mL). Samples were mounted with Aquatex Mounting Media (Merck) and examined under an Olympus FV1000 Confocal Laser Scanning Biological Microscope. In control sections the primary antibody was substituted by incubation buffer.

3. Results and discussion

In a previous work, using iTRAQ combined with LC–MS/MS we found several differentially expressed proteins in early response of Tc larvae to the coleopteran active Cry3Ba spore–crystal mixture (Contreras et al., 2013). Among the increased proteins, ApoLp-III showed a statistically significant 1.6-fold change in treated larvae compared to non-intoxicated larvae.

In the present work, to validate at the transcription level the increased ApoLp-III protein change detected in the iTRAQ analysis, we used quantitative real time PCR (qRT-PCR) to compare mRNA expression in untreated control larvae and larvae treated with the Cry3Ba spore–crystal mixture reported active against Tc or the non-toxic spore–crystal mixture of the Cry1Ac toxin producing strain (Contreras et al., 2013) (Fig. 1A). A 2.1-fold up-regulation of the *ApoLp-III* gene was found after Cry3Ba spore–crystal treatment relative to non-treated control larvae, whereas no induction of *ApoLp-III* gene was observed upon Cry1Ac spore–crystal treatment since non-significant differences between the amount of ApoLp-III transcript induced by Cry1Ac spore–crystal treatment and in control larvae were detected ($p > 0.05$, Student's *t*-test). *ApoLp-III* gene was also described to be up-regulated after larval exposure to Cry3Aa toxin in the beetle *Tenebrio molitor* (Oppert et al., 2012), suggesting that the induction of this protein expression might be relevant in Bt mode of action in coleopteran insects.

In order to assess whether Tc ApoLp-III is involved in insect defense against Bt, its expression was knocked down by means of RNAi. We employed qRT-PCR to examine mRNA levels of Tc ApoLp-III in dsRNA-injected larvae and buffer-injected larvae (control) and the analysis showed a statistically significant 98 ± 2% reduction of Tc ApoLp-III transcript compared to control larvae ($p < 0.05$, Student's *t*-test) (Fig. 1B). Regarding the effect of gene silencing on Tc larvae, results indicated that *ApoLp-III* gene's normal expression must not be essential for larvae viability since knockdown of the *ApoLp-III* gene's transcript did not induce larval mortality.

We performed toxicity assays using approximately a LC_{50} of Cry3Ba spore–crystal mixture (12.5 μg spore–crystal mixture/μL flour disk) or the same concentration of the non-active Cry1Ac spore–crystal mixture on ApoLp-III dsRNA-injected and buffer-injected larvae. As a control, buffer-injected and dsRNA-injected larvae that were not treated with Bt spore–crystal mixtures were also analyzed. In Fig. 1C, mortality increase in silenced larvae relative to buffer-injected larvae was represented for each treatment as well as in control larvae. Silenced larvae treated with the non-toxic Cry1Ac spore–crystal mixture did not show mortality increase, ruling out that the injection of dsRNA by itself could be activating an immune response that could impose a fitness cost leading to mortality. In contrast, silenced larvae treated with Cry3Ba spore–crystal mixture showed a statistically significant 19.4 ± 2.0% mortality increase compared to non-treated larvae ($p < 0.05$, Student's

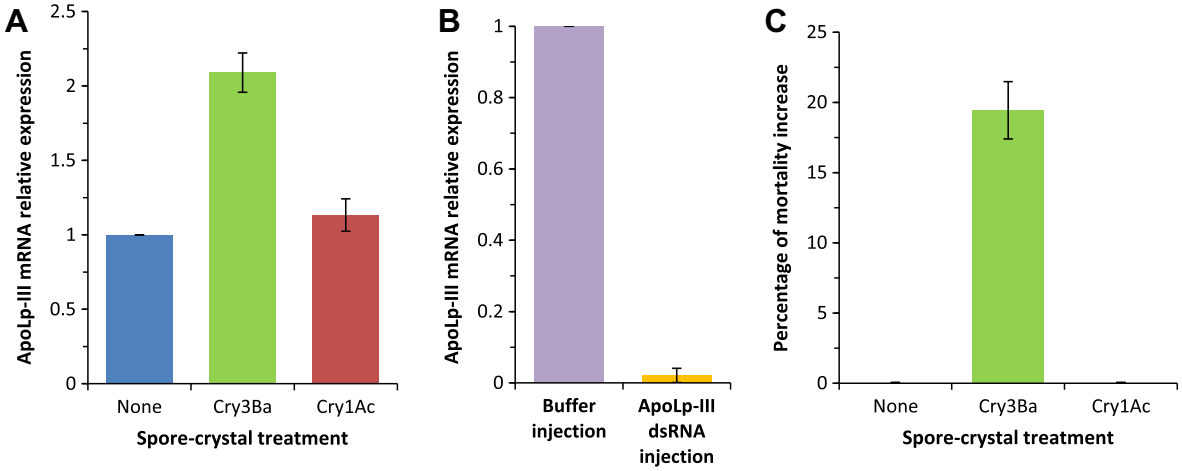

Fig. 1. Tc ApoLp-III transcriptional analysis. (A) qRT-PCR analysis of mRNA expression levels of Tc ApoLp-III in larvae exposed to Cry3Ba or Cry1Ac spore–crystal mixtures (12.5 μg spore–crystal mixture/μL flour disk) relative to untreated control larvae, normalized to RPS18 mRNA. Error bars indicate standard errors of the means from two biological replicates of twenty-four individuals per replicate. (B) ApoLp-III silencing in Tc larvae in response to injection of dsRNA against Tc ApoLp-III. The amount of Tc ApoLp-III transcript, normalized to the expression of *RPS18* gene, was estimated by qRT-PCR in silenced larvae relative to buffer-injected larvae. Error bars represent standard error of the mean of two biological replicates of thirty larvae. (C) Percentage of mortality increase of ApoLp-III dsRNA-injected larvae versus buffer-injected larvae following no treatment or treatment with Cry3Ba or Cry1Ac spore–crystal mixtures (12.5 μg spore–crystal mixture/μL flour disk). Error bars represent standard error of the mean of three biological replicates of thirty Tc larvae.

t-test), evidencing that ApoLp-III protein is involved in Tc defense against Cry3Ba spore–crystal intoxication. This result is consistent with the reported immune role of ApoLp-III in other insects, in which this protein was found to be associated with the activation of the ProPO cascade (Mullen and Goldsworthy, 2003; Park et al., 2005), a key component of the insect humoral immune response (González-Santoyo and Córdoba-Aguilar, 2012).

In Lepidoptera, ProPO synthesis occurs in hemocytes and mostly appears in hemolymph (González-Santoyo and Córdoba-Aguilar, 2012) from where it has been described it can be transported through the gut lining into the gut lumen (Ma et al., 2005). To examine the localization of ProPO in whole gut tissue of Tc larvae, first Tc larvae were analyzed to assess the possibility of detecting ProPO using antibodies against ProPO from *M. sexta*. Western blot of Tc larvae extracts showed that *M. sexta* ProPO antibody cross-reacted mainly with a protein band of around 77 kDa (Fig. 2A), which corresponds to the expected molecular mass of the 682 amino acid residues of Tc ProPO subunit 1 (NCBI accession number NP_001034493) or the 683 amino acid residues of ProPO subunit 2 (NCBI accession number NP_001034522). A band of around 48 kDa also observed in western blot might correspond to the 362 amino acid fragment of ProPO subunit 2 that is found in NCBI database as EFA05755. Next, confocal microscopy analysis was performed on Tc larvae using the antibodies against ProPO from *M. sexta* and Alexa Fluor 488-labeled secondary antibody detection. Control larvae and larvae treated with the non-toxic Cry1Ac spore–crystal mixture showed intact gut tissue and the same green fluorescence distribution corresponding to ProPO antibody localization in the haemocoel and in the gut, where the staining was visible as bright green color in the basement membrane of the gut and light green in the microvilli and the peritrophic membrane (a representative image of a Cry1Ac treated larva is shown in Fig. 2B). In larvae treated with Cry3Ba spore–crystal mixture, despite gut tissue appeared disorganized and disrupted as a consequence of the toxic effect, no differences in fluorescence distribution in comparison to control larvae were observed in regions that were not extensively damaged (Fig. 2C). For each treatment, microscope slides were also prepared in the absence of ProPO primary antibody and no fluorescence signal was observed (data not shown).

The inactive precursor ProPO is activated to PO by zymogenic serine proteinases, serine proteinase homologs and pattern recognition proteins as part of the melanisation cascade following the recognition of foreign compounds (Cerenius et al., 2008). To test whether the ProPO activation system might be involved in the response to Cry3Ba spore–crystal intoxication, we measured PO activity in hemolymph extracts of non-treated Tc larvae and larvae treated with Cry3Ba or Cry1Ac spore–crystal mixtures. As ProPO breaks down into PO naturally over time, PO activity was recorded for less than 25 min to minimize hydrolysis not dependent on specific activation induced by treatments. Fig. 2D shows no statistically significant differences in PO activity between non-treated larvae and Cry1Ac spore–crystal mixture treated larvae that were both used as controls. In contrast, when compared to controls, PO activity significantly increased after Cry3Ba spore–crystal treatment. In controls, the slight increase in PO activity observed after 13 min is suggestive of spontaneous activation of the proPO cascade in the hemolymph *in vitro*. Therefore, we chose an incubation time of 13 min to establish comparison of PO activity among treatments. Consistent with Cry3Ba spore–crystal mixture being the only tested treatment able to induce *ApoLp-III* gene expression in Tc larvae (Fig. 1A), PO activity measured in Cry3Ba spore–crystal treated sample at 13 min was significantly increased in relation to controls. Additionally, the specificity of the PO enzyme activity determination was assessed using the general oxidase enzyme inhibitor PTU and the PO selective inhibitor, tropolone. In the presence of these inhibitors, no PO activity was detected in hemolymph extracts from non-treated control larvae (Fig. 2D), showing that measurements at 490 nm were indicative of melanin formation due to PO activity.

Finally, by using RNAi against ApoLp-III we confirmed this protein is involved in regulation of ProPO cascade activation in Tc. PO activity after 13 min incubation time was compared in hemolymph extracts prepared from ApoLp-III silenced larvae challenged with a spore–crystal mixture of Cry3Ba (12.5 μg spore–crystal mixture/μL flour disk) with control extracts from buffer injected larvae treated with the same concentration of Cry3Ba spore–crystal mixture. A statistically significant activity decrease of 71 ± 14% was observed in extracts from ApoLpIII dsRNA injected larvae treated with Cry3Ba spore–crystal mixture relative to buffer-injected challenged

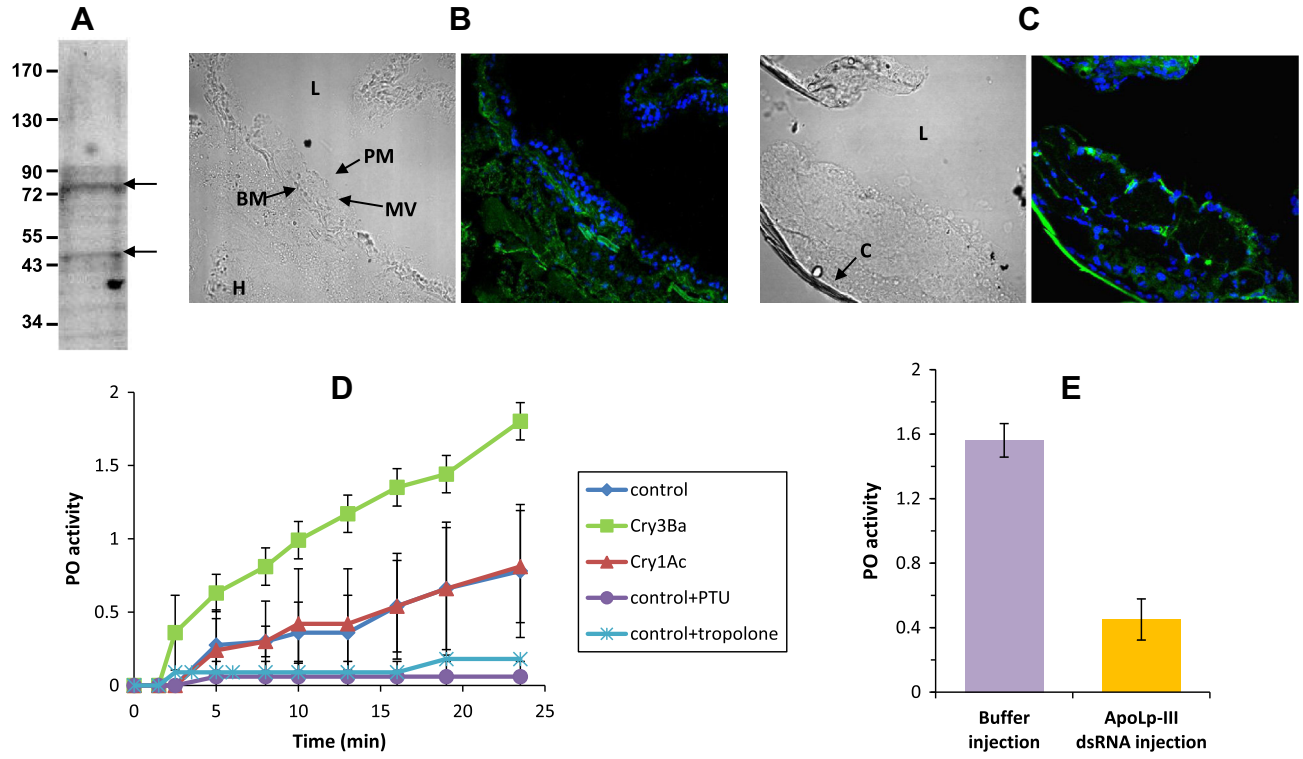

Fig. 2. Tc ProPO analysis. (A) Western blot of Tc larvae extract probed with *M. sexta* ProPO antibody. Molecular weight markers are shown on the left. Arrows indicate 77 kDa and 48 KDa bands. (B) Confocal microscopy of sections of Tc larvae after treatment with 12.5 μg Cry1Ac spore–crystal mixture/μL flour disk. Tissue was stained with *M. sexta* ProPO antibody and counterstained with Alexa Fluor 488-conjugated secondary antibodies (green) to detect ProPO and DAPI staining (blue) to depict nuclei. On the left, the corresponding white-light transmission image is shown in which C stands for cuticula, H for haemocoel, L for lumen, BM for gut basement membrane, MV for microvilli and PM for peritrophic membrane. (C) Confocal microscopy of sections of Tc larvae after treatment with 12.5 μg Cry3Ba spore–crystal mixture/μL flour disk, as described in panel B. (D) Activation of ProPO by Tc larvae treatment with Cry1Ac or Cry3Ba spore–crystal mixtures and in untreated larvae (control) in the presence or absence of 0.01% PO inhibitors, PTU or tropolone. The points represent the mean of at least two individual measurements ± SE. (E) PO activity in hemolymph extracts of ApoLp-III dsRNA-injected larvae treated with 12.5 μg Cry3Ba spore–crystal mixture/μL flour disk and buffer-injected larvae treated with the same concentration of Cry3Ba spore–crystal mixture after 13 min incubation with L-DOPA. Bars represent the mean of at least two individual measurements ± SE.

larvae ($p < 0.05$, Student's *t*-test) (Fig. 2E), evidencing that ApoLpIII participates in the Tc immune response to Bt through activation of the ProPO cascade.

ProPO activation has been reported to occur following recognition of pathogen associated molecular patterns (PAMPs) such as peptidoglycans or lipopolysaccharides from bacteria and β-1,3-glucans from fungi as well as after tissue damage inflicted by mechanical wounding and possibly by enzymes released from pathogens (Cerenius et al., 2008). In immune-induced insects lipophorin particles have been described to be associated with PAMPs and regulatory proteins that activate ProPO in hemolymph (Rahman et al., 2006). Particularly, ApoLp-III has been described to act as a multifunctional immune stimulating activity in insects involving activation of the ProPO system as well as synthesis of antimicrobial peptides (Zdybicka-Barabas and Cytryńska, 2011). A trade-off between lysozyme/antibacterial activity and PO activity has been observed in some insects (Rao et al., 2010), which has been correlated with the nature of the immune induction and the fitness costs associated with immunity (Freitak et al., 2007; Erler et al., 2011). Illustrating the complexity of the role of ApoLp-III in insect immunity, in the lepidopteran insect *G. mellonella*, ApoLp-III improved antibacterial activity of cecropin against *Escherichia coli*, played a role in the activation of the ProPO cascade (Park et al., 2005) and it has been described to be involved in the antibacterial response in this insect (Zdybicka-Barabas and Cytryńska, 2011), but injection of ApoLp-III protein in *G. mellonella* larvae depressed the activation of PO induced by lipoteichoic acid from *Bacillus subtilis* (Halwani et al., 2000). Here, we show that ApoLp-III induction leading to PO activity increase in Tc larvae hemolymph was caused by Cry3Ba spore–crystal mixture treatment, which is toxic against Tc (Contreras et al., 2013) and damaged the insect gut (Fig. 2C), but not by Cry1Ac spore–crystal mixture that is not a coleopteran active strain (Contreras et al., 2013) and was unable to disrupt the larval gut (Fig. 2B). These results are consistent with the well-known effect of Bt toxins that target, and cause the disruption of, the epithelial cells lining the midgut, thus allowing Bt cells or spores to enter the haemocoel where to be infectious they must avoid destruction by the innate humoral immune system and circulating haemocytes (Raymond et al., 2010). Understanding the role of insect immunity on the response to Bt might be instrumental to improve the efficacy of pest control and counteract the development of resistance.

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Artículo 3: Sodium solute symporter and Cadherin protins act as *Bacillus thuringiensis* Cry3Ba toxin functional receptors in *Tribolium castaneum*

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Sodium Solute Symporter and Cadherin Proteins Act as *Bacillus thuringiensis* Cry3Ba Toxin Functional Receptors in *Tribolium castaneum*[*]

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Background: Interaction with insect midgut receptors is required for *Bacillus thuringienesis* (Bt) toxicity.
Results: RNAi knockdown of E-cadherin and sodium solute symporter (SSS) genes dramatically decreases *Tribolium castaneum* (Tc) larval susceptibility to Cry3Ba. A SSS fragment enhances Cry3Ba toxicity.
Conclusion: E-cadherin and SSS but not aminopeptidase N are Cry3Ba receptors in Tc.
Significance: For the first time, SSS was demonstrated as a Bt functional receptor.

Understanding how *Bacillus thuringiensis* (Bt) toxins interact with proteins in the midgut of susceptible coleopteran insects is crucial to fully explain the molecular bases of Bt specificity and insecticidal action. In this work, aminopeptidase N (TcAPN-I), E-cadherin (TcCad1), and sodium solute symporter (TcSSS) have been identified by ligand blot as putative Cry3Ba toxin-binding proteins in *Tribolium castaneum* (Tc) larvae. RNA interference knockdown of TcCad1 or TcSSS proteins resulted in decreased susceptibility to Cry3Ba toxin, demonstrating the Cry toxin receptor functionality for these proteins. In contrast, TcAPN-I silencing had no effect on Cry3Ba larval toxicity, suggesting that this protein is not relevant in the Cry3Ba toxin mode of action in Tc. Remarkable features of TcSSS protein were the presence of cadherin repeats in its amino acid sequence and that a TcSSS peptide fragment containing a sequence homologous to a binding epitope found in *Manduca sexta* and *Tenebrio molitor* Bt cadherin functional receptors enhanced Cry3Ba toxicity. This is the first time that the involvement of a sodium solute symporter protein as a Bt functional receptor has been demonstrated. The role of this novel receptor in Bt toxicity against coleopteran insects together with the lack of receptor functionality of aminopeptidase N proteins might account for some of the differences in toxin specificity between Lepidoptera and Coleoptera insect orders.

The development of improved bioinsecticides based on toxin-producing *Bacillus thuringiensis* (Bt)[3] bacteria requires new toxins with increased activity and wider insecticidal spectrum, as well as more effective toxin delivery methods. Identifying molecules that confer toxin susceptibility in target insects is essential to understand how Bt toxins interact with their hosts, facilitating a more rational design of Bt products. Several membrane components in the insect midgut epithelium capable of binding Bt toxins have been identified; however, not all appear to be functionally relevant (1).

In Lepidoptera, it has been demonstrated that interaction of Cry1 toxins with aminopeptidase N (APN) and cadherin (CAD)-like) midgut proteins (2, 3) is required for toxic action, and alkaline phosphatase and ABC transporter have also been proposed as Cry1 receptors (4, 5). In mosquito larvae, homologous APN, CAD-like, and alkaline phosphatase proteins have been described as Cry11 and Cry4 receptor proteins (6), and in Coleoptera a cadherin-like protein has been demonstrated to act as a Cry3Aa receptor (7). In coleopteran insects, other molecules, such as an ADAM-like metalloprotease (8) and alkaline phosphatase (9), have been proposed as putative Cry receptors. The best characterized Cry receptors, APN and CAD-like proteins, have been unequivocally involved in a Bt mode of action by gene silencing, resulting in a reduced sensitivity to toxin (10, 11).

The APN proteins belong to a family of zinc-binding metalloprotease/peptidase enzymes inserted into the midgut microvillar membrane via a C-terminal glycosylphosphatidylinositol anchor that play an essential role in insect digestion (12). APNs are highly variable in sequence and have been grouped into five phylogenetic classes based on their amino acid sequences (13). They have distinct *N*- and *O*-glycosylation patterns, which seem to be determinant for Cry toxin binding (12).

The CAD-like proteins that function as Cry receptors contain several cadherin repeats but, unlike classical cadherins, are not primarily located within adherens junctions involved in cell-cell adhesion but on the apical membrane of midgut columnar epithelial cells (12). In this type of Cry receptors, the membrane-proximal cadherin repeats have been reported to be key interaction sites that mediate toxin susceptibility (14–16).

In Lepidoptera, a sequential toxin interaction with CAD-like and APN proteins in the midgut membrane has been proposed,

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[3] The abbreviations used are: Bt, *B. thuringiensis*; APN, aminopeptidase N; CAD, cadherin; Tc, *T. castaneum*; SSS, sodium solute symporter; dsRNA, double-stranded RNA; BBMV, brush border membrane vesicle; qRT-PCR, quantitative RT-PCR.

resulting in toxin recruitment on the membrane surface and conformational changes that promote toxin insertion into the membrane, leading to cell osmotic disequilibrium and eventually to cell death (17).

Through proteomic approaches, other proteins have been identified as Cry binding proteins, although their role as Bt toxin receptors has not been demonstrated yet. Among them are actin and V-ATP-synthase in Lepidoptera (18–20) and Diptera (21), heat shock cognate protein in Lepidoptera (20), and flotillin and prohibitin in Diptera (21). Actin, V-ATP-synthase, and prohibitin have been also identified as Cry binding partners in Coleoptera through targeted mass spectrometry protein analysis.[4] Whether these molecules act facilitating the toxic process or contributing to the insect response against Cry toxins remains to be investigated and constitutes an important issue to reveal the entire picture of the Bt mode of action.

The coleopteran model insect *Tribolium castaneum* (Tc) is a major global pest of stored products for human consumption for which many genetic and genomics tools have been developed (22), so it constitutes an ideal subject for the identification of new biopesticide targets based in Bt. In this work, we carried out receptor binding and ligand blot experiments in Tc larvae with the coleopteran-specific toxin Cry3Ba, previously shown to be active against this insect pest (23). Using LC-MS/MS spectrometry, among other proteins, we have identified as putative Cry3Ba toxin receptors an APN (NP_001164285, in this work denoted as TcAPN-I), an E-cadherin protein (XP_971388, denoted as TcCad1), and a sodium solute symporter protein (EFA03129, denoted as TcSSS) containing cadherin repeats. RNA interference experiments with dsRNA of the corresponding genes demonstrated that TcCad1 and TcSSS proteins were Cry3Ba toxin functional receptors in Tc, whereas TcAPN-I was not related to Cry3Ba susceptibility.

EXPERIMENTAL PROCEDURES

Cry3 Toxin Production—BTS1 and BTS00125L Bt Cry3Aa and Cry3Ba-producing strains were grown in solid sporulation medium (24) at 30 ± 1 °C until complete autolysis. Lysed bacteria were resuspended in 2× PBS (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl), pH 7.4, and washed twice with 0.02% Triton X-100 in 2× PBS, pH 7.4, and twice with water. Following centrifugation at 6000 × *g* for 10 min at 4 °C, spore-crystal mixtures were resuspended in water and stored at −20 °C until use. Crystal inclusions were purified from spores by centrifugation in discontinuous sucrose gradients as described in Rausell *et al.* (25).

Insects—A laboratory colony of Tc founded from Ga-2 strain adults kindly provided by Dr. Beeman (U.S. Department of Agriculture) was used. Insects were reared on whole grain flour with 5% brewer yeast powder at 30 ± 1 °C in darkness.

Tc Brush Border Membrane Vesicle (BBMV) Preparation—Tc BBMV were prepared from 10–14-day-old larvae (after egg laying) according to the method of Wolfesberger *et al.* (26), as modified by Reuveni and Dunn (27). APN enzyme activity was monitored as described by Hafkenscheid (28) to assess BBMV preparation quality.

Binding Assays on Tc BBMV—Purified Cry3Ba toxin was biotinylated using biotinyl-*N*-hydroxysuccinimide ester (Amersham Biosciences; protein biotinylation module, GE Healthcare) according to the manufacturers' indications. Cry3Ba biotinylated toxin (1.4 nM) was incubated with 10 μg of Tc BBMV in PBS buffer, pH 7.4, 0.1% BSA, for 1 h in the presence or absence of 1000-fold excess unlabeled Cry3Ba or Cry3Aa toxins. Subsequently, unbound toxin was removed by centrifugation (10 min at 14,000 × *g*), and BBMV with bound toxin were washed twice with the same buffer (100 μl). BBMV were suspended in 15 μl of PBS and the corresponding volume of 8× Laemmli sample loading buffer. Samples were 10% SDS-PAGE electrophoresed and electrotransferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences). The biotinylated toxins that were bound to the protein vesicles were visualized by incubating with streptavidin-peroxidase conjugate (Amersham Biosciences; 1:4000 dilution) for 1 h, followed by the addition of Amersham Biosciences ECL Prime Western blotting reagents (GE Healthcare), as recommended by the manufacturers.

Ligand Blot Analysis on Tc BBMV—Tc BBMV proteins (15 μg) were separated in 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) that was incubated overnight with PBS buffer, pH 7.4, 3% BSA, 0.1% Tween 20. After washing with PBS buffer, pH 7.4, 2% Tween 20, the membrane was incubated with 10 μg/ml Cry3Ba toxin in PBS buffer, pH 7.4. The blot was then washed twice with PBS buffer, pH 7.4, 2% Tween 20 and incubated with Cry3 rabbit polyclonal antibody (1:10,000) for 1 h. Following two washes with PBS buffer, pH 7.4, 2% Tween 20, the membrane was incubated with alkaline-phosphatase conjugated anti-rabbit secondary antibody (Sigma, 1:30,000), and the immunoreactive proteins were visualized using the ECL detection system Immobilon Western (Millipore). The corresponding bands in a Coomassie-stained gel of Tc BBMV (10% SDS-PAGE) were excised, trypsinized, and analyzed by LC-MS/MS.

Toxicity Assays—Toxicity assays on Tc larvae were performed using preweighed 10 to 14-day-old larvae (after egg laying), fed for 7 days on 20-μl flour discs (20% flour, w/v), prepared as described by Xie *et al.* (29), containing 12.5 μg/μl Cry3Ba spore-crystal mixture for treatments or water in control assays. The assays were performed in 96-well polystyrene plates (Sterilin, Thermofisher) with one flour disc and one larva per well. Thirty larvae were used in each assay, and at least two replicates were carried out. Mortality was recorded after 7 days under laboratory rearing conditions.

For toxicity assays in gene silencing experiments, 4 days after dsRNA or control buffer injection, larvae were weighed and exposed to 12.5 μg/μl Cry3Ba spore-toxin mixtures in flour discs. Flour discs prepared with water were used as controls of the toxicity assays.

Toxicity assays with a 29-mer peptide containing amino acids 1110–1138 of TcSSS protein (PepTcSSS peptide, Ac-KVDAAGSATVELKDTIELITILTPKLTFT-NH_2) were carried out exposing groups of 20 larvae of 0.33–0.50-mg weight per larva to flour discs containing 8.8 μg/μl Cry3Ba spore-crystal mixture with or without 10 μg/μl peptide (final concentration).

[4] M. D. Real and C. Rausell, unpublished results.

TABLE 1
Primers used in qRT-PCR to analyze the expression of genes corresponding to TcCad1, TcSSS, TcAPN-I, TcAPN-II, and TcAPN-III proteins and to generate dsRNA in RNAi experiments

Accession number and name	Sequence
XP_971388	
TcCad1 RNAi Fw	5′-GAATTGTAATACGACTCACTATAGGGAACTGACCAAATCACCTTCG-3′
TcCad1 RNAi Rv	5′-GAATTGTAATACGACTCACTATAGGTGTCTCCAACATCTTTATCGGT-3′
TcCad1 qRT-PCR Fw	5′-AACAACCCGAGTGGCGAAT-3′
TcCad1 qRT-PCR Rv	5′-TCTGCCATTGATGAGTTCTTGGT-3′
EFA03129	
TcSSS RNAi Fw	5′-GAATTGTAATACGACTCACTATAGGCTTCCAAACTTACAGTAAAACTAG-3′
TcSSS RNAi Rv	5′-GAATTGTAATACGACTCACTATAGGAACACTGATAAGATATTGGTCC-3′
TcSSS qRT-PCR Fw	5′-AAACCGCGATTCTGGTAAACC-3′
TcSSS qRT-PCR Rv	5′-TGACCGAATTGTGGTATGGTGAT-3′
NP_001164285	
TcAPN-I RNAi Fw	5′-GAATTGTAATACGACTCACTATAGGAGTCCACGATGTTTCTAGAGC-3′
TcAPN-I RNAi Rv	5′-GAATTGTAATACGACTCACTATAGGCAGTCAGTATGTTCAACGTCAG-3′
TcAPN-I qRT-PCR Fw	5′-CAAGTGGCGGTCCCAGAT-3′
TcAPN-I qRT-PCR Rv	5′-TCAACAATCCCCAATTTTCCA-3′
EEZ99297	
TcAPN-II RNAi Fw	5′-GAATTGTAATACGACTCACTATAGGCGATAACAAGTTTGGTAACACG-3′
TcAPN-II RNAi Rv	5′-GAATTGTAATACGACTCACTATAGGAGATATTTGTTAAGTACGGCTTC-3′
TcAPN-II qRT-PCR Fw	5′-CGCCCTATTCGCCACTGA-3′
TcAPN-II qRT-PCR Rv	5′-CGACTATTTGTGCCCGGTTT-3′
EEZ99296	
TcAPN-III RNAi Fw	5′-GAATTGTAATACGACTCACTATAGGCTGAAGTACTACTAGCAGCGG-3′
TcAPN-III RNAi Rv	5′-GAATTGTAATACGACTCACTATAGGCATCGCTTGCTAGAACAGG-3′
TcAPN-III qRT-PCR Fw	5′-CACCTGGTCTGTTCACGAAATG-3′
TcAPN-III qRT-PCR Rv	5′-CCCAACTTTTTCGGCCAAT-3′
EFA04159	
TcRPS18 qRT-PCR Fw	5′-TGATGGCAAACGCAAAGTCA-3′
TcRPS18 qRT-PCR Rv	5′-TCGGCCGACACCTTTGA-3′

As a control, larvae were challenged with PepTcSSS peptide alone.

RNAi—RNA isolation and cDNA synthesis were performed as described before (23), using 10–14-day-old larvae (after egg laying). cDNA was used as template for PCR amplification using Prime Star polymerase (Takara) and specific primers generated from TcAPN-I, two other APN proteins (EEZ99297, denoted as TcAPN-II, and EEZ99296, denoted as TcAPN-III), TcCad1 and TcSSS protein NCBI gene sequences, containing a T7 polymerase promoter sequence at their 5′ end (see Table 1). The PCR products (1 μg) were used for *in vitro* transcription to prepare dsRNA using Ambion MEGAscript T7 kit (Applied Biosystems, Austin, TX) according to the manufacturer's protocol. Purified dsRNA was stored at −20 °C until injected into Tc larvae.

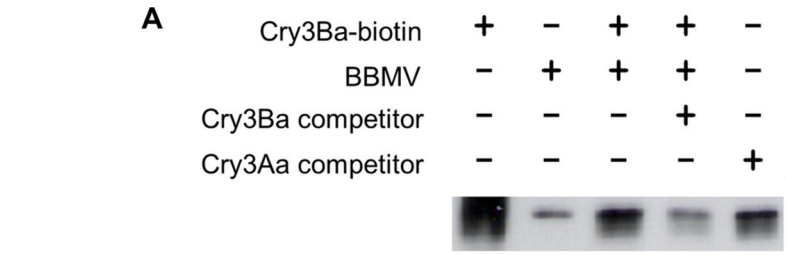
Larvae were anesthetized for 5 min on ice before ventral injection of ~0.2 μl of 1 μg/μl dsRNA in injection buffer (1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl), using thin wall capillars (World Precision Instruments) in a microinjection system (Narishige). Control larvae were injected with injection buffer. Following injection, larvae were grown under standard rearing conditions.

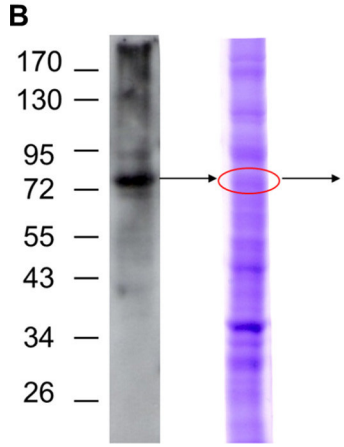
Quantitative Real Time PCR—TcCad1, TcAPN-I, TcAPN-II, TcAPN-III, and TcSSS transcript levels were evaluated 8 days after dsRNA injection by quantitative real time PCR. qRT-PCR amplification was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems) thermocycler, following the manufacturer's recommendations, using Power SYBR Green PCR Master Mix (Applied Biosystems), 100 ng of cDNA, and gene-specific forward and reverse primers (Table 1), designed with Primer Express software (Applied Biosystems). For each sample, two biological replicates were analyzed using the mean values of three technical replicates. Gene expression was normalized using RPS18 (ribosomal protein S18, EFA04159) expression as an endogenous control (30) (primers included in Table 1). The data were analyzed by Student's *t* test for statistically significant differences ($p < 0.05$). To analyze the expression of TcCad1, TcAPN-I, and TcSSS genes during larval development, qRT-PCR amplification was performed as described above on RNA obtained from larvae of different weight within a range of 0.32–1.8 mg.

RESULTS

Aminopeptidase N, E-cadherin, and Sodium Solute Symporter Are Putative Cry3Ba Toxin-binding Proteins in Tc—Purified Cry3Ba toxin was biotin-labeled and homologous, and heterologous competition binding assays were performed on BBMV of Tc (Fig. 1*A*). Binding experiments showed that Cry3Ba toxin was able to bind to Tc BBMV, and this interaction was specific because it was competed with 1000-fold excess of unlabeled Cry3Ba toxin but not with 1000-fold unlabeled Cry3Aa.

Tc BBMV proteins were blotted onto a nitrocellulose membrane, and binding of proteins to the Cry3Ba toxin was visualized by immunodetection of the bound toxin. The major protein band recognized by this toxin was ~75 kDa (Fig. 1*B*). The corresponding band in a Coomassie-stained gel of Tc BBMV (10% SDS-PAGE) was not among the most intense bands in the gel, indicating that the ligand blotting was performed in optimal conditions so that nonimmunoreactive proteins present in high concentration did not adsorb the primary antibody nonspecifically. The 75-kDa band excised from the Coomassie-stained gel was analyzed by LC-MS/MS, and the NCBInr database (taxonomic restriction to Tc) was searched with peptide mass finger-





NCBI Accession Number	Protein Identified	Mascot Score	Peptides Identified
XP_976188	V-ATPase subunit A isoform2	1531	36
NP_001164285	Aminopeptidase N	1270	30
EFA05794	Apolipoprotein LI-II precursor	996	21
EFA03129	Sodium solute symporter	905	19
XP_971388	E Cadherin	74	2

FIGURE 1. **Cry3Ba toxin binds TcAPN-I, TcCad1, and TcSSS.** *A*, competition binding assays on Tc BBMV. Biotin-labeled Cry3Ba toxin was incubated with Tc BBMV in the absence or presence of an excess of unlabeled toxin competitor. After 1 h of incubation, unbound toxins were removed by centrifugation, and vesicles containing bound toxins were loaded onto a SDS-PAGE and blotted to a nitrocellulose membrane. Labeled proteins were visualized by means of streptavidin-peroxidase conjugate. *B*, Cry3Ba toxin ligand blot of Tc BBMV proteins immunodetected with anti-Cry3 antibody is shown on the *left*. The *arrow* points to the band at ~75 kDa that was excised for LC-MS/MS analysis from the corresponding Coomassie-stained gel shown on the *right*. The most relevant identifications obtained after searching the NCBInr database (taxonomic restriction to Tc) with peptide mass fingerprinting data using Mascot (MatrixScience) are shown in the *box*.

printing data using Mascot (MatrixScience) to establish the best protein matches (a score value higher than 57 was considered a significant hit). The most relevant identifications are shown in Fig. 1*B*. The highest Mascot score corresponded to a predicted protein similar to V-ATPase subunit A isoform 2 (XP_976188), which has been reported to bind Cry toxins in other insects (19–21). Among the identified proteins in the 75-kDa band, we detected aminopeptidase N and E-cadherin that have been demonstrated to act as Cry toxin receptors (12), and with a high Mascot score, the reported Cry binding protein apolipoprotein LI-II precursor (31) and a novel putative Cry receptor, the sodium solute symporter protein, were also found (Fig. 1*B*).

To demonstrate whether these proteins act as functional Bt receptors in Tc midgut epithelial membrane, we used RNAi to assess the effect of expression knockdown of the corresponding genes in Cry3Ba toxin insecticidal activity against Tc larvae. Because it has been described that V-ATPase silencing compromises Tc viability (32), and apolipoprotein has been proposed to be involved in toxin sequestration by a coagulation reaction inside the gut lumen (31), we focused on gene silencing of aminopeptidase N NP_001164285 (in this work denoted as APN-I), E-cadherin XP_971388 (TcCad1), and the sodium solute symporter protein EFA03129 (TcSSS).

RNA Interference Knockdown of TcCad1 or TcSSS Proteins Resulted in Decreased Susceptibility to Cry3Ba Toxin—To choose the appropriate larval size for silencing, we obtained the transcription profile of the TcAPN-I, TcCad1, and TcSSS protein genes in different Tc larval developmental stages using qRT-PCR (Fig. 2*A*), with RPS18 mRNA as an internal control. TcCad1 and TcSSS showed similar expression profile. In contrast, TcAPN-I displayed a completely different pattern. The optimal larval weight range that allowed toxicity assays to be performed following silencing, so that pupation is not reached during bioassay time course, was 0.25–0.80 mg. Because within that range the abundance of TcCad1 and TcSSS transcripts showed a maximum in larvae of 0.64-mg weight, we selected this larval stage to examine mRNA levels of the corresponding transcripts by qRT-PCR, in dsRNA-injected larvae and control (buffer-injected) larvae (Fig. 2*A*). Because the TcAPN-I expression profile remained stable during the analyzed larval weight range, we selected the same larval stage as in TcCad1 and TcSSS to assess gene knockdown efficiency (Fig. 2*A*). Additionally, we performed a gene silencing experiment injecting a mixture of dsRNAs of TcAPN-I and the closely related APN EEZ99297 (in this work denoted as TcAPN-II) and APN EEZ99296 (in this work denoted as TcAPN-III) (Fig. 2*B*). As shown in Fig. 2*C*, following dsRNA injection, reductions of 80.5, 76.8, and 84.2% compared with control buffer-injected larvae were observed in TcCad1, TcSSS, and TcAPN-I transcript abundance, respectively. In the triple silenced larvae, reductions of 84.7, 37.7, and 90.9% compared with control buffer-injected larvae were observed in TcAPN-I, TcAPN-II, and TcAPN-III transcripts, respectively (Fig. 2*D*). Single or multiple gene silencing did not cause larval mortality in any of the assayed genes, suggesting

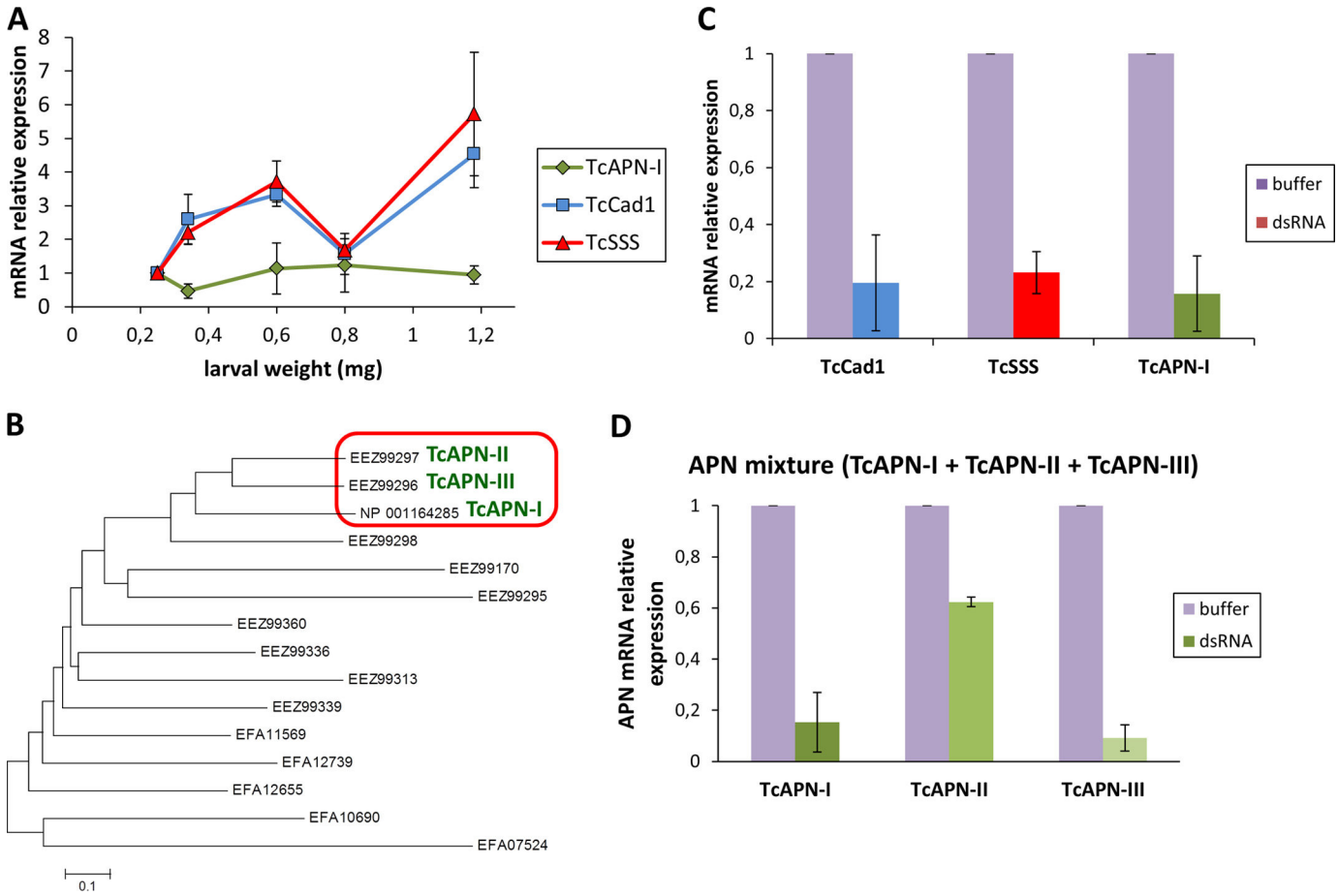


FIGURE 2. **TcAPN-I, TcCad1, and TcSSS mRNA expression is reduced in response to injection of dsRNA.** *A*, qRT-PCR analysis of TcAPN-I, TcCad1, and TcSSS mRNA expression levels in Tc larvae of different weight. RPS18 mRNA abundance was used to normalize gene expression. *B*, unrooted phylogenetic tree generated with Mega 5 (46) of 15 Tc aminopeptidases N and aminopeptidases-like amino acid sequences. The neighbor-joining method (47) for reconstructing the phylogenetic tree was used. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (48) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. *C*, gene silencing in Tc larvae in response to injection of TcAPN-I or TcCad1 or TcSSS dsRNA. The relative amount of Tc gene transcripts estimated by qRT-PCR in buffer-injected control larvae and silenced larvae was compared, normalized to the expression of *RPS18* gene. The statistical significance of the gene expression between the two samples was evaluated using Student's *t* test, and significant knockdown was observed ($p < 0.05$) for all genes. The *error bars* represent standard errors of the mean of two biological samples and three technical replicates each. *D*, gene silencing in Tc larvae in response to injection of a mixture of TcAPN-I and TcAPN-II and TcAPN-III dsRNA. The relative amount of each individual Tc gene transcript corresponding to TcAPN-I, TcAPN-II, or TcAPN-III estimated by qRT-PCR in buffer-injected control larvae, and silenced larvae was compared normalized to the expression of *RPS18* gene. The statistical significance of the gene expression between the two samples was evaluated using Student's *t* test, and significant knockdown was observed ($p < 0.05$) for all genes. The *error bars* represent the standard error of the mean of two biological samples and three technical replicates each.

that these genes' normal expression must not be essential for Tc larvae viability.

Prior to toxicity assays in silenced larvae, a control assay was performed to investigate the influence of injection on Cry3Ba toxicity. Tc larvae of different weight were injected with injection buffer and 4 days post-injection were fed with flour discs containing Cry3Ba spore-crystal mixture. Mortality was recorded after 7 days and compared with that of noninjected larvae (Fig. 3*A*). In both cases, increased larval weight correlated with lower Cry3Ba mortality showing similar mortality profiles, indicating that injection had no effect on the mortality caused by Cry3Ba toxin.

We next carried out toxicity assays with Cry3Ba toxin on dsRNA-injected and buffer-injected larvae in a weight range of 0.64–0.79 mg (Fig. 3*B*). TcCad1 and TcSSS dsRNA treatments led to statistically significant mortality decrease compared with buffer-injected larvae (~87.8 and 67.0% mortality decreases, respectively) (Student's *t* test, $p < 0.05$), consistent with a role of both proteins as Cry3Ba toxin functional receptors. In contrast, TcAPN-I dsRNA and a mixture of TcAPN-I, TcAPN-II, and TcAPN-III dsRNA did not show significant mortality differences when compared with buffer-injected larvae (Student's *t* test, $p > 0.05$) (Fig. 3*B*). The results demonstrated that TcCad1 and TcSSS but not aminopeptidase N proteins are Cry3Ba receptors in Tc.

Cry3Ba Toxicity Is Enhanced by a TcSSS Peptide Fragment Containing a Putative Binding Epitope Found in Other Bt Cadherin Functional Receptors—PROSITE patterns database was searched with Motif Scan to identify known motifs in TcSSS and TcCad1 sequences, and the corresponding domain figures were generated using MyDomains-Image Creator PROSITE tool (Fig. 4*A*). Similar to the CAD-like functional Bt receptor in *Tenebrio molitor* (TmCad1, ABL86001) (7), in the TcCad1sequence, extracellular, transmembrane, and intracel-

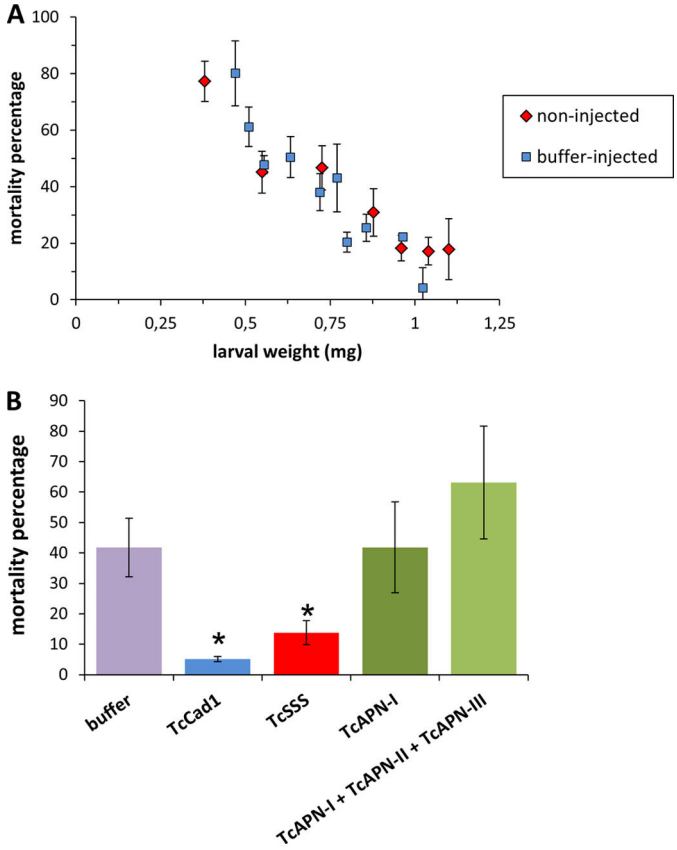


FIGURE 3. **Knockdown of TcCad1 or TcSSS enhances survival of Cry3Ba toxin challenged *T. castaneum* larvae.** *A*, mortality percentage following Cry3Ba spore-crystal treatment on either noninjected or buffer-injected Tc larvae of different weight. For each experiment, mortality was recorded using 30 Tc larvae. The *error bars* represent standard error of the mean of at least two biological replicates. *B*, mortality percentage following Cry3Ba spore-crystal treatment on either buffer-injected (control) or dsRNA-injected larvae. Mortality experiments were performed using 30 Tc larvae. The *error bars* represent standard error of the mean of two biological samples and three technical replicates each. *Asterisks* indicate that the mortality increase observed upon Bt treatments in silenced larvae with respect to buffer-injected larvae was statistically significant using Student's *t* test ($p < 0.05$).

lular domains were found using the TMHMM server. In the extracellular domain, a signal sequence (using SignalP server) (33) and 12 extracellular repeat domains were predicted. TcSSS contains the common topological motif of an arrangement of 11 transmembrane domains present in the sodium solute symporter family (34) and 3 predicted cadherin repeats in the extracellular domain. TcSSS and TcCad1 sequences were scanned to identify conserved patterns using the PRATT 2.1 tool that allows discovering patterns of the PROSITE database conserved in sets of unaligned protein sequences (35). Fig. 4*A* shows the Clustal alignment corresponding to the PRATT identified pattern in TcSSS and TcCad1 sequences. This alignment was then used to identify the previously described cadherin Bt toxin binding epitopes in *Manduca sexta* CAD-like protein (^{1416}GVLTLNIQ^{1423}) and in *T. molitor* TmCad1 (^{1359}GDITINFE^{1366}) (7, 36). Sequence alignments of *M. sexta* and *T. molitor* Cry receptor binding regions showed a high degree of similarity to the TcSSS ^{1115}GSATVELK^{1122} sequence corresponding to the homology region in TcCad1 identified using PRATT (Fig. 4*B*).

It has been demonstrated that peptides containing the above mentioned binding epitopes of *M. sexta* and *T. molitor* enhanced the activity of Cry1A and Cry3Aa toxins in different lepidopteran and coleopteran larvae, respectively (36, 37). Therefore, consistent with TcSSS being a functional receptor in Tc, it would be expected that a peptide containing the homologous fragment identified in the TcSSS protein would also enhance the toxicity of Cry3Ba against Tc larvae. A 29-mer peptide Ac-^{1110}KVDAAGSATVELKDTIELITILTPK-LTFT^{1138}-NH_2 (PepTcSSS) containing the putative binding epitope in TcSSS was synthesized and, when used in bioassays, significantly enhanced Cry3Ba toxicity against Tc larvae, resulting in 54.4% mortality in contrast to 28.4% mortality with Cry3Ba toxin alone (Fig. 4*C*). As shown in Fig. 4*C*, PepTcSSS itself did not cause larval mortality.

DISCUSSION

It is well known that Bt toxins insecticidal activity relies on the interaction with midgut epithelial membrane components of the target insect. In the case of Cry3Ba toxin, reported active against Tc (23), as expected, the toxin specifically bound to Tc larvae BBMV (Fig. 1*A*). By ligand blot analysis, a protein band of 75 kDa was mainly recognized by Cry3Ba toxin and several putative binding proteins were identified using mass spectrometry (Fig. 1*B*). The considered genuine Cry receptors APN and CAD-like proteins were found among them and also other proteins reported as Cry binding proteins, such as V-ATPase (19–21), with the highest score, and apolipoprotein LI-II precursor (31) (Fig. 1*B*). Additionally, a novel Cry binding protein, TcSSS, was identified with a high score (Fig. 1*B*).

A substantial amount of evidence suggests that toxin-binding APNs found in various insect larvae act as Bt receptors (12). It has been proposed that the main significance of toxin binding to these proteins might be to concentrate the prepore toxin structure at the cell membrane surface prior to membrane insertion (17). However, our RNAi results with TcAPN-I in Tc did not support a role for this protein in Cry3Ba toxicity because no change in mortality was observed in Tc silenced larvae treated with Cry3Ba toxin relative to nonsilenced larvae challenged with the toxin. Failure to demonstrate the receptor function of TcAPN-I made us consider the possibility that in Tc larvae lacking TcAPN-I gene expression, other APN proteins might functionally replace it. We then performed a multiple gene silencing experiment in which TcAPN-I and the closely related TcAPN-II and TcAPN-III genes were simultaneously knockdown. Following Cry3Ba intoxication in Tc triple silenced larvae, no mortality decrease was observed relative to control larvae, ruling out the involvement of these APN proteins in Cry3Ba toxin action in Tc. Although not statistically significant, Cry3Ba intoxicated triple silenced larvae showed a mortality increase relative to intoxicated control larvae, probably evidencing the detrimental effect of the multiple silencing. Because the effectiveness of TcAPN-II silencing (37.7%) was significantly lower than that of the other two Tc APNs (84.7% for TcAPN-I and 90.9% for TcAPN-III) (Fig. 2*D*), it is not possible to infer whether all three APN proteins equally contributed to this effect.

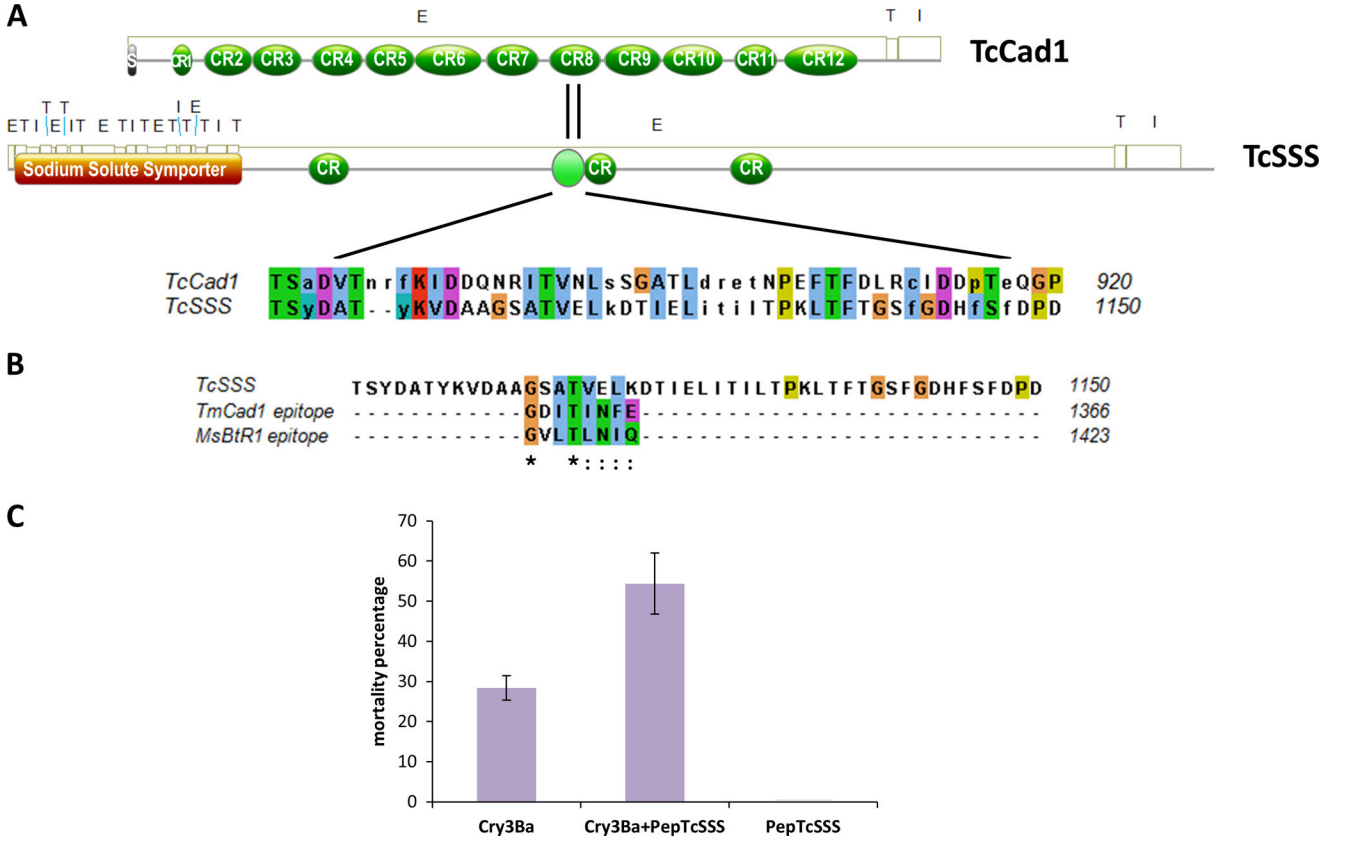


FIGURE 4. **TcSSS contains cadherin repeats and a putative binding epitope homologous to other Bt cadherin functional receptors, which enhances Cry3Ba toxicity in Tc larvae.** *A*, schematic representation of TcCad1 and TcSSS receptors secondary structure obtained with Motif Scan showing the Clustal alignment corresponding to an identified pattern in TcSSS and TcCad1 sequences using PRATT 2.1 (33). The extracellular (*E*), transmembrane (*T*), and intracellular (*I*) domains and numbered cadherin repeat regions (*CR1–CR12*) are illustrated. *B*, clustal alignment of the previously described cadherin Bt toxin binding epitopes in *M. sexta* MsBtR1 (^{1416}GVLTLNIQ^{1423}) and in *T. molitor* TmCad1 (^{1359}GDITINFE^{1366}) (7, 36) and residues 1103–1150 of the TcSSS sequence corresponding to the homology region in TcCad1 identified using PRATT. In this TcSSS fragment, a putative binding epitope (^{1115}GSATVELK^{1122}) was found. *C*, enhancement of Cry3Ba toxicity to Tc larvae by a 29-mer peptide (PepTcSSS) spanning amino acids 1110–1138, containing the identified putative binding epitope in TcSSS protein.

Functional studies have turned midgut CAD-like proteins into one of the most likely Cry toxin receptor molecules in lepidopteran, dipteran, and coleopteran larvae (7, 12, 38). It has been proposed that they play the role of the first receptor of Cry toxins, binding toxin monomer and facilitating further processing required for the prepore oligomer formation (17). In the beetle *T. molitor*, lower expression of the CAD-like gene *TmCad1* in larvae directly correlated with survival on Cry3Aa-treated diet, demonstrating the functional role of this protein as Cry3Aa receptor (7). In this report we have demonstrated that the TmCad1 ortholog CAD-like protein TcCad1, identified as a Cry3Ba binding protein in Tc larvae, also acts as a functional Cry3Ba receptor in this insect. Tc silenced larvae intoxicated with a Cry3Ba spore-crystal mixture concentration that corresponds approximately to LC_{50} in nonsilenced larvae showed a dramatic decrease in susceptibility because 95 ± 1% larvae survived. This result adds to those of others highlighting the critical role of CAD-like receptors in Bt mode of action.

In this work, we have demonstrated that the novel Cry3Ba binding protein TcSSS also functions as a toxin receptor in Tc larvae. As in the case of TcCad1, reduction of TcSSS expression by RNA interference resulted in increased larval survival on a Cry3Ba spore-crystal-treated diet (86 ± 4% Tc larvae survived when treated with a concentration that corresponds approximately to LC_{50}). Interestingly, the TcCad1 and TcSSS proteins that act as functional Cry3Ba receptors in Tc larvae exhibited the same gene expression profile during larval development (Fig. 2*A*) and have cadherin repeats (Fig. 4*A*), supporting that CAD-like proteins are relevant to Cry3Ba toxicity in Tc. Consistent with the role of TcSSS as a CAD-like Cry receptor, Cry3Ba toxicity was enhanced by a TcSSS peptide fragment containing a putative binding epitope found in other Bt cadherin functional receptors, such as in *M. sexta* and *T. molitor* CAD-like proteins.

These features, while suggesting a parallel role of TcCad1 and TcSSS mode of action, are in contrast with the Cry3Ba mortality data obtained in silenced larvae. If both proteins were fully redundant in their participation in Cry3Ba toxic action, the dramatic decrease in mortality observed after toxin challenge in larvae in which each of the corresponding genes were independently knocked down would not be expected (Fig. 3*B*). The results are more in accordance with a complementary function of both receptor molecules in a stepwise mechanism of action.

SSSs are Na^+-dependent transport proteins responsible for the absorption of nutrients, vitamins, osmolytes, and ions across the plasma membrane of pro- and eukaryotic cells (34). SSS proteins constitute a family belonging to the amino acid-

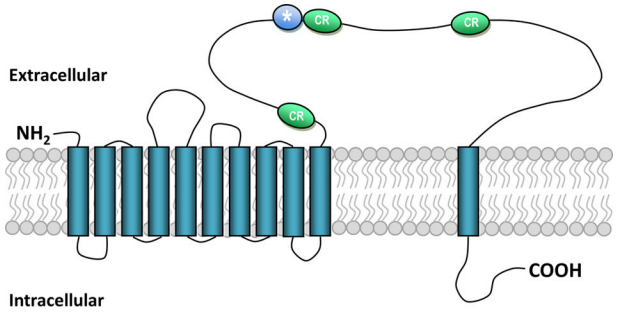


FIGURE 5. **Schematic structure of the *T. castaneum* TcSSS transporter.** Using TMHMM version 2.0, 11 transmembrane domains were predicted based on the amino acid sequence of *T. castaneum* EFA03129. In the schematic diagram, cadherin repeat regions (*CR*) and localization of the TcSSS fragment (*asterisk*) containing the region homologous to cadherin binding epitopes in *M. sexta* MsBtR1 and *T. molitor* TmCad1 (7, 36) are also illustrated.

polyamine-organocation superfamily Transporter Classification Database (TCDB) (39), which is composed of 11 families (40). Members of the SSS family generally share a core topology characterized by two inverted structural repeats of five transmembrane α-helices each containing the binding site for substrate and ion, and a periplasmic N terminus and a cytoplasmic C terminus.

Very recently, the functionality for ATP-binding cassette transporter subfamily C member 2 (ABCC2) as Cry1 toxin receptor has been reported in *Bombyx mori* larvae (5), in accord with the role of this protein in the mechanism of Cry1 toxin resistance (41, 42). Remarkably, *B. mori* ABCC2 protein (42) structurally resembles TcSSS protein in that both are multimembrane-spanning transporters (Fig. 5).

There are two main classes of active transmembrane transport systems: primary active transporters, such as ABC proteins, which rely on ATP hydrolysis to actively pump their substrates across membranes; and secondary active transporters, such as SSS, in which transport is driven by proton or sodium transmembrane gradients (43). The common element of primary and secondary transporters is that transporter-mediated movement of solutes across membranes involves the alternating access mechanism proposed by Jardetzky (44), in which transport occurs by binding of substrate to the "open-to-in" state followed by isomerization of the transporter to the "open-to-out" state, allowing the release of the substrate to the cytoplasm. In relation to Bt mode of action, it has been proposed that toxin oligomer insertion might be coupled to the transport cycle of the ABCC2 protein (45).

The biological role of the TcSSS protein is not known, but results of the RNAi experiments together with its functional similarity to ABCC2 transporter, suggest that this novel Cry3Ba binding protein could also be implicated in Cry3Ba toxin insertion. Alternatively, because of the essential role of ion gradients in active transport in almost any cell type, natural products and toxins that collapse the ion gradients across cellular membranes are poisons, and therefore the interaction of Cry3Ba toxin with the TcSSS protein might result in enhanced activity caused by transporter-related toxicity. That could also be the case of the V-ATPase subunit A isoform 2, identified in this work as a putative Cry3Ba binding protein (Fig. 1*B*), that couples the energy of ATP hydrolysis to proton transport across intracellular and plasma membranes of eukaryotic cells.

Our data suggest a complex mechanism underlying the toxicity process of Cry3Ba toxin in Tc; therefore, more research is needed to understand its mode of action. In contrast with the Bt mode of action described for lepidopteran insects, in which APN plays a relevant role in toxicity, in coleopteran insects APN protein has never been involved in Bt mode of action, and our results support the possibility that these proteins might not act as a Cry functional receptor in this insect order. On the other hand, TcCad1 and TcSSS, and probably other midgut proteins identified in this work, are determinant in coleopteran-specific Cry toxicity. The model coleopteran insect Tc represents an ideal experimental subject to obtain a complete picture of the complexity of Bt interactions and the molecular bases of insect toxin specificity.

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Artículo 4: Functional significance of membrane associated proteolysis in the toxicity of *Bacillus thuringiensis* Cry3Aa toxin against Colorado potato beetle

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Functional significance of membrane associated proteolysis in the toxicity of *Bacillus thuringiensis* Cry3Aa toxin against Colorado potato beetle

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ABSTRACT

Bacillus thuringiensis Cry toxins are widely used as biocontrol agents in bioinsecticides and transgenic plants. In the three domain-Cry toxins, domain II has been identified as an important determinant of their highly specific activity against insects. In this work, we assessed the role in membrane associated proteolysis and toxicity in Colorado potato beetle (CPB) of a previously reported ADAM recognition motif present in Cry3Aa toxin domain II. We used site-directed mutagenesis to modify the *Bacillus thuringiensis cry3A* gene in amino acid residues 344, 346, 347, 351 and 353 of the ADAM recognition motif in Cry3Aa toxin. Cry3Aa toxin mutants displayed decreased toxicity when compared to the wild type toxin and impaired ability to compete CPB brush border membrane associated cleavage of an ADAM fluorogenic substrate. Although the proteolytic profile of Cry3Aa toxin mutants generated by brush border membrane associated proteases was similar to that of Cry3Aa toxin, the metalloprotease inhibitor 1,10-phenanthroline was less efficient on the proteolysis of mutants than on that of the wild type toxin. The relevance of the Cry3Aa–ADAM interaction through the predicted recognition sequence was further confirmed by analyzing the effect of membrane integrity disturbance on Cry3Aa toxin membrane associated proteolysis and CPB larvae toxicity. Data support that Cry3Aa proteolysis, as a result of the interaction with ADAM through the Cry3Aa recognition motif, is essential for Cry3Aa toxic action in CPB. Detailed knowledge of Cry3Aa interaction with CPB midgut membrane should facilitate the development of more effective Bt based products against this devastating pest and other Coleoptera.

1. Introduction

Bacillus thuringiensis (Bt) is an invertebrate pathogen that exerts its virulence by means of large crystalline inclusions produced during sporulation, which contain pore-forming proteins known as crystal (Cry) toxins. As these toxins affect only a narrow range of target species, leaving vertebrates and most beneficial invertebrates unaffected, they are currently being used worldwide as biopesticides formulated in commercial sprays or introduced in transgenic plants (Sanahuja et al., 2011). The Cry protein family provides a rich diversity of toxin variants which share a high degree of structural conservation but have different spectra of activity (Crickmore et al., 2012). Bt toxin structures solved by X-ray crystallography have revealed a major group of Cry toxins characterized by a three domain structure organization (de Maagd et al., 2003). The conserved three-dimensional structures of these toxins have provided considerable insight into the mechanism of toxin function and have helped to explain differences in toxin specificity (Pigott and Ellar, 2007).

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Domain I is required for pore-formation, since it is the only one that contains alpha-helices long enough to span the plasma membrane bilayer, and domains II and III are implicated in receptor binding and insect specificity. Site directed mutagenesis in domain II hypervariable loops affected receptor binding affinity, recognition sites and toxicity (Dean et al., 1996; Smedley and Ellar, 1996) and domain III swapping between toxins targeting different insect species demonstrated the involvement of domain III in toxin specificity (de Maagd et al., 1996). Structural and biochemical data evidenced that receptor interaction through domains II and III facilitates a conformational change of domain I enabling the toxin to insert into the membrane triggering the toxicity process (Li et al., 2001).

Numerous physiological and biochemical studies with various toxins and target animals support that Cry toxins fundamental mode of action is a multistep mechanism which initiates with the ingestion of the toxin by susceptible larvae. Upon ingestion, the environment of the midgut promotes crystal solubilization and the released protoxin is cleaved by host proteases generating active toxin that subsequently binds to specific receptors on the midgut epithelium. Then toxin oligomerizes and inserts into the membrane forming pores, which eventually lead to death of the target insect (Bravo et al., 2007).

The activity or composition of gut proteases influences insects susceptibility to Cry toxins and may provide the basis for the specificity of Bt in different insect species and development of insect resistance (Loseva et al., 2002; Oppert, 1999; Oppert et al., 2011). Nevertheless, while there is reasonable understanding of the effect of soluble midgut digestive proteases in toxin activation, little is known about the role of membrane associated protease processing of Bt toxins in relation to toxin–receptor complex and pore formation events of the Bt mode of action.

In previous work we have reported that the coleopteran active Bt Cry3Aa toxin interacts with ADAM like membrane associated metalloproteases in the midgut of the Colorado potato beetle (CPB) through a region of the toxin domain II and it is proteolytically processed in regions of domain III (Ochoa-Campuzano et al., 2007; Rausell et al., 2007).

ADAMs (A Disintegrin And Metalloproteases) are modular transmembrane zinc-dependent metalloproteinases ubiquitously found from protozoans to mammals with key roles in many cellular processes such as proliferation, differentiation, migration and apoptosis through shedding of transmembrane substrates (Seals and Courtneidge, 2003). Given that most ADAMs can potentially cleave many substrates, a major issue is how specificity is achieved *in vivo*. Rather than unique cleavage site sequences in the substrate, it has been proposed that it is the accessibility and structure of the cleavage site, the distance from the membrane, and interactions distal to the cleavage site mediated by ADAM non-catalytic ancillary domains, either alone or in combination, that are important determinants for the recognition and processing by membrane-bound ADAMs (Takeda, 2009).

Structural studies have revealed that ADAMs extracellular metalloproteinase/disintegrin/cysteine-rich domain architecture has a unique C-shaped structure, which suggests that the molecular mechanism for ADAMs target recognition for shedding implies interplay between distal protein sites and the enzyme catalytic core (Takeda et al., 2006). A model has been proposed in which for catalytic ADAMs, when the cysteine domain is sterically incompatible with substrate access to the catalytic site, a protein recognition module of the substrate protein may bind ADAMs first, resulting in a conformational change of ADAMs so that the cysteine domain as part of the extracellular module, moves away from the catalytic site, allowing the peptide to be cleaved to enter the catalytic grove (Liu et al., 2009). Interfering with such conformational transitions may affect catalysis and might represent an alternative inhibition approach different from the traditional strategies targeting the catalytic site (Tape et al., 2011).

In this study we have generated mutant toxins in the ADAM recognition motif identified in domain II of Bt Cry3Aa toxin, tested the effect of the ADAM inhibitor 1,10-phenanthroline on their proteolytic processing and confirmed the relevance of this interaction motif by disturbing the integrity of the CPB midgut cell membrane. Results demonstrate that this motif is required for Cry3Aa membrane associated proteolytic processing and is functionally relevant for the insecticidal activity of this toxin.

Cry3Aa is the only Bt coleopteran active toxin that has been tested against a broad range of coleopteran species (23 species, 60% of which were susceptible) (van Frankenhuyzen, 2009). Detailed knowledge of Cry3Aa interaction with CPB midgut membrane should facilitate the development of more effective Bt based products against this devastating pest and other target Coleoptera.

2. Materials and methods

2.1. Insects

A laboratory colony of *Leptinotarsa decemlineata* (Colorado potato beetle) founded from eggs taken from the field was used. Larvae and adults were reared on potato leaves at 25 °C and with a photoperiod of 16:8 (light/dark).

2.2. Preparation of brush border membrane vesicles (BBMV)

BBMV were prepared from last instar *L. decemlineata* larvae according to the method of Wolfesberger et al. (1987), as modified by Reuveni and Dunn (1991). Larvae were dissected in storage buffer (300 mM mannitol, 20 mM 2-mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 10 mM HEPES, pH 7.5) and the insect midguts obtained were immediately frozen and stored at −80 °C until use. Frozen midguts were mechanically homogenized in homogenization buffer (200 mM mannitol, 10 mM ascorbic acid, 5 mM EDTA, 2 mM DTT, 10 mM HEPES, pH 7.4) for 10 s. One volume of 24 mM $MgCl_2$ was added and the mixture incubated for 10 min. Following centrifugation of the mixture (10 min, 6000 × *g* at 4 °C), the supernatant was further centrifuged (30 min, 30,000 × *g* at 4 °C) and the final pellet suspended in 200 mM mannitol, 1 mM DTT, 1 mM HEPES–Tris, pH 7.4, frozen and stored at −80 °C until use. The protein concentration of BBMV was measured by Bradford's procedure (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

2.3. Toxins purification

Cry3Aa, Cry3Ba and Cry3Ca crystals were produced in Bt strains BTS1, BTS00125L and BTS02109P, respectively. Crystal inclusions were separated from spores and cell debris by centrifugation in discontinuous 67%, 72%, 79%, and 84% (w/v) sucrose gradients in 50 mM Tris–HCl, pH 7.5, as described by Thomas and Ellar (1983). Crystal proteins were solubilized in 50 mM Na_2CO_3, pH 10.5, at 37 °C for 2 h. Purity of the crystal preparation was monitored by phase contrast microscopy and analyzed by 10% SDS-PAGE. Protein concentration was measured by the protein-dye method of Bradford (1976), using BSA as a standard.

2.4. Cry3 toxins cleavage assays

Proteolysis assays on wild type Cry3 toxins or Cry3Aa mutant toxins were performed as described before (Rausell et al., 2007). Toxins (2 μM) were incubated with 20 μg BBMV in a final volume of 30 μL PBS (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.4), for 10 min at room temperature, and centrifuged 20 min at 12,000 × *g*. The supernatants were loaded in 10% SDS-PAGE gels and the resolved proteins were transferred onto a nitrocellulose membrane (Millipore) and immunoblotted against anti-Cry3Aa polyclonal antibody. The secondary antibody was alkaline phosphatase-conjugated anti-rabbit antibody (Sigma). The immunoreactive proteins were visualized using the ECL detection system Immobilon Western (Millipore).

In toxin cleavage inhibition assays, CPB BBMV were incubated with 1,10-phenanthroline (final concentration 10 mM) or methyl-β-cyclodextrin (MCD) (2.5, 5 and 10 mM, final concentration) for 30 min at room temperature prior to toxin addition.

2.5. Midgut epithelium cells (MECs) isolation and confocal microscopy analysis

Midguts isolated from 20 s instar larvae of *L. decemlineata* were placed on a Petri dish containing 4 mL PBS buffer, pH 7.4, and stirred for 1 h at 25 °C. Dissociated cells were recovered by centrifugation at 470 × *g* for 3 min and the pellet was resuspended in 350 μL PBS buffer, pH 7.4.

To visualize the cell distribution pattern on CPB MECs of a peptide representative of the ADAM10 substrate recognition motif previously identified in Cry3Aa toxin domain II (Ochoa-Campuzano et al., 2007), referred as pep-rec peptide (Ac-FHTRFQPGYYGNDSFN-NH2), confocal microscopy analysis was performed as follows. Freshly dissociated MECs (approximately 5×10^6 in 10 μL) were incubated for 30 min with 0.84 μL of 30 mM MCD or PBS, followed by 15 min incubation with 0.25 μL of 10.25 mM FITC-labeled pep-rec peptide diluted 1:10 (v:v) in DMSO:PBS (final volume 11.09 μL). For microscope analysis, 12 μL of VECTASHIELD Hard Set mounting medium (Vector Laboratories) was added to treated CPB MECs (approximately 2.5×10^6 in 5 μL) and stained samples were mounted on glass slides.

Fluorescence analysis was performed on a Leica TCS SP2 laser confocal microscope using a 40× oil immersion objective. Images were captured and processed using an HP workstation with Leica Microsystems TCS SP2 software.

2.6. Synthetic fluorogenic peptide substrate cleavage assays

ADAM proteolytic activity was monitored using a fluorescent cleavage assay with the synthetic peptide Mca-PLAQAV-Dpa-RSSSR-NH2 (BIOMOL International LP), an ADAMs substrate. Mca ([7-methoxycoumarin-4-yl]acetyl) fluorescence is quenched by the Dpa (3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl) group until cleavage separates them.

Fluorescent assays were carried out with the supernatant obtained after centrifugation of 400 μL BBMV (10 μg/μL) at 20,000 × *g* for 15 min at 4 °C. Incubations were performed in 1 mL of PBS buffer, pH 11.0, with 20 μL of BBMV supernatant and subsequent addition of 1 μL of substrate solution (10 mM) at 25 °C into the assay cuvette. Wild type Cry3Aa or Cry3Aa toxin mutants were used as competitors in the fluorogenic substrate cleavage assay with BBMV supernatant at a final toxin concentration of 0.27 μM and 10 μM of fluorogenic substrate. Hydrolysis rates were monitored for 30 min at λ excitation 328 nm and λ emission 393 nm using a VARIAN Cary Eclipse Fluorescence Spectrophotometer equipped with a constant-temperature water bath.

2.7. Insect toxicity assay

Toxicity assays were performed on newly molted third instar larvae starved for 2 h prior to treatment. Sixteen larvae were exposed to 3.5 cm^2 potato leaf disks with 300 ng of Cry3Aa wild type toxin or Cry3Aa mutant toxins and upon ingestion of the entire leaf disk larvae were fed fresh potato leaves and reared under standard laboratory conditions. Mortality was recorded 7 days after the initial treatment. Assays were performed in triplicate and in each case a control with larvae fed on leaf disks without toxin was included.

Toxicity assays in the presence of MCD were carried out exposing larvae to a mixture of 0.3 μL 1 μg/ml Cry3Aa toxin with 0.7 μL 30 mM MCD deposited in 3.5 cm^2 potato leaf disks.

2.8. Site-directed mutagenesis

Plasmid phT305 (Lereclus et al., 1989) including *cry3Aa* gene sequence was used as a template for site-directed mutagenesis. Six pairs of complementary mutagenic oligonucleotides primers were designed (Table 1) and synthesized (Integrated DNA Technologies, Leuven, Belgium).

Mutations were generated following the QuikChange® Site-Directed Mutagenesis (Stratagene) procedure and confirmed by sequencing.

Plasmids with each construct were used to transform the acrystalliferous Bt strain 4D7 (*Bacillus* Genetic Stock Center). Electroporation was carried out in a Bio-Rad GenePulse II with settings of 2.5 kV, 25 μF, and ∞ Ω. Cells were then mixed with 1.6 mL of brain heart infusion medium plus 0.5% glycerol and incubated at 30 °C

Table 1
Oligonucleotides used in site-directed mutagenesis.

Amino acid substitution	Primer	Amino acid sequence
R345 to A345	5′CATAGAATTCAATTTCACACG**GCG**TTCCAACCAGGATAT3′	HRIQFHTAFQPGY
Y351 to F351	5′CAGGATATTTTGGAACTGACTCT**TTT**AATTATTGGTCCGG3′	GYFGTDSFNYWS
T344 to S344	5′TATCTGCATAGAATTCAATTTCAC**TCG**CGG**CTC**CAACCAGGA3′	YLHRIQFHSRLQPG
F346 to L346		
T344 to S344	5′TATCTGCATAGAATTCAATTTCAC**TCGGCGCTC**CAACCAGGA3′	YLHRIQFHSALQPG
F346 to L346		
R345 to A345		
Q347 to R347	5′ATTCAATTTCACACGCGGTTC**CGA**CCAGGATATTATGGAAATG3′	IQFHTRFRPGYYGN
N353 to T353	5′CAGGATATTATGGA**ACT**GACTCTTTTAATTATTGGTCCGG3′	GYYGTDSFNYWS

Bold letters indicate mutated nucleotide residues and the corresponding amino acid changes are underlined.

for 1–3 h, and 50–100 μL were plated on LB plates with 25 μg/mL erythromycin. Crystals containing toxins were recovered using sucrose density gradients as described in Section 2.3.

3. Results and discussion

3.1. CPB membrane associated proteolysis of Bt Cry3 toxins

In a previous work we have reported the membrane associated metalloprotease proteolytic processing of Bt Cry3Aa toxin upon incubation with CPB BBMV and we have proposed that interaction with a CPB ADAM-like metalloprotease is essential in Cry3Aa mode of action (Ochoa-Campuzano et al., 2007; Rausell et al., 2007). To gain more insight into the role of membrane associated proteolysis in relation to Cry3 toxins mode of action, we extended the analysis to the Cry3Aa phylogenetically related toxins, Cry3Ba and Cry3Ca. Proteolysis assays on Cry3 toxins were performed as described before (Rausell et al., 2007). Fig. 1 shows distinct proteolytic patterns for each toxin, suggesting variations of the specific Cry3Aa determinants of substrate recognition or cleavage sites in Cry3Ba and Cry3Ca.

Studies on toxin determinants of binding to receptors are key to understand its molecular mechanism of action. We previously identified in Cry3Aa domain II sequence a recognition motif F342HTRFQPG349, which is also present in other ADAM10 substrates outside the cleavage site (Ochoa-Campuzano et al., 2007; Hattori et al., 2000). A peptide representative of this motif in Cry3Aa toxin sequence, referred as pep-rec (Ac-FHTRFQPGYYGNDSFN-NH2), prevented Cry3Aa proteolytic processing and nearly abolished pore formation, evidencing the functional significance of the Cry3Aa–ADAM interaction in relation to this toxin mode of action (Ochoa-Campuzano et al., 2007).

Here we first examined the binding of FITC-labeled pep-rec peptide to dissociated CPB MECs. Pep-rec peptide fluorescence preferentially localized in the apical surfaces of columnar cells (Fig. 2A) and additionally, in cells that were not completely dissociated, pep-rec peptide accumulated in basolateral contact regions between cells (Fig. 2B). Controls were performed in the absence of fluorescently labeled pep-rec peptide, which showed no fluorescence signal.

Consistent with the cellular detection of pep-rec in important potential sites for the action of Bt toxins, ADAM10 has also been reported to localize at the apical and basolateral plasma membrane of polarized epithelial cells, primarily at cell–cell contacts (Wild-Bode et al., 2006), consistent with the pep-rec sequence representing a major receptor recognition motif in Cry3Aa.

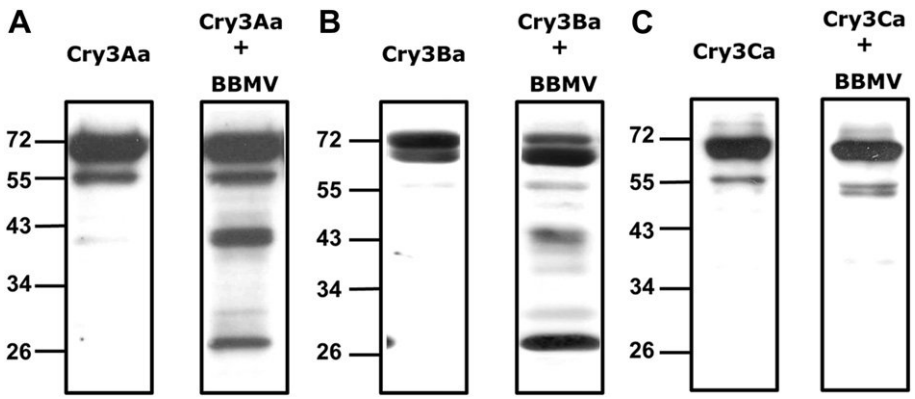

Fig. 1. Polypeptide profile of Cry3 toxins cleaved by CPB BBMV proteases. Each panel shows western blots of Cry3 toxins (lane 1) and supernatants obtained after incubation of Cry3 toxins with CPB BBMV (lane 2). A) Cry3Aa toxin. B) Cry3Ba toxin. C) Cry3Ca toxin. Molecular weight markers are shown on the left.

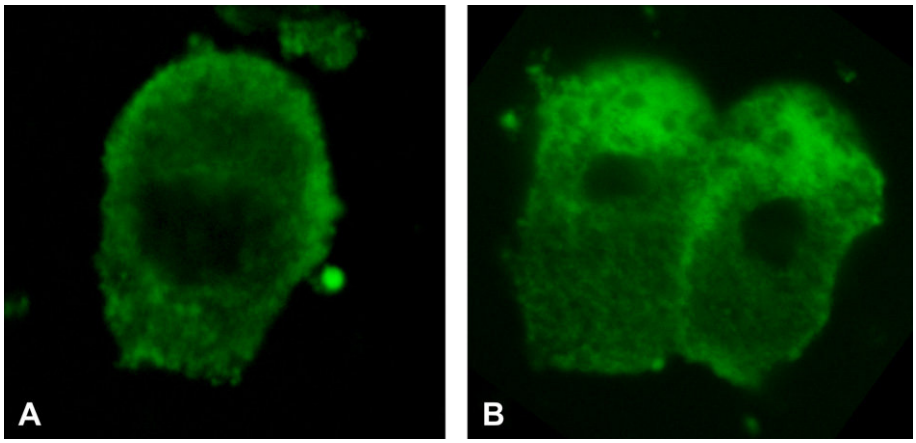

Fig. 2. Confocal laser microscopy analysis of CPB MECs completely (A) or partially (B) dissociated incubated with 180 μM FITC-labeled pep-rec peptide. Apical cell edge is oriented to the top and basal to the bottom, in all images.

3.2. Loop I Cry3Aa domain II mutant toxins

We next compared the recognition motif identified in Cry3Aa toxin domain II with the corresponding sequences of Cry3Ba and Cry3Ca toxins (Fig. 3A) and we detected amino acid changes that might account for differences in the specific interaction between protease and substrate leading to the variation observed in the toxins proteolytic pattern. Accordingly, we performed site directed mutagenesis on specific residues of the recognition sequence in Cry3Aa toxin to incorporate substitutions in amino acids found in Cry3Ba and Cry3Ca corresponding region that are

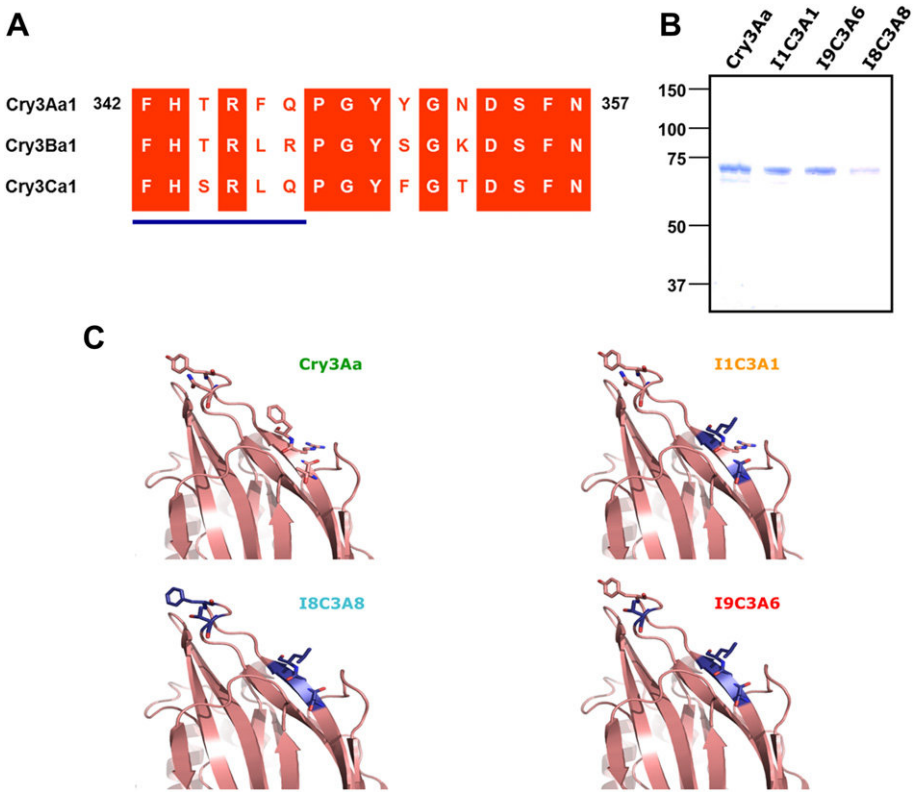

Fig. 3. A) Amino acid sequence alignment of residues 342 to 357 in Bt Cry3Aa toxin domain II with the corresponding sequences in Cry3Ba and Cry3Ca toxins using Clustal X. Underline indicates the region corresponding to the ADAM recognition motif predicted in Cry3Aa toxin sequence. B) Coomassie blue-stained SDS-10% PAGE of Cry3Aa wild type toxin and Cry3Aa mutant toxins I1C3A1, I8C3A8 and I9C3A6 expressed in Bt 4Q7 cells. Molecular weight markers are shown on the left. C) Mutation sites on Cry3Aa model structure. The 3-D models were prepared using PyMOL (Delano Scientific Inc., CA).

Table 2
Cry3Aa toxin mutants in domain II recognition motif and their effect on expression and toxicity against CPB larvae.

Name	Amino acid changes	Amino acid sequence	Expression	Mortality (%)[a]
Cry3Aa	None	YLHRIQFHTRFQPGYYGNDSFNYW	Yes	57 ± 6
I03CA0	R345A	YLHRIQFHTAFQPGYYGNDSFNYW	No	–
I1C3A1	T344S, F346L	YLHRIQFHSRLQPGYYGNDSFNYW	Yes	50 ± 0
I2C3A2	T344S, R345A, F346L	YLHRIQFHSALQPGYYGNDSFNYW	No	–
I3C3A3	Y351F, N353T	YLHRIQFHTRFQPGYFGTDSFNYW	No	–
I4C3A4	Y351F, R345A, N353T	YLHRIQFHTAFQPGYFGTDSFNYW	No	–
I5C3A5	T344S, F346L, N353T	YLHRIQFHSRLQPGYYGTDSFNYW	No	–
I9C3A6	T344S, R345A, F346L, N353T	YLHRIQFHSALQPGYYGTDSFNYW	Yes	22 ± 3
I7C3A7	T344S, F346L, Y351F, N353T	YLHRIQFHSRLQPGYFGTDSFNYW	No	–
I8C3A8	T344S, R345A, F346L, Y351F, N353T	YLHRIQFHSALQPGYFGTDSFNYW	Yes[b]	ND[c]
I6C3A9	Q347R	YLHRIQFHTRFRPGYYGNDSFNYW	No	–

[a] Mortality was estimated using a toxin dose of 300 ng.
[b] Degraded soon after solubilization in 50 mM sodium carbonate buffer, pH 10.5.
[c] ND, not determined.

not present in Cry3Aa toxin. Domain II is the most variable of the Bt Cry toxins domains, especially in the apex loops, which differ in length, conformation and sequence. Therefore, domain II is believed to be an important determinant of toxin specificity and extensive mutagenesis studies have provided evidence for this hypothesis (Pigott and Ellar, 2007). In CPB, it has been described that specific changes in loop 1 of the receptor binding domain II either affected dissociation and enhanced toxicity (R345A, Y350F, Y351F and R345A, ΔY 350, ΔY 351) (Wu et al., 2000) or caused the loss of binding affinity and toxicity (N353A, D354A) (Wu and Dean, 1996).

Mutations in the region containing the predicted ADAM recognition motif in Cry3Aa toxin were made and the effect on membrane associated proteolysis and toxicity was analyzed. Residues T344, F346 and Q347 in Cry3Aa toxin domain II were mutated to the corresponding residues in Cry3Ca and/or Cry3Ba toxin sequences, S344, L346 and R347, respectively. Moreover, residue Y351 as well as N353 in loop I of Cry3Aa were also changed to the corresponding residues in Cry3Ca toxin sequence, F351, and T353, respectively, because mutations in these amino acids of Cry3Aa toxin (Y351F, N353A) had been shown critical for toxin binding and insecticidal activity against CPB in previous work by Dr. Dean's group (Wu and Dean, 1996; Wu et al., 2000). To suppress unstable mutants, a nearby mutation R345A was made as reported by Wu and Dean (1996).

A total of 9 mutant toxins were produced, expressed in Bt (Table 2) and analyzed by SDS-PAGE (Fig. 3B). Although residues chosen for substitution were not expected to have significant effect on protein folding, 6 mutants did not produce structurally stable proteins. As shown in Fig. 3C, the residue substitutions within domain II region in the 3 Cry3Aa toxin mutants that expressed proteins were predicted not to alter the toxin main-chain conformation. Toxin mutants I1C3A1 and I9C3A6 were expressed at a level comparable to that of wild type Cry3Aa, and mutant I8C3A8 was readily degraded. Residue Y351 that had been previously identified to be relevant for Cry3Aa toxin binding to CPB (Wu et al., 2000) seemed to be also essential for the toxin stability since toxin mutants I8C3A8 and I9C3A6 differed only in residue 351, phenylalanine in toxin mutant I8C3A8 and tyrosine in toxin mutant I9C3A6, the latter as in the wild type Cry3Aa toxin sequence.

Mutational effects on Cry3Aa toxicity against CPB larvae were analyzed showing that mutant I1C3A1 produced slightly less mortality than Cry3Aa toxin, whereas mutant I9C3A6 reduced Cry3Aa mortality 2.6-fold (Table 2).

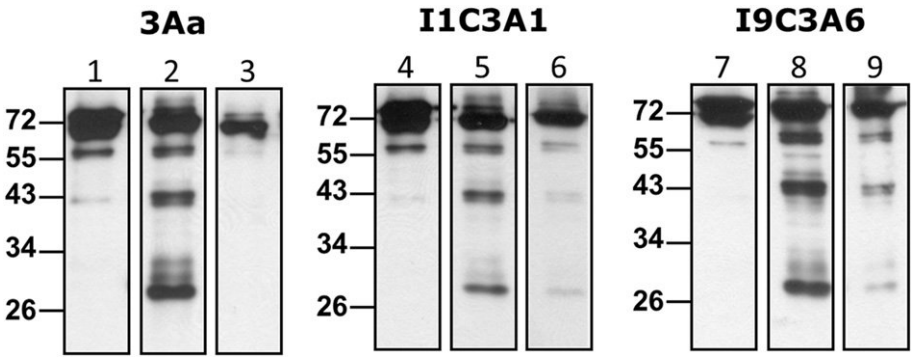

Fig. 4. Inhibition effect of 1,10-phenantroline on the proteolytic cleavage of Cry3Aa wild type toxin and Cry3Aa mutant toxins I1C3A1 and I9C3A6 catalyzed by CPB BBMV. Each panel shows western blot analysis. Lanes 1, 4 and 7 correspond to Cry3Aa wild type and mutant toxins; lanes 2, 5 and 8 correspond to supernatant obtained after incubation of toxins with CPB BBMV; lanes 3, 6 and 9 correspond to supernatant obtained after incubation of toxins with CPB BBMV in the presence of 1,10-phenantroline. Molecular weight markers are shown on the left.

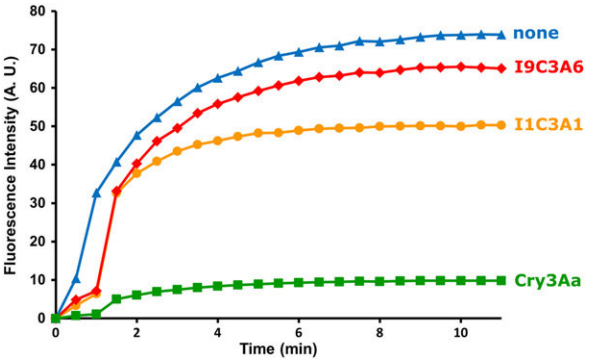

Fig. 5. ADAM10 Synthetic fluorogenic peptide substrate cleavage assays. Time course of fluorescence released by CPB BBMV supernatant in the presence and absence of 26.5 μM Cry3Aa wild type and mutant toxins I1C3A1 and I9C3A6 upon peptide cleavage. Each experiment was performed in triplicate and one representative curve is shown. Competitors of peptide cleavage present in each experiment are indicated on the graph.

We then used I1C3A1 and I9C3A6 domain II mutants to assess the proteolytical processing by CPB BBMV. Fig. 4 shows that the proteolytic profile of Cry3Aa toxin mutants was similar to that of Cry3Aa toxin. However, a differential effect of the ADAM inhibitor 1,10-phenanthroline on the proteolysis of Cry3Aa mutant toxins was observed at an inhibitor concentration of 10 mM, previously reported to significantly inhibit Cry3Aa toxin proteolysis by CPB BBMV (Rausell et al., 2007). As shown in Fig. 4, 1,10-phenanthroline cleavage inhibition decreased as the number of mutations introduced in the recognition region increased, suggesting that the altered interaction with CPB ADAM metalloproteases through the mutated motif in turn diminished the effectiveness of the inhibitor to chelate the zinc ion required for catalytic activity in the active site. In the case of ADAM17 (TNF-α converting enzyme, TACE) it has been reported that interaction of the substrate with distal protein side chains induce discrete charge transitions at the catalytic zinc–protein complex that are required for substrate catalysis (Solomon et al., 2007).

The hypothesis that interaction of Cry3Aa toxin mutants in the recognition region affects metalloprotease substrate specificity was tested by analyzing the ability of Cry3Aa mutants to compete CPB BBMV cleavage of an ADAM fluorogenic substrate. Fig. 5 shows that I9C3A6 mutant competed less efficiently the cleavage of the ADAM fluorogenic substrate than I1C3A1 mutant, both toxin mutants showing significantly less competition capacity than the wild type Cry3Aa toxin, which almost abolished substrate cleavage. These results correlate with the progressive toxicity reduction from the I1C3A1 double mutant to the I9C3A6 quadruple mutant relative to Cry3Aa toxicity (Table 1), supporting our hypothesis and evidencing that the specificity of the Cry3Aa–ADAM interaction through the predicted recognition sequence is essential for toxicity.

3.3. Functional significance of Cry3Aa toxin proteolysis

Finally, we have obtained further evidence of the relevance of the Cry3Aa–ADAM interaction on Cry3Aa insecticidal activity by analyzing the effect of membrane integrity disturbance on Cry3Aa toxin membrane associated proteolysis and CPB larvae toxicity. We tested the effect of the cholesterol extracting compound MCD on CPB BBMV Cry3Aa proteolysis (Fig. 6). MCD action was dose-dependent with Cry3Aa toxin cleavage completely inhibited in the presence of 5 mM MCD. We selected MCD to disturb membrane integrity because coleopteran microvilli cholesterol content is 5–6.5-fold higher than in Lepidoptera (Terra et al., 2006), in which integrity of cholesterol enriched domains has shown to be essential for efficient binding of Cry1A lepidopteran specific toxins (Zhuang et al., 2002). To confirm the effect of MCD on CPB midgut membrane integrity, we incubated MCD treated dissociated CPB MECs with FITC-labeled pep-rec peptide. Results showed that MCD treatment led to a distortion of pep-rec peptide fluorescence distribution in the cell (Fig. 6B) when comparing with pep-rec peptide fluorescence localization in non-treated cells (Fig. 2A), indicating that membrane integrity was being altered. Regulation of ADAM activity may occur at different levels and among them localization within the plasma membrane and availability of the ADAM substrates might be key determining features of how ADAM specificity is achieved *in vivo* (Huovila et al., 2005). Some ADAM-mediated shedding events have been associated with a dependency of a common localization of ADAM and substrates within lipid rafts and removing cellular cholesterol and disruption of these local cholesterol-enriched lateral assemblies in the plasma membrane altered shedding of various ADAM substrates (Reiss et al., 2006).

To investigate whether cholesterol depletion affects Cry3Aa toxicity on CPB, we intoxicated CPB larvae with a mixture of Cry3Aa toxin with MCD (Table 3). Results show that when cell membrane integrity was disturbed by MCD treatment, Cry3Aa proteolysis was impaired and toxicity significantly reduced.

This collective data strongly suggests that Cry3Aa toxin proteolysis as a result of the interaction with ADAM through the Cry3Aa recognition motif is essential for Cry3Aa toxin action in CPB.

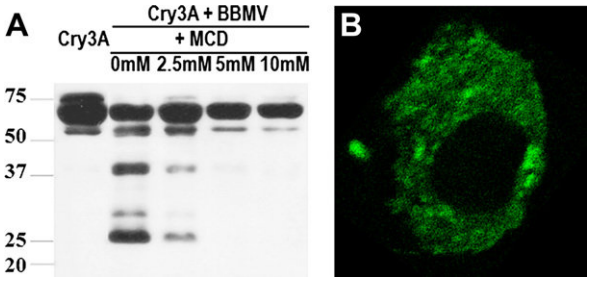

Fig. 6. Effect of MCD treatment on Cry3Aa proteolysis by CPB BBMV. A) Western blot of the supernatants obtained after incubation of Cry3Aa toxin with CPB BBMV in the absence or presence of increasing concentrations of MCD. Molecular weight markers are shown on the left. B) Confocal laser microscopy analysis of CPB dissociated MECs incubated with 180 μM FITC-labeled pep-rec peptide following MCD cells treatment. Apical cell edge is oriented to the top and basal to the bottom. Conditions and concentrations are described in Material and methods.

Table 3
Effect of CPB larvae treatment with 2.8 μg MCD on the mortality caused by 300 ng Cry3Aa toxin. Assays were performed in triplicate and the corresponding mocks are included.

CPB larvae treatment	Mortality
Cry3Aa toxin (300 ng)	65 ± 1
Mock (Na_2CO_3 50 mM)	0
MCD (2.8 μg) + Cry3Aa (300 ng)	26 ± 4
Mock (2.8μg + 50 mM Na_2CO_3)	0

CPB is well known by its impressive ability to develop insecticide resistance (Alyokhin, 2009). Studies carried out by Loseva et al. (2002) demonstrated that CPB resistance to Cry3Aa toxin correlated with reduced binding of Cry3Aa toxin to insect midgut epithelial cells along with changes in the composition as well as activity of proteases present in the insect midgut juice and BBMV. These authors proposed that these features reflect adaptive responses that render the insect refractory to toxin action. Therefore, knowledge on how Cry3Aa toxin membrane associated proteolysis modulates toxicity in CPB might set up the basis for an effective management of resistance, which remains a challenging issue regarding this destructive pest. Our data indicate that subtle differences in domain II Cry3Aa toxin structure might have a significant effect on toxin proteolysis and toxicity, so we foresee engineered Cry3Aa toxins might represent a sound strategy to counteract resistance.

In conclusion, results demonstrate that Cry3Aa toxin domain II contains a recognition sequence required for Cry3Aa membrane associated proteolytic processing, which is functionally relevant for the insecticidal activity of this toxin against CPB.

Ethical statement

The submitted manuscript entitled "Functional significance of membrane associated proteolysis in the toxicity of *B. thuringiensis* Cry3Aa toxin against Colorado potato beetle larvae" has not been published before and it is not under consideration for publication elsewhere. Its publication has been approved by all authors and tacitly by the responsible authorities in the laboratory where the work was carried out and, if accepted, it will not be published elsewhere in the same form, in either the same or another language, without the consent of the editors and publisher.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Artículo 5: Prohibitin, an essential protein for Colorado potato beetle larval viability, is relevant to *Bacillus thuringiensis* Cry3Aa toxicity

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Prohibitin, an essential protein for Colorado potato beetle larval viability, is relevant to *Bacillus thuringiensis* Cry3Aa toxicity

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ABSTRACT

Bacillus thuringienesis (Bt) Cry toxins constitute the most extensively used environmentally safe biopesticide and their mode of action relies on the interaction of the toxins with membrane proteins in the midgut of susceptible insects that mediate toxicity and insect specificity. Therefore, identification of Bt Cry toxin interacting proteins in the midgut of target insects and understanding their role in toxicity is of great interest to exploit their insecticidal action. Using ligand blot, we demonstrated that Bt Cry3Aa toxin bound to a 30 kDa protein in Colorado potato beetle (CPB) larval midgut membrane, identified by sequence homology as prohibitin-1 protein. Prohibitins comprise a highly conserved family of proteins implicated in important cellular processes. We obtained the complete CPB prohibitin-1 DNA coding sequence of 828 pb, *in silico* translated into a 276-amino acid protein. The analysis at the amino acid level showed that the protein contains a prohibitin-homology domain (Band7_prohibitin, cd03401) conserved among prohibitin proteins. A striking feature of the CPB identified prohibitin-1 is the predicted presence of cadherin elements, potential binding sites for Cry toxins described in other Bt susceptible insects. We also showed that CPB prohibitin-1 protein partitioned into both, detergent soluble and insoluble membrane fractions, as well as a prohibitin-2 homologous protein, previously reported to form functional complexes with prohibitin-1 in other organisms. Prohibitin complexes act as membrane scaffolds ensuring the recruitment of membrane proteases to facilitate substrate processing. Accordingly, sequestration of prohibitin-1 by an anti-prohibitin-1 antibody impaired the Cry3Aa toxin inhibition of the proteolytic cleavage of a fluorogenic synthetic substrate of an ADAM-like metalloprotease previously reported to proteolize this toxin. In this work, we also demonstrated that prohibitin-1 RNAi silencing in CPB larvae produced deleterious effects and together with a LD50 Cry3Aa toxin treatment resulted in a highly efficient short term response since 100% larval mortality was achieved just 5 days after toxin challenge. Therefore, the combination of prohibitin RNAi and Cry toxin reveals as an effective strategy to improve crop protection.

1. Introduction

Microbial insecticides constitute an alternative to chemical pesticides, offering the possibility to avoid the environmental and health drawbacks associated with the utilization of chemical compounds for pest control. *Bacillus thuringiensis* (Bt) is currently the most widely used environmentally compatible biopesticide, representing around 2% of the worldwide insecticide market [1]. It is safe for humans and although it has been traditionally employed as sprayable insecticide, Bt genes have also been incorporated in transgenic plants to confer inherent insect resistance [2].

Bt mechanism of toxicity relies on insecticidal crystal inclusions produced during sporulation that may contain one or several proteins of the Cry (for Crystal) or Cyt (for Cytotoxic) type. Cry toxins are classified based on primary sequence similarity into 72 types and many sub-types [3], and are specifically active only against a limited number of susceptible insects, including lepidopterans, coleopterans, dipterans, or against nematodes. A major group of Cry-toxins is the three domain 3D-Cry family, members of which share similarities in sequence and structure [4]. Upon ingestion by susceptible insects, 3D-Cry toxins develop the entomopathogenic action through a number of sequential events, including crystal solubilization and the subsequent release of the protoxin, activation to toxin by midgut proteases, interaction of the toxin with midgut epithelium, binding to midgut specific receptors, toxin oligomerization

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and finally formation of pores in the epithelial cells of the midgut, which eventually results in cell death [4].

Receptor binding has been recognized as an important step mediating toxicity and determining insect specificity [5]. The best characterized Bt toxin receptors are from Lepidoptera, where it has been described that Cry1 toxins bind to cadherin-like and aminopeptidase-N molecules [6,7], although other candidates, such as alkaline phosphatase and glycolipids, have been reported in several insect orders and nematodes [8,9]. For coleopteran specific toxins, a cadherin-like receptor has been identified in *Tenebrio molitor* [10], as it was earlier proposed in *Diabrotica virgifera* (Western Corn Rootworm, WCRW) [11], and very recently, an E-cadherin and a sodium solute symporter have been demonstrated as functional receptors in *Tribolium castaneum* (Tc) [12]. In *Leptinotarsa decemlineata* (Colorado Potato Beetle, CPB) an ADAM metalloprotease has been involved in Cry3Aa mechanism of action [13].

In addition, through high throughput proteomic approaches, other proteins have been identified in the midgut membrane of several insects that are capable of binding to Bt toxins. Actin and ABC transporter have been identified as Cry1Ac binding proteins in *Manduca sexta* [14]. Cry1Ac was also reported to bind actin, V-ATP-synthase subunit A and desmocollein in *Heliothis virescens* [15] and in *Helicoverpa armigera* this toxin bound to actin, V-ATP-synthase subunit B, heat shock cognate protein and ABC transporter [16]. In midgut membranes of *A. aegypti*, actin, V-ATP-synthase subunit B, flotillin and prohibitin were proteins identified to bind to Cry4Ba toxin [17]. The role of these proteins as Bt toxins receptors has not been demonstrated yet, but interestingly, some of these proteins such as V-ATP-synthases have been reported to show altered expression in Cry1Ac-resistant *Plodia interpunctella* [18] and in Cry4Aa, Cry4Ba and Cry11Aa-resistant *Aedes aegypti* LiTOX strain [19]. Likewise, an ABC transporter mutation was correlated with insect resistance to Cry1Ac toxin in *H. virescens* [20] and evidence that it is a functional receptor in *Bombyx mori* has been provided [21]. Moreover, in comparative proteomic analyses, actin, HSP90 and ATP synthase subunit B have been reported to differentially express after *H. armigera* intoxication with Cry1Ac [22] and in *A. aegypti* larvae treated with Cry11Aa toxin [23].

The way these proteins identified to bind Bt Cry toxins influence toxin action might be relevant to understand Bt Cry toxins mode of action. Very recently, Cancino-Rodezno et al. [23] demonstrated that when the expression of ATP synthase subunit B and actin was knockdown by RNAi silencing *A. aegypti* larvae became hypersensitive to toxin action, whereas mosquito larvae displayed a resistant phenotype when the heat shock protein was silenced, providing evidence of a functional role of these proteins in the insect response to Cry toxin action.

The multiple midgut membrane proteins reported to bind Cry toxins in different insects through proteomic analyses suggest Bt toxins mode of action might be more complex than originally envisioned, with potential toxin binding partners that can assist the toxin in its path to membrane insertion or with novel roles other than direct participation in promoting membrane insertion [24]. Therefore, identification of Bt toxins interacting proteins in the midgut of target insects and understanding of their role in toxicity might provide tools for enhancing Bt toxins action against major crop pests and delay resistance development.

In this work, searching for Bt Cry3Aa toxin binding proteins in CPB we identified prohibitin (PHB) protein and demonstrated that it is an essential protein for CPB larvae viability. PHB silencing together with Cry3Aa toxin treatment enhanced CPB larval mortality while reducing the timing of the effects and therefore might represent a novel strategy to efficiently control this devastating insect pest.

2. Materials and methods

2.1. Cry3Aa toxin purification

Cry3Aa crystals were produced in Bt strain BTS1. Crystal inclusions were separated from spores and cell debris by centrifugation on discontinuous 67%, 72%, 79%, and 84% (w/v) sucrose gradients in 50 mM Tris–HCl, pH 7.5, as described by Thomas and Ellar [25]. Crystal proteins were solubilized in 50 mM Na_2CO_3, pH 10.5, for 12 h at 37 °C. Purity of the crystal preparation was monitored by phase contrast microscopy and analyzed by SDS-10%PAGE. Protein concentration was measured by the protein-dye method of Bradford [26], using BSA as a standard.

2.2. Isolation of lipid rafts

Midguts isolated from CPB second instar larvae were placed on a Petri dish containing 4 mL PBS buffer (10 mM Phosphate buffer, 150 mM NaCl), pH 7.4, and stirred for 1 h at 25 °C. Dissociated midgut epithelial cells (MECs) were recovered by centrifugation at 470*g* for 3 min and the pellet was resuspended in 350 μL PBS buffer, pH 7.4.

Triton X-100 insoluble membrane fractions from CPB MECs were isolated following a modification of the protocols by Riedle et al. [27] and, Krauss and Altevogt [28]. Dissociated MECs (approximately 1.5×10^7) were incubated in 100 mM Tris/HCl, pH 7.5, containing 1% Triton-X100, 150 mM NaCl, 0.2 mM EGTA, 1 mM PMSF and 1 tablet of Complete Mini (Roche) protease inhibitor cocktail, at 4 °C. The mixture was incubated for 30 min on ice, homogenized, transferred to an Ultra-Clear centrifuge tube (Beckman) and mixed with an equal volume of sucrose solution (85%, w/v, in TNE buffer containing 100 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2 mM EGTA). The mixture was overlaid with 3.5 mL of 35% sucrose in TNE buffer, followed by 1 mL of 5% sucrose in TNE buffer. A step gradient was generated by centrifugation for 18 h at 200,000*g* in a Beckman SW55Ti rotor, at 4 °C, and fractions (0.2 mL each) were collected from the top of the gradient. Proteins of fractions were separated by SDS-10% PAGE and analyzed by Western blotting.

Purity of lipid rafts fractions was confirmed by immunodetection of fasciclin GPI-anchored and fasciclin transmembrane protein markers. Membranes were blocked with powdered milk 3% (w/v) in TBS buffer (100 mM Tris/HCl, pH 7.5, 150 mM NaCl), for 1 h at room temperature. The blocked membranes were overnight incubated at 4 °C with polyclonal antibodies anti *M. sexta* fasciclin II-GPI anchored (1:5000 dilution) and fasciclin II transmembrane (1:2000 dilution), kindly provided by Philip Copenhaver (Oregon Health and Science University), in TBS buffer. Membranes were subsequently washed three times with 2% (v/v) Tween 20 in TBS buffer, pH 7.4, incubated with goat anti-guinea pig horseradish peroxidase-conjugated secondary antibody (GE Healthcare), 1:5000 dilution in 2% (v/v) Tween 20 in TBS buffer, pH 7.4, for 1 h, and ECL (Immobilon Western, Millipore) detected.

2.3. Ligand blot experiments in lipid raft fractions

For immunodetection of Cry3Aa in ligand blotting experiments, blocked nitrocellulose membranes were incubated with 75 nM Cry3Aa toxin in PBS buffer for 1 h, washed three times with 2% (v/v) Tween 20 in PBS buffer, pH 7.4, and incubated with a Cry3Aa polyclonal antibody for 1 h. Following 1 h incubation with alkaline phosphatase-conjugated anti-rabbit antibody (Sigma), 1:30,000 dilution in 0.1% (v/v) Tween 20 in PBS buffer, pH 7.4, the immunoreactive proteins were visualized using the ECL detection system Immobilon Western (Millipore).

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Protein bands of interest were excised from the corresponding Coomassie stained gels, and digested with sequencing grade trypsin (Promega) as described elsewhere [29]. The digestion mixture was dried in a vacuum centrifuge, resuspended in 7 μL of 0.1% TFA, and 1 μL was spotted onto the MALDI target plate. Droplets were air-dried at room temperature and 0.5 μL of matrix (5 mg/mL CHCA (Sigma) in 0.1% TFA-ACN/H_2O (1:1, v/v)) was added and allowed to air-dry at room temperature. The resulting mixtures were analyzed in a 4700 MALDI TOFTOF (AB Sciex) in positive reflectron mode. Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10; minimum cluster area: 500; maximum precursor gap: 200 ppm; maximum fraction gap: 4) were selected for every position for the MSMS analysis. MS/MS data was acquired using the default 1 kV MS/MS method. The MS and MS/MS information was sent to MASCOT via the Protein Pilot (ABSciex). Database search was performed on Swiss-Prot and NCBInr**.** Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications. Proteins showing score higher than homology or significance threshold were identified with confidence ⩾95%.

2.4. Prohibitin-1 immunodetection in plasma membrane fractions

For immunodetection of PHB-1 in CPB MECs fractions, membranes were blocked overnight at 4 °C with 3% (w/v) BSA in PBS buffer, pH 7.4, containing 0.1% (v/v) Tween 20. After blocking, the membranes were incubated with a polyclonal anti-human prohibitin-1 antibody (H-80) (Santa Cruz Biotechnology) (dilution 1:200 in 0.1% (v/v) PBS buffer), for 1 h at room temperature. Following three times washing with 2% (v/v) Tween 20 in PBS buffer, the membranes were subsequently incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit antibody (Sigma), 1:30,000 dilution in 0.1% (v/v) Tween 20 in PBS buffer, and immunoreactive proteins were visualized using the ECL detection system Immobilon Western (Millipore).

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from dissected midguts of CPB second instar larvae using TRIZOL LS Reagent (Invitrogen), following the manufactureŕs protocol, and the purified RNA was treated with DNase I (Ambion, Inc.). RNA quality was assessed by 1% agarose gel electrophoresis and quantification was carried out with a spectrophotometer (NanoDrop 2000, Thermo Scientific). AMV reverse transcriptase (Roche) was used for first strand cDNA synthesis using oligo $(dT)_{15}$ (Promega).

2.6. Amplification of CPB prohibitin-1 coding sequence

The CPB larvae cDNA was used to PCR amplify the *prohibitin-1* coding sequence, using degenerate primers designed from peptide fragments S_{83}KDLQNV$_{89}$ **(**F: 5′ AG(C/T) AA(A/G) GAT TTG CA(G/A) AAT GT 3′); I_{91}TLRIL$_{96}$ (F: 5′ AAC ATC AC(A/G) CT(G/C) CGT AT(T/C) CT 3′); V_{184}ELKQVA$_{190}$ (R: 5′ GCC AC(C/T) TG(C/T) TTC ATT TC(C/G) AC 3′); I_{242}EAAEDI$_{248}$ (R: 5′ AT(A/G) TCC TCG GCG GC(T/C) TC 3′) and Paq5000TM DNA Polymerase (Stratagene). PCR reaction was carried out in a Mastercycler gradient (Eppendorf) in the following conditions: 1 cycle of 94 °C for 2 min, 10 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min followed by 5 s additional after each cycle, and a final elongation step of 72 °C for 10 min. The PCR product was purified from a 1% agarose gel using the NucleoSpin Extract II PCR purification kit (Macherey-Nagel). Purified DNA products were sequenced at the University of Valencia Genomics Facility using an ABI 3730XL Automated Sequencer (Applied Biosystems).

The complete coding sequence of CPB *prohibitin-1* gene was obtained by RACE with SMARTER RACE cDNA Amplification kit (Clontech), following manufacturer's protocol, using RNA isolated from second instar larvae midguts. 5′ and 3′ RACE first strand cDNAs were produced by reverse transcription and used, together with gene specific primers (GSP), for PCR amplification of overlapping 5′ and 3′ RACE fragments. GSP were designed considering the sequence of the *prohibitin-1* amplified fragment (GSP3′F: 5′ CCATCGATCACCACTGAAGTTCTCAAAGCCG 3′ and GSP5′R: 5′ CCCAAGCTG CTATTCTGTTGGCCAAAGC 3′). The PCR RACE 5′ and 3′products were analyzed by 1% agarose gel electrophoresis and sequenced at the University of Valencia Genomics Facility using an ABI 3330XL Automated Sequencer (Applied Biosystems).

2.7. dsRNA synthesis

Initial PHB-1 DNA template was obtained by amplifying cDNA with two gene-specific primers tailed with the T7 polymerase promoter sequence (F: 5′ TAATACGACTCACTATAGGGAGAAGGT CAGGACTACGAAGAAAGAG 3′ and R: 5′ TAATACGACTCACTATAG GGAGAGCTTTGGCCAACAGAATAGCAG 3′), using a high fidelity DNA Polymerase (PrimeSTARTM HS, Takara). PCR conditions were: 98 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 55 °C for 10 s and 72 °C for 40 s. The PCR product was purified from a 1% agarose gel using the NucleoSpin Extract II PCR purification kit (Macherey-Nagel). The purified PCR product was used as a template for dsRNA synthesis with the MEGAscript® RNAi Kit (Ambion, Inc.), according to manufactureŕs protocol. For dsRNA precipitation, the dsRNA was incubated with 60% ethanol and 1 M ammonium acetate, pH 7.0, for 1 h, at −20 °C, followed by centrifugation for 30 min, at 20,817*g*, at 4 °C. The dsRNA pellet was washed with 70% ethanol, diluted in nuclease-free water, quantified using a spectrophotometer (NanoDrop 2000, ThermoScientific) and stored at −20 °C until use.

As a control, *T. castaneum* odorant binding protein [GenBank: EEZ97740] dsRNA was synthesized using gene-specific primers tailed with the T7 polymerase promoter sequence (F: 5′ GAATTGTAATACGACTCACTATAGGATGAAAGTCTTTGTGTGTCTCG 3′ and R: 5′GAATTGTAATACGACTCACTATAGGCTAATGGGAGTGAACTTTCTTG 3′) in the same conditions as described above.

2.8. dsRNA feeding bioassays

Cry3Aa toxicity assays were carried out in CPB second instar larvae by voluntary feeding. Two groups of 20 larvae were placed into individual wells of 25-well boxes and starved for 2 h. After starvation, one group of larvae was fed with a 0.24 cm^2 discolor of fresh potato leaves containing 0.8 μL drop with 10 μg of dsRNA diluted in nuclease-free water (dsRNA treated). The second group (control) was fed in the same conditions with 0.8 μL of nuclease-free water. After 2 h of treatment, both groups of CPB larvae were transferred to plastic containers with fresh potato leaves and reared at 25 °C with a photoperiod of 16:8 (light/dark). After 48 h of treatment, following 2 h starvation, CPB dsRNA treated and control larvae were fed with a 0.24 cm^2 discolor of fresh potato leaves containing a drop of 0.3 μL with 100 or 300 ng of Cry3Aa toxin diluted in 50 mM Na_2CO_3. As control, CPB larvae were fed with 0.3 μL of 50 mM Na_2CO_3 in the same conditions. Upon ingestion of the entire leaf discolor, larvae were fed fresh potato leaves and reared at 25 °C with a photoperiod of 16:8 (light/dark). Mortality was recorded on days 4, 7, 9, 11, 14 and 18 after the initial dsRNA treatment. Assays were performed three times ($n = 3$).

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Two types of dsRNA were used in the experiments: PHB-1 dsRNA and *T. castaneum* odorant binding protein dsRNA, the latter as a control of RNA interference (RNAi) experiments.

2.9. Quantitative real-time PCR (qRT-PCR)

In RNAi experiments, transcript levels in CPB larvae were evaluated 5 days after dsRNA treatment by quantitative real-time PCR. Total RNA from dsRNA treated and control larvae was obtained as described in Section 2.4, and cDNA synthesis was performed using AMV reverse transcriptase (Roche), and oligo $(dT)_{15}$ (Promega) and random hexamers (Applied Biosystems). Ten ng of cDNA were used as templates with Power SYBR Green PCR Master Mix (Applied Biosystems), and forward (5′ GTTCTCAAAGCCGTAGTAGCTCAGT 3′) and reverse (3′ CCAAGTCACGCTGGGTGAT 5′) primers, designed with Primer Express software (Applied Biosystems). A StepOnePlus Real-Time PCR system (Applied Biosystems) was utilized, under the conditions recommended by the manufacturer. For each experiment, two biological replicates were analyzed using the mean values of three technical replicates. Relative-fold calculations were made using ribosomal protein *RP4* as reference gene (F: 5′ CCCTACAGTGTCAGCGACAAAG 3′ and R: 3′ CGACCGGTACCCCAAGACT 5′) to normalize gene expression as reported by Zhu et al. [30]. Data were analyzed by Student's *t*-test for statistically significant differences ($p < 0.05$).

2.10. Insects and brush border membrane vesicles (BBMV) preparation

A laboratory colony of CPB founded from eggs taken from the field was used. Larvae and adults were reared on potato leaves at 25 °C and with a photoperiod of 16:8 (light/dark).

CPB brush border membrane vesicles (BBMV) were prepared from last instar CPB larvae as described in Rausell et al. [31].

2.11. Synthetic fluorogenic peptide substrate cleavage assays

ADAM metalloprotease proteolytic activity assays were performed as previously described by Ochoa-Campuzano et al. [13] by means of fluorescent cleavage assays using the synthetic peptide Mca-PLAQAV-Dpa-RSSSR-NH2 (BIOMOL International LP) substrate of ADAMs. Assays were carried out with 60 μL of BBMV containing 200 μg of proteins, which were previously incubated in the absence or the presence of 10 μL of the polyclonal antibody Prohibitin (H-80) (Santa Cruz Biotechnology) raised against human PHB-1. In both cases, Cry3Aa toxin was used as competitor in the cleavage assays at a final concentration of 26.5 μM.

3. Results

3.1. Identification of Cry3Aa toxin binding proteins in CPB plasma membrane fractions

Membrane lipid rafts are cholesterol enriched platforms shown to be essential for Cry1 toxin-receptor interaction and the subsequent toxicity events in lepidopteran insects [32]. In coleopteran insects, in which microvilli cholesterol content is 5–6.5fold higher than in Lepidoptera [33], the distribution between the lipid raft and non-raft phases of specific membrane proteins relevant for Cry3 toxin action has not been reported. We isolated Triton X-100 insoluble membrane fractions from CPB MECs using a sucrose density gradient to identify, in these midgut membrane fractions, CPB proteins that interact with Cry3Aa protein, toxin previously reported active against CPB [34]. The CPB insoluble detergent band was collected from just below the interface of 5% and 35% sucrose layers, whereas the soluble fraction was collected from the bottom of the sucrose gradient. Insoluble and soluble fractions were identified by immunoblotting using the markers *M. sexta* fasciclin GPI-anchored and fasciclin transmembrane, respectively (Fig. 1A and B).

Proteins of detergent insoluble and soluble fractions were SDS-10%PAGE separated, blotted onto a nitrocellulose membrane and incubated with Cry3Aa toxin. Binding of proteins to Cry3Aa toxin was visualized on the membrane by immunodetection of the bound toxin using an anti-Cry3Aa toxin antibody. In the Cry3Aa toxin ligand blot of the soluble fraction (Fig. 1C), among the putative Cry3Aa binding proteins, an intense signal was detected in a molecular mass around 250 kDa and a band with an apparent molecular mass of approximately 30 kDa was observed. In the insoluble fraction, a band of an apparent molecular mass around 37 kDa was immunodetected, which was also detected in the soluble fraction, although with less intensity (Fig. 1C).

The protein bands around 250 kDa and 30 kDa in the soluble membrane fraction, and 37 kDa in the soluble and insoluble fractions, which gave the most intense signals in the ligand blot experiments, were excised from the corresponding SDS-10% PAGE gels (S-250.1-2, S-37.1-3, S-30.1-3 and I-37.1-3, in Fig. 1D), trypsin digested and analyzed by mass spectrometry (MALDI TOF/TOF). Since the CPB genome is not sequenced, identifications were performed by sequence similarity, searching the non-redundant protein database at NCBI (taxonomic restriction to insects) with PMF data using Mascot (MatrixScience, UK) to establish the best protein match for the bands. Identifications with a significant score are shown in Table 1.

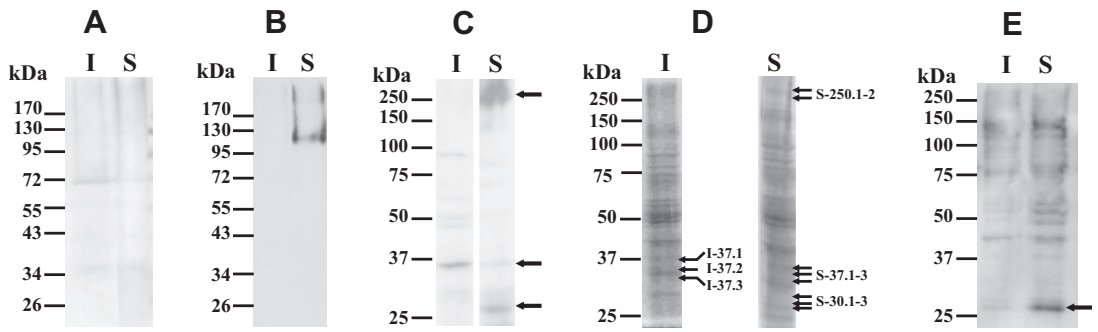

Fig. 1. Isolation of 1% Triton X-100 soluble (S) and insoluble (I) fractions from CPB MECs. Panels (A) and (B) show western blots of identical gradient fractions probed with *M. sexta* antibodies to detect fasciclin GPI-anchored and fasciclin transmembrane proteins, respectively. (C) Cry3Aa toxin ligand blots of S and I gradient fractions immunodetected with anti-Cry3Aa antibody. Arrows point to the bands around 30, 37 and 250 kDa. (D) SDS-10%PAGE Coomassie stained gel of S and I gradient fractions. Arrows point to the bands around 30, 37 and 250 kDa that were excised for mass spectrometry analysis. (E) Western blots of S and I gradient fractions immunodetected with anti-human prohibitin-1 (H-80) antibody. Arrow indicates the 30 kDa band. In each panel molecular weight markers are shown.

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Table 1
Mass Spectrometry analysis of proteins in the bands pointed with arrows in Fig. 1D that were excise from gels of detergent soluble and insoluble fractions of CPB MECs.

Band	Accession number	Mascot score	Protein identified	Predicted mass (kDa)	Peptides identified	Species
Soluble fraction (non-rafts)						
S-30.1	XP_001848603	85	Prohibitin-1	30	10	*C. quinquefasciatus*
S-30.2	XP_968561	158	Similar to adenine nucleotide translocase	33.28	20	*T. castaneum*
S-30.3	ABU68467	381	Similar to adenine nucleotide translocase	33.35	9	*M. alternatus*
	XP_972721	337	Similar to mitochondrial ATP synthase gamma chain	32.28	9	*T. castaneum*
	ACY71283	289	S4 ribosomal protein	29.58	6	*C. tremulae*
	XP_001864030	268	Mitochondrial tricarboxylate transport protein	35.15	10	*C. quinquefasciatus*
	P31402	223	V-ATPase subunit E	26.13	6	*M. sexta*
	XP_968561	88	Similar to adenine nucleotide translocase	32.28	2	*T. castaneum*
S-37.1	XP_974101	131	Prohibitin-2	36.04	19	*T. castaneum*
S-37.2	XP_970163	252	Similar to pyruvate dehydrogenase	39.41	7	*T. castaneum*
	EEZ97412	232	Hypothetical protein	34.32	6	*T. castaneum*
	XP_311527	152	Pyruvate dehydrogenase (E1-PDHc)	38.65	4	*A. gambiae*
	AAK51552	106	RACK1	36.48	3	*H. virescens*
	XP_974101	100	Prohibitin-2	36.04	2	*T. castaneum*
	XP_968561	88	Similar to adenine nucleotide translocase	32.28	2	*T. castaneum*
S-37.3	XP_968064	423	Similar to S3Ae ribosomal protein	30	10	*T. castaneum*
	XP_001959032	309	Mitochondrial transport protein	40.79	11	*D. ananassae*
	ABU68467	259	Adenine nucleotide translocase	33.35	6	*M. alternatus*
	XP_966490	114	Succinyl -CoA synthetase	35.05	3	*T. castaneum*
	CAA28192	99	Actin A3		1	*B. mori*
	XP_001601256	63	Similar to annexin IX-A	36.14	1	*N. vitripennis*
	XP_623470	62	Similar to tropomyosin	39.79	2	*A. mellifera*
S-250.1	XP_973750	2272	Similar to spectrin	282.64	52	*T. castaneum*
S-250.2	XP_001813466	3251	Similar to myosin (heavy chain)	224.57	67	*T. castaneum*
Insoluble fraction (rafts)						
I-37.1	XP_397306	247	Similar to Transaldolase 1	36.24	6	*A. mellifera*
	XP_966771	142	Similar to mitochondiral receptor Tom40	34.49	3	*T. castaneum*
	ABM55485	134	Digestive cysteine protease intestain D-5	36.76	3	*L. decemlineata*
	EEB12070	127	Malate dehydrogenase	37.50	2	*P. humanus corporis*
	XP_976064	88	Similar to ribose phosphate pyrophosphokinase	38.12	3	*T. castaneum*
I-37.2	XP_975007	415	Similar to glycerol phosphate dehydrogenase	39.46	9	*T. castaneum*
	XP_974101	311	Prohibitin-2	36.04	7	*T. castaneum*
	XP_970675	303	Similar to nascent polypeptide-associated complex subunit alpha	22.72	6	*T. castaneum*
	XP_970131	240	Similar to guanine nucleotide-binding protein subunit β	37.95	5	*T. castaneum*
	XP_397306	191	Similar a transaldolase 1	36.24	4	*A. mellifera*
	CAD58931	184	Similar to acidic ribosomal protein p0	34.09	4	*T. balearica*
	XP_001659012	167	Malate dehydrogenase	45.04	3	*A. aegypti*
	AAS20591	161	Digestive cysteine protease intestain B-11	36.67	5	*L. decemlineta*
	XP_001844836	130	Aldehyde reductase 1	35.68	3	*C. quinquefasciatus*
	XP_966482	124	Similar to conserved hypothetical protein	28.9	3	*T. castaneum*
	XP_973445	114	Similar to vesicular mannose-binding lectin	37.28	2	*T. castaneum*
	AAK51552	86	Receptor for activated protein kinase C (RACK1)	36.48	2	*H. virescens*
I-37.3	BAD52259	516	Homolog to RACK1	36.37	13	*P. xylostella*
	ABC61672	152	Electron-transferring flavoprotein subunit alpha	35.26	4	*P. americana*
	XP_967051	131	Pyrophosphatase	32.74	4	*T. castaneum*
	XP_968656	113	Similar to CAAX prenyl protein 1 homolog	50.02	3	*T. castaneum*
	XP_967931	99	Similar to annexin IX	36.03	3	*T. castaneum*

Among the putative Cry3Aa toxin binding proteins, we detected PHB, V-ATPase and actin that have been previously reported as Cry toxin binding molecules in other insects [14–17]. We focused on PHB because we have previously found PHB-1 as putative CPB larvae Cry3Aa binding protein, using an alternative cross-linking approach [35], and in the present work these proteins were identified in four out of the eleven protein bands analyzed, being the only hit in two of them, S-30.1 and S-37.1, corresponding to PHB-1 of *Culex quinquefasciatus* [GenBank: XP_001848603], and PHB-2 of *T. castaneum* [GenBank: XP_974101], respectively (Table 1). PHB-1 and -2 comprise a highly conserved family of proteins ubiquitously present in divergent species from prokaryotes to eukaryotes, described to form a high molecular mass complex that represents the physiologically active structure [36]. The molecular mass of both PHB predicted identification for each band in CPB corresponded to the size of these proteins in other organisms, around 30 and 37 kDa, respectively for PHB-1 and PHB-2 [37]. Binding of Cry3Aa toxin to both proteins (Fig. 1C) is not unexpected since it has been reported that there is approximately 53% amino acid sequence identity between PHB1 and PHB2 proteins and allowing for conservative substitution, sequence similarity approaches 74% [37].

Since a 29.8 kDa molecular mass PHB was previously identified as a Bt Cry4Ba toxin binding protein in *A. aegypti* [17], to confirm the identity of PHB-1 in the 30 kDa band recognized by Cry3Aa toxin in CPB, filters were also probed with anti-human prohibitin-1 (H-80) antibody (Santa Cruz Biotechnology) (Fig. 1E). Anti-human prohibitin-1 (H-80) antibody was raised against the region comprised between amino acids 193 and 272 of the highly conserved C-terminal region of the protein. Consistent with the 30 kDa Cry3Aa binding protein being a PHB-1 protein, a band with an apparent molecular mass of 29 kDa was strongly immunodetected in the soluble fraction, which was also present in the insoluble fraction, although it showed lower intensity.

3.2. CPB Prohibitin-1 protein

Fig. 2 shows the alignment using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo) of several insect PHB-1 sequences

retrieved from the NCBInr database in which peptides obtained from the mass spectrometry analysis of the CPB 30 kDa band are indicated. Degenerate primers were designed from the DNA sequences corresponding to peptide fragments S_{83}KDLQNV$_{89}$, I_{91}TLRIL$_{96}$, V_{184}ELKQVA$_{190}$ and I_{242}EAAEDI$_{248}$ (positions of the coleopteran insect *T. castaneum* PHB-1 sequence), representing highly conserved regions in the alignment, and used for PCR amplification and sequencing. Two overlapping DNA fragments were amplified yielding a final sequence of 395 bp that following a NCBI BLASTn search showed homology with a number of PHB-1 genes from insects. To obtain the complete CPB PHB-1 DNA coding sequence, 5′ and 3′ RACE were performed. Sequence analysis of the amplified cDNA revealed an open reading frame of 828 bp long that was *in silico* translated into a protein of 276 amino acids with a predicted molecular mass of 30.2 kDa (Fig. 3A), named as CPB PHB-1 [GenBank: JX275964]. The analysis at the amino acid level showed that the CPB protein belongs to the prohibitin family since it contains a prohibitin-homology domain (cd03401, Band_7_prohibitin) (aa: 30–205) conserved in other prohibitin proteins. Common structural features, such as a signal peptide (aa: 1-26), a putative transmembrane domain (aa: 16-37) and a coiled-coil region (aa: 181-210) were also identified (Fig. 3B) using InterProScan Sequence Search (www.ebi.ac.uk/Tools/pfa/iprscan) and COILS (www.ch.embnet.org/software/COILS_form.html). When PRINTS database was searched with "MOTIF Search" (www.genome.jp/tools/motif) the protein signature of prohibitin (PR00679) with its entire sevsn-element fingerprint was found (scores from 1325 to 1068, depending on the element). Additionally, the following fingerprints with scores higher than 1005, were obtained: elements one, four and seven out the seven elements that compose the rhodopsin-like GPCR superfamily signature (PR00237), in three possible sequence positions; elements four and five out of the five elements constituting the FAD-dependent pyridine nucleotide reductase signature (PR00368), in two possible sequence positions; elements two and six out of the seven elements of the cadherin signature (PR00205), in four possible sequence positions; element three out of the four elements that provides the signature for the fumarate lyase superfamily (PR00149), in four possible sequence positions; element four out of the four elements that compose the DnaJ domain signature (PR00625), in four possible sequence positions (Fig. 3B). It is intriguing to note that cadherin signature elements were identified in the CPB Cry3Aa binding protein analyzed, since cadherin-like proteins are reported to be Bt Cry toxins receptors [5].

We have predicted the three-dimensional homology model of CPB PHB-1 using the automated comparative protein modeling server SWISS MODEL Workspace (http://swissmodel.expasy.org/workspace/) [38], based on the theoretical structure of the predicted human prohibitin-1fold by Kurshid and coworkers (PDB accession No. 1LU7) provided as template by user. As seen in Fig. 3C and in agreement with the predicted human PHB-1, it is composed of seven β strands in three β sheets arranged as two antiparallel clusters, and six α helices with helices one, two, three forming a cluster perpendicular to helices four, five and six.

A molecular phylogenetic tree was constructed including 10 insect PHB-1 proteins (http://www.megasoftware.net) [39] (Fig. 3D). The CPB PHB-1 [GenBank: JX275964] clustered together in one group in the phylogenetic tree with *T. castaneum* PHB-1 [GenBank: XP_974606.1], confirming they are evolutionary closely related.

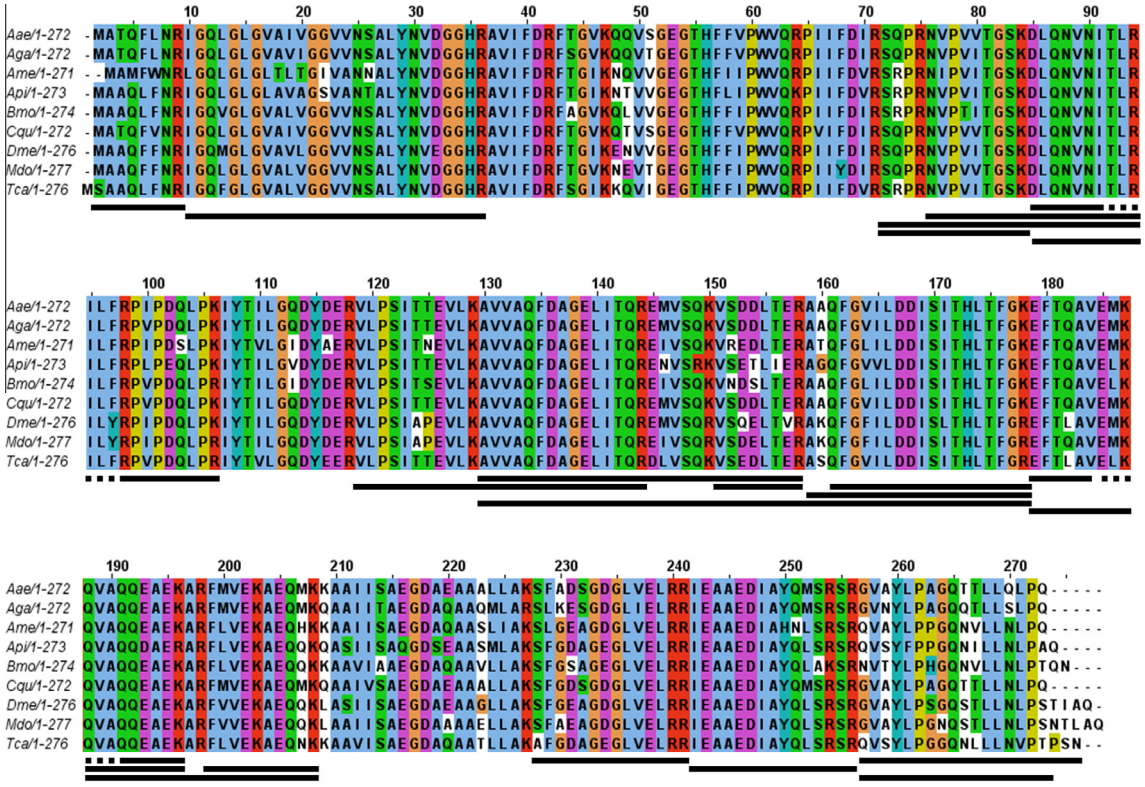

Fig. 2. Multiple sequence alignment of Prohibitin-1 amino acid sequences of several insects using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo). *Aedes aegypti* (Aae, [GenBank: XP_001653792.1]), *Anopheles gambiae* (Aga, [GenBank: XP_309992.2]), *Apis mellifera (*Ame, [GenBank: XP_391959.1]), *Acyrthosiphon pisum* (Api, [GenBank: NP_001119688.1]), *Bombyx mori* (Bmo, [GenBank: NP_001040289.1]), *Culex quinquefasciatus* (Cqu, [GenBank: XP_001848603.1]), *Drosophila melanogaster* (Dme, [GenBank: NP_476607.2]), *Musca domestica* (Mdo, [GenBank: ADT92002.1]) and *T. castaneum* (Tca, [GenBank: XP_974606.1]). Lines depict mass spectrometry peptides identified in the 30 kDa band named as S.30-1 in Fig. 1D. The color scheme used in the alignment corresponds to that of Clustal X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Pairwise percentage of sequence identity among all PHB-1 proteins was always higher than 80%, being 92% in the case of CPB and *T. castaneum*.

3.3. PHB-1 gene silencing in CPB larvae

RNAi is a powerful molecular tool in the study of gene function, which we have used to assess whether PHB-1 gene expression knockdown has an effect on Cry3Aa toxin insecticidal activity against CPB larvae.

Double-stranded RNA targeting PHB-1, corresponding to nucleotides 333–683 of the CPB cDNA sequence, was provided to CPB larvae in potato leaf disks. To confirm gene expression silencing, qRT-PCR was conducted on individuals sampled 5 days after dsRNA treatment using *RP4* gene as constitutive internal control (Fig. 4A). Statistically significant silencing of the PHB-1 gene was observed (16-fold reduction in CPB dsRNA treated larvae *versus* non-treated larvae) and remained stable during the time period of experimentation (data not shown). Knockdown of the PHB-1 gene's transcripts was sufficient to induce high levels of mortality 18 days post PHB-1 dsRNA treatment (Fig. 4B), suggesting that the gene's normal expression must be essential for CPB larvae viability. As a control, a non-relevant dsRNA derived from *T. castaneum* odorant binding protein [GenBank: EEZ97740] was assayed, yielding no CPB larval mortality (Fig. 4B).

2 days after PHB-1 dsRNA treatment, CPB larvae were challenged with two doses of Cry3Aa toxin and evaluated during 14 days for their ability to survive. Fig. 4B shows that as early as 2 days after PHB-1 dsRNA treated larvae were intoxicated with 300 ng Cry3Aa toxin 13% mortality was recorded, despite toxin treatment alone or PHB-1 silencing did not cause larval mortality.

5 days after 300 ng Cry3Aa toxin ingestion 31% mortality was achieved whereas no mortality was observed in larvae challenged with 100 ng Cry3Aa toxin. When PHB-1 silenced larvae were intoxicated with 300 or 100 ng Cry3Aa toxin 100% or 78% mortality was recorded while in non-intoxicated PHB-1 silenced larvae only 30% mortality was achieved (Fig. 4B), showing that PHB-1 silencing enhances Cry3Aa toxin action. As a control, CPB larvae fed with *T. cas-*

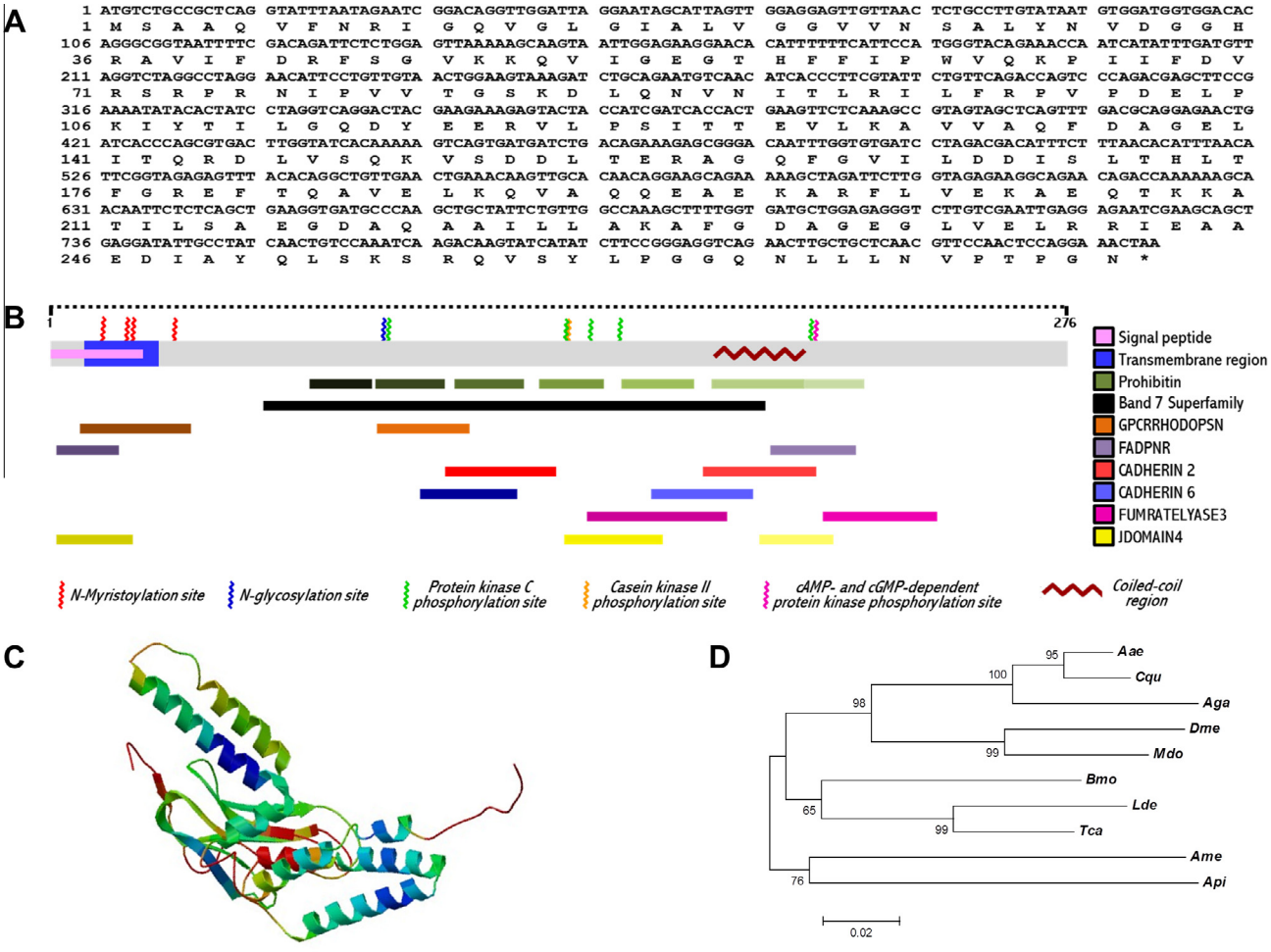

Fig. 3. CPB prohibitin-1. (A) cDNA nucleotide sequence and deduced amino acid sequence for CPB PHB-1 (http://www.fr33.net/translator.php). (B) Relative location of conserved structural and functional protein domains, according to InterProScan Sequence Search (www.ebi.ac.uk/Tools/pfa/iprscan) and COILS (www.ch.embnet.org/software/COILS_form.html). PRINTS database was searched with "MOTIF Search" (www.genome.jp/tools/motif) for protein fingerprints. Gray boxes represent protein backbones; colored boxes represent different protein structural features; horizontal colored bars below protein backbones represent different conserved elements that provide protein signatures. (C) Predicted three-dimensional homology model of the CPB PHB-1 protein, based on 1LU7.pdb and SWISS-MODEL workspace server (http://swissmodel.expasy.org/workspace/) [38]. Estimated per-residue accuracy is visualized using a color gradient from blue (more reliable regions) to red (potentially unreliable regions). (D) Unrooted phylogenetic tree generated with Mega 5 (http://www.megasoftware.net) [39] of the 9 insect PHB-1 amino acid sequences included in the alignment of Fig. 2 and the CPB PHB-1 [GenBank: JX275964]. The Neighbor-Joining method [56] for reconstructing the phylogenetic tree was used. In the optimal tree the sum of branch length was 0.70441585 and the number of bootstrap test replicates was 1000 [57]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [58] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 271 positions in the final dataset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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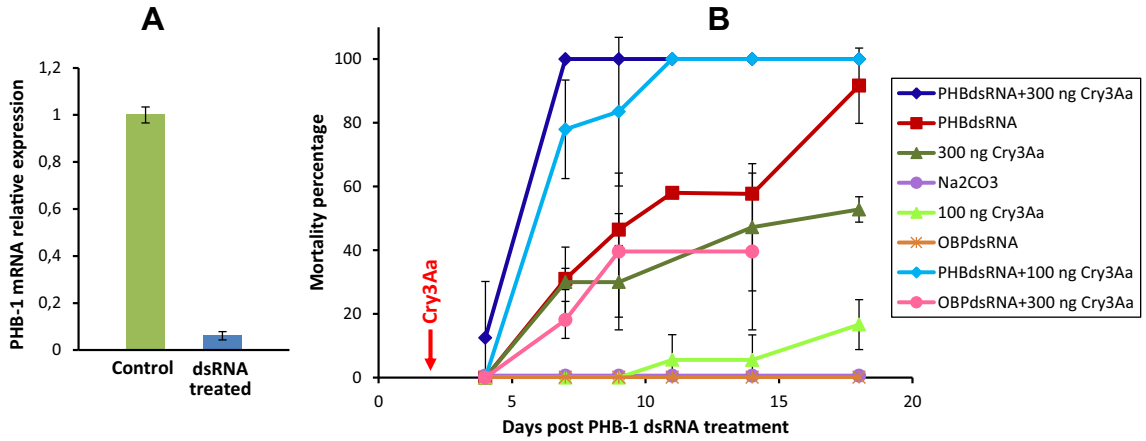

Fig. 4. CPB PHB-1 gene silencing. (A) Reduction in PHB-1 mRNA abundance in CPB larvae in response to ingestion of dsRNA against PBH-1. The relative amount of CPB PHB-1 transcript estimated by qRT-PCR in control and silenced larvae was compared normalized to the expression of *RP4* gene. The statistical significance of the gene expression between the two groups was evaluated using Student's *t*-test and significant knockdown was observed ($p < 0.05$). In each group, experiments were performed three times in triplicate ($n = 9$) and error bars represent standard error of the mean. (B) Mortality percentage following bioassay of CPB larvae from treatment groups (either control or dsRNA-fed larvae) with control or Cry3Aa-treated potato leaf disks, as indicated in the inset. Twenty second instar CPB larvae were used in each bioassay group. Each experiment was performed in duplicate ($n = 2$) and error bars represent standard error of the mean. Arrow points to day two post dsRNA treatment when larvae were challenged with Cry3Aa toxin. PHB-1 dsRNA and *T. castaneum* odorant binding protein (abbreviated as OBP) dsRNA were used in the experiments. Two doses of Cry3Aa toxin (300 and 100 ng) were used in the experiments.

taneum odorant binding protein dsRNA were also intoxicated with the highest Cry3Aa toxin dose and no significant differences in mortality were observed compared to those challenged only with 300 ng Cry3Aa toxin (Fig. 4B). These results demonstrated that combination of PHB-1 dsRNA treatment with Cry3Aa toxin challenge potentiated CPB larval mortality.

3.4. Involvement of PHB-1 protein in Cry3Aa toxin interaction with CPB BBMV

In a previous work we have reported the membrane associated metalloprotease proteolytic processing of Bt Cry3Aa toxin upon incubation with CPB BBMV and we have proposed that interaction with a CPB ADAM-like metalloprotease is essential in Cry3Aa mode of action [13,40].

To investigate the involvement of CPB PHB-1 protein in relation to the events of Cry3Aa interaction with the insect midgut membrane, we analyzed the ability of Cry3Aa toxin to inhibit CPB BBMV cleavage of an ADAM fluorogenic substrate in the presence of anti-human prohibitin-1 (H-80) antibody. Fig. 5 shows that Cry3Aa toxin almost abolished the cleavage of the ADAM fluorogenic substrate, as previously reported [13], whereas no Cry3Aa inhibition of substrate cleavage was observed when anti-human prohibitin-1 (H-80) antibody was present, indicating that Cry3Aa toxin binding to CPB PHB-1 might be required for efficient Cry3Aa toxin interaction with the CPB ADAM-like membrane associated metalloprotease putative receptor.

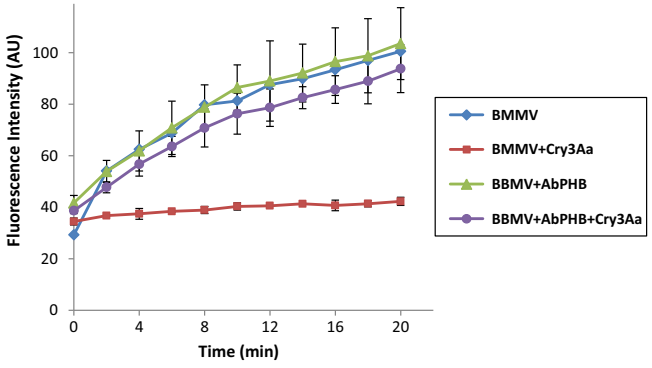

Fig. 5. ADAM10 synthetic fluorogenic peptide substrate cleavage assays. Time course of fluorescence released upon peptide cleavage by CPB BBMV supernatant in the presence and absence of 26.5 μM Cry3Aa toxin and/or with anti-human prohibitin-1 (H-80) antibody (AbPHB), as indicated in the inset. Each experiment was performed three times independently ($n = 3$) and error bars represent standard error of the mean.

4. Discussion

By ligand blot, PHB proteins were identified as putative Cry3Aa binding proteins. PHB has been reported to be present in different cellular compartments playing an important biological role in mitochondrial function, cell proliferation and development [37]. In human intestinal epithelial cells, prohibitin functions as a binding site for the Vi capsular polysaccharide of *Salmonella typhi* [41] and in human microglial cells, very recently, this protein has been described as a receptor molecule of chikungunya virus [42]. In *A. aegypti*, PHB acts as a receptor protein mediating Dengue virus two entry into insect cells [43] and in this insect a 29.8 kDa molecular mass PHB has also been identified as a Bt Cry4Ba toxin binding protein, although its role as receptor or as a protein involved in this toxin mode of action has not been established [17,44].

In mass spectrometry fingerprint analysis, we found MS peptides conserved in the sequence of several insect 30 kDa PHB-1 proteins (Fig. 2), covering approximately 77% of the full length CPB PHB-1 sequence, which supports the identity of the Cry3Aa toxin interacting protein identified in CPB as PHB-1. We obtained its full cDNA sequence and the analysis of the predicted protein sequence revealed a domain that is conserved (Band_7_prohibitin, cd03401) among other known PHB proteins (Fig. 3B). We constructed a phylogenetic tree with 10 PHB-1 protein sequences including CPB PHB-1. The phylogeny agrees well with their evolutionary branches and, as expected, CPB PHB-1 clustered together with the coleopteran representative *T. castaneum* (Fig. 3D). A striking feature of the identified CPB PHB-1 is the presence of putative cadherin motifs (CADHERIN2 and CADHERIN6 of the cadherin signature) in the amino acid sequence of the protein. Cadherin-like proteins have been reported as Bt Cry toxin receptors in lepidopteran, dipteran and coleopteran insects [45] but in CPB, to date, no cadherin-like proteins have been identified to bind Cry tox-

ins. Nevertheless, Park et al. [46] demonstrated that a fragment spanning cadherin repeats CR8 to CR10 from WCRW cadherin significantly enhanced the toxicities of Cry3Aa and Cry3Bb toxins to CPB larvae. Interestingly, the predicted cadherin motifs in CPB PHB-1 sequence aligned to CR8-CR9 sequence of WCRW (aa: 1088-1125; data not shown). In *A. aegypti*, PHB-1 protein has been reported to bind to Cry4Ba toxin [17]. We have analyzed the *A. aegypti* PHB-1 protein sequence against PRINTS database searching with "MOTIF Search" (www.genome.jp/tools/motif) and we found that it also contains a putative CADHERIN2 motif of the cadherin signature in the same regions of the sequence than that of CPB PHB-1 (data not shown). It is therefore tempting to speculate that in *A. aegypti*, as well as in CPB, cadherin motifs in PHB-1 proteins might act as Cry toxins binding sites.

PHB-1 forms hetero-oligomers with PHB-2 as functional units exhibiting a mutual interdependence of the two proteins [47]. PHB complexes have been reported to have a scaffolding function, defining the spatial membrane organization. This is also a feature of other members of the stomatin/prohibitin/flotilin/HflK/C-domain (SPFH) family of proteins that form microdomains at the plasma membrane often anchored by interaction with the actin cytoskeleton [48]. SPFH proteins have been typically classified as lipid raft proteins based on their enrichment in detergent resistant membranes [49], although PHB have been localized in both, detergent soluble and insoluble fractions [50]. This is the case of CPB PHB-1, which was immunodetected in both fractions (Fig. 1E) as it occurred with its functional partner, CPB PHB-2, identified by mass spectrometry in the same membrane fractions (Fig. 1D). Consistent with the potential role of cadherin motifs in the PHB sequence as Cry toxin binding sites in CPB, in *M. sexta*, the cadherin-like 210 kDa Cry1Ab-binding protein also partitioned into both, lipid rafts and the soluble fraction [32].

RNAi experiments to knockdown PHB-1/PHB-2 expression in different human cell lines showed that reduction of cellular PHB-1/PHB-2 amounts were cytotoxic within 36–48 h post-transfection of the RNAi constructs [51]. Likewise, depletion of PBH compromised survival in wild type *C. elegans* [52] and deletion of a PHB homologue in *D. melanogaster* resulted in lethality during larval development [53]. As for CPB, PHB-1 silencing also caused larval mortality evidencing that PHB-1 is an essential protein for insect survival. The potential of RNAi in the practical application to control insect pests relies on the identification of interesting target genes whose loss of function caused larval mortality and significantly decreased insect growth. Three target genes (β-tubulin, V-ATPase A and V-ATPase E) demonstrated an effective RNAi response in the coleopteran pests WCR, SCR (*Diabrotica undecimpunctata*) and CPB that resulted in high larval mortality [54]. Interesting to note is that V-ATPases have been reported in the midgut membrane of several insects as capable of binding to Bt toxins [12,15–17] and when the expression of ATP synthase subunit B was knockdown by RNAi silencing, *A. aegypti* larvae became hypersensitive to toxin action [23]. The identification of PHB-1 as an essential protein for CPB viability might develop it in a novel RNAi target of tremendous biotechnological interest, but the slow rate of killing of PHB-1 silencing alone would limit this technology for field uses. However, when used in combination with a LD_{50} Cry3Aa treatment (300 ng Cry3Aa toxin dose) enhanced Cry3Aa toxic action as demonstrated by the threefold mortality increase in CPB larvae, corresponding to 100% mortality that was achieved just 5 days after Cry3Aa toxin treatment (Fig. 4B).

PHB complexes acting as membrane scaffolds might recruit proteases, or ensure a specific lipid environment that facilitates substrate processing [55], so their role in Cry3Aa toxic action might be relevant to toxin cleavage by ADAM-like metalloproteases, which has been reported to be important for Cry3Aa toxic action in CPB [13,40]. However, whether they also act a Cry receptor promoting pore formation or as a mediator of toxin interaction with other proteins directly involved in the toxicity mechanism or, alternatively, with membrane proteins involved in defense response still remains to be elucidated.

5. Conclusions

Our results demonstrated that CPB PHB-1 protein is essential for CPB larval viability and showed that this protein might be relevant for Bt Cry3Aa toxic action. The combination of PHB-1 silencing with Cry3Aa toxin treatment potentiated CPB larval mortality evidencing the feasibility of utilizing RNAi strategies to complement existing Bt based crop protection methods. Further knowledge on the physiological function and regulation of CPB PHB-1, as well as the molecular mechanisms of its involvement in Cry3Aa toxin mode of action might broaden the biotechnological insect control strategies by utilizing multiple targets.

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Resúmenes de los artículos

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Proteome Response of *Tribolium castaneum* Larvae to *Bacillus thuringiensis* Toxin Producing Strains

Estefanía Contreras, Carolina Rausell*, M. Dolores Real

Respuesta a nivel proteómico de las larvas de *Tribolium castaneum* a cepas de *Bacillus thuringiensis*

Objetivos: Evaluación de la actividad insecticida de diferentes cepas de *Bt* en el coleóptero *T. castaneum*. Realización de un análisis proteómico global en las larvas del insecto después de ser tratadas con las cepas de *Bt* que resultaron tóxicas, Cry3Ba y Cry23/37, con el objetivo de identificar proteínas diferencialmente reguladas tras los tratamientos respecto a las larvas control sin tratar.

Metodología: Los ensayos de toxicidad se realizaron alimentando larvas de *T. castaneum* con discos de harina que contenían mezclas de esporas y cristales de las cepas de *Bt* a evaluar. Se utilizó la técnica proteómica iTRAQ para identificar y determinar la abundancia diferencial de proteínas en extractos de larvas de *T. castaneum* tratadas y sin tratar con *Bt*. La expresión de algunas de las proteínas diferencialmente expresadas se evaluó mediante PCR cuantitativa. El análisis funcional de la proteína que mostró una regulación diferencial mayor se realizó mediante silenciamiento del correspondiente gen por RNAi, utilizando RNA de doble cadena que se inyectó a la cavidad corporal de la larva.

Conclusiones: Las proteínas de *T. castaneum* que aumentaron tras el tratamiento con la cepas de *Bt* productoras de Cry3Ba o Cry23/37 fueron la proteína de unión a odorantes C12, la apolipoforina III y la proteína quimiosensora 18, las cuales pueden participar en la defensa del insecto y la respuesta inmune. Las proteínas que disminuyeron en respuesta a los tratamientos fueron el precursor de la apolipoproteína I/II, la proteína ribosomal L13a, una proteína cuticular y la subunidad Eα de la piruvato deshidrogenasa, las cuales pueden estar implicadas en el metabolismo y desarrollo del insecto.

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Short Communication

Tribolium castaneum Apolipophorin-III acts as an immune response protein against *Bacillus thuringiensis* Cry3Ba toxic activity

Estefanía Contreras, Carolina Rausell, M. Dolores Real *

La apolipoforina III de *Tribolium castaneum* actúa como una proteína de respuesta inmune contra la acción tóxica de la toxina Cry3Ba de *Bacillus thuringiensis*

Objetivos: Análisis de la expresión de la proteína apolipoforina III (apoLpIII) y la actividad del enzima fenoloxidasa (PO) en las larvas de *T. castaneum*, tras el tratamiento con la cepa de *Bt* productora de Cry3Ba. Demostración de la implicación de esta proteína en la respuesta inmune del insecto y su participación en la activación de la cascada de la profenoloxidasa (proPO) mediante el silenciamiento del gen codificante de apoLpIII en las larvas del insecto.

Metodología: La expresión de apoLpIII se evaluó mediante PCR cuantitativa y el silenciamiento del correspondiente gen se realizó mediante RNAi, utilizando RNA de doble cadena que se inyectó a la cavidad corporal de la larva. El ensayo de la actividad PO se realizó mediante detección espectrofotométrica de la formación de dopacromo. Utilizando anticuerpos anti-PO se analizó la localización del enzima en cortes de tejido intestinal por microscopía confocal.

Conclusiones: El silenciamiento de apoLpIII produjo un aumento de la sensibilidad de las larvas de *T. castaneum* al tratamiento con la cepa de *Bt* productora de Cry3Ba y una disminución de la actividad PO. Estos resultados demuestran la implicación de esta proteína en la respuesta inmune de *T. castaneum* frente a la acción tóxica de la cepa de *Bt* productora de Cry3Ba y la participación de la misma en la activación de la cascada proPO.

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Sodium Solute Symporter and Cadherin Proteins Act as *Bacillus thuringiensis* Cry3Ba Toxin Functional Receptors in *Tribolium castaneum**

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Un simportador de sodio-soluto y una cadherina actúan como receptores funcionales de la toxina Cry3Ba de *Bacillus thuringiensis* en *Tribolium castaneum*

Objetivo: Identificación de receptores funcionales de la toxina Cry3Ba en el intestino de las larvas de *T. castaneum*.

Metodología: La unión de Cry3Ba a vesículas de membrana del epitelio intestinal de las larvas de *T. castaneum* se analizó mediante ensayos de unión ligando-receptor con la toxina purificada y vesículas de membrana del epitelio intestinal de las larvas de *T. castaneum*. Las proteínas de unión a Cry3Ba se identificaron mediante "ligand blot" y la implicación de algunas de estas proteínas como receptores funcionales de Cry3Ba se demostró mediante silenciamiento génico a través de la inyección de RNAi a la cavidad corporal de las larvas.

Conclusiones: El silenciamiento de la expresión de la cadherina TcCad1 así como el del simportador TcSSS produjo la disminución de la susceptibilidad de larvas de *T. castaneum* a Cry3Ba. El silenciamiento de la expresión de tres genes de APN no tuvo ningún efecto. Estos resultados indican que TcCad1 y TcSSS son receptores funcionales de Cry3Ba en *T. castaneum*. Se identificó un fragmento de TcSSS que contenía el epítopo de unión a toxinas Cry en otros insectos y que suministrado a las larvas conjuntamente con la toxina Cry3Ba incrementó la susceptibilidad de las mismas, confirmando la implicación por primera vez de un simportador de sodio en el modo de acción de las toxinas de *Bt*.

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Functional significance of membrane associated proteolysis in the toxicity of *Bacillus thuringiensis* Cry3Aa toxin against Colorado potato beetle

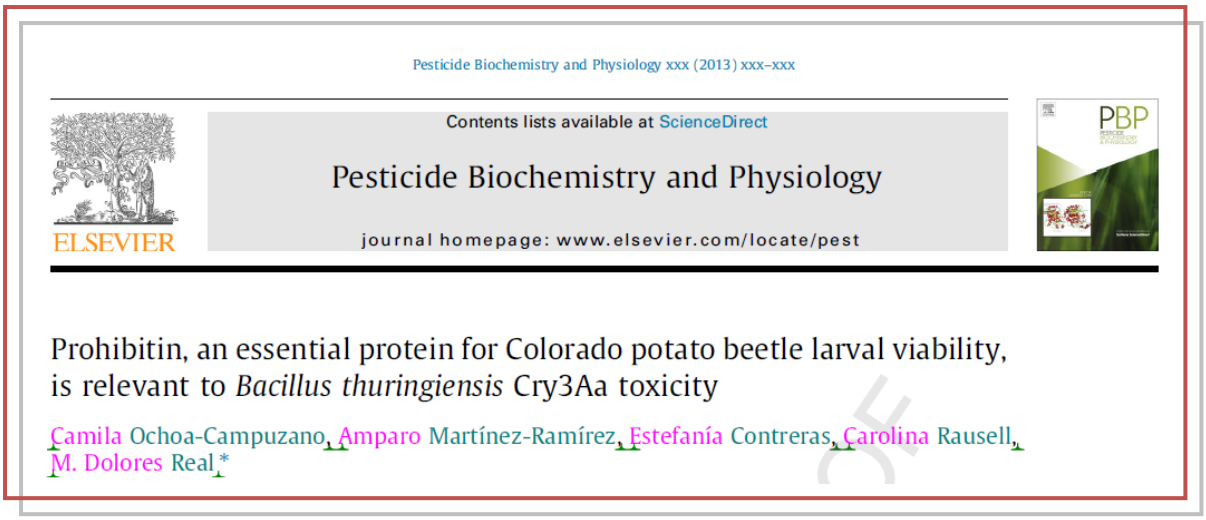
Inmaculada García-Robles, Camila Ochoa-Campuzano, Jorge Sánchez [1], Estefanía Contreras, M. Dolores Real, Carolina Rausell*

Significado funcional de la proteólisis asociada a membrana en la toxicidad de la toxina Cry3Aa de *Bacillus thuringiensis* en el escarabajo de la patata

Objetivos: Evaluación del papel del motivo de reconocimiento de la metaloproteasa ADAM presente en la toxina Cry3Aa en la proteólisis asociada a membrana del epitelio intestinal de *L. decemlineata* y en la toxicidad, mediante el diseño toxinas mutantes que presentaron cambios aminoacídicos en dicho motivo.

Metodología: Las toxinas modificadas se produjeron mediante mutagénesis dirigida del gen *cry3Aa* clonado en un vector que se introdujo y se expresó en una cepa acristalífera de *Bt*. Los ensayos de toxicidad en *L. decemlineata* se llevaron a cabo por contaminación de discos de patata con las toxinas. Se analizaron los patrones de proteólisis asociada a membrana de las toxinas Cry3Aa silvestres y mutantes mediante incubación de las toxinas con vesículas de membrana del epitelio intestinal de las larvas de *L. decemlineata* e inmunodetección de los fragmentos. En el análisis de la accesibilidad a la metaloproteasa de las proteínas mutantes se utilizó un sustrato fluorogénico de metaloproteasa como competidor de proteolisis.

Conclusiones: Se confirmó la importancia de la interacción de las toxinas Cry3 a través del motivo de reconocimiento por la metaloproteasa ADAM, ya que las toxinas mutantes que presentaron cambios aminoacídicos en este motivo resultaron ser menos tóxicas que las silvestres e interaccionaron de manera menos eficiente con la metaloproteasa ADAM.



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Prohibitin, an essential protein for Colorado potato beetle larval viability, is relevant to *Bacillus thuringiensis* Cry3Aa toxicity

Camila Ochoa-Campuzano, Amparo Martínez-Ramírez, Estefanía Contreras, Carolina Rausell, M. Dolores Real*

La prohibitina, una proteína esencial para la viabilidad del escarabajo de la patata, es relevante para la toxicidad de Cry3Aa de *Bacillus thuringiensis*

Objetivos: Estudio de la implicación de la proteína prohibitina-1 (Phb1) del escarabajo de la patata en el modo de acción de la toxina Cry3Aa.

Metodología: Las fracciones ricas y no ricas en colesterol de la membrana epitelial *de L. decemlineata* se separaron mediante gradiente de sacarosa. Las proteínas de unión a Cry3Aa presentes en dichas fracciones se identificaron mediante "ligand blot". La secuencia de DNA codificante de Phb1 se determinó mediante RACE. El silenciamiento de Phb1 en larvas de *L. decemlineata* se llevó a cabo por contaminación de la dieta con dsRNA. En los estudios funcionales se analizó la proteolisis de Cry3Aa por la metaloproteasa ADAM utilizando un sustrato fluorogénico de ésta.

Conclusiones: Phb1 del escarabajo de la patata presentó unión a la toxina Cry3Aa de *Bt* y la interacción de ambas proteínas podría ser importante para la proteolisis de Cry3Aa por la metaloproteasa ADAM. La proteína Phb1 es esencial para la viabilidad de las larvas ya que el silenciamiento del correspondiente gen provocó efectos deletéreos en las larvas de *L. decemlineata.* La combinación del silenciamiento de Phb1 junto con el tratamiento con Cry3Aa produjo un efecto tóxico sinérgico que puede constituir una estrategia prometedora para el control de plagas.

Resultados y discusión

1. Toxicidad de las toxinas Cry de *Bt* en *T. castaneum*

A pesar de la importancia de *T. castaneum* como plaga de grano almacenado y como especie modelo en el orden de los coleópteros, apenas existen datos de toxicidad producida por *Bt* y sus toxinas en esta especie. El escarabajo rojo de la harina, que ha desarrollado resistencia a muchos insecticidas químicos, ha resultado también refractario al efecto de las toxinas Cry, y su modo de vida subterráneo y su dieta basada en harina complica aún más el diseño de los ensayos de toxicidad.

En estudios previos, en larvas de *T. castaneum*, se evaluaron por contaminación de la dieta las toxinas Cry1F, Cry8E, Cry8F, Cry8G y Cry34/35Ab, que resultaron inactivas, y Cry23/37 y Cyt2C presentaron actividad insecticida (van Frankenhuyzen y Nystrom, 2009; Donovan *et al*., 2000; Rupar *et al*., 2000; Oppert *et al*., 2010). La toxina Cry3Aa, activa contra coleópteros como *L. decemlineata* o *T. molitor*, apenas lo es contra las larvas de *T. castaneum*, aunque sí produce un efecto deterrente y una deceleración en el desarrollo (Oppert *et al*., 2011). Hasta la fecha, ninguna toxina del grupo de las toxinas Cry de tres dominios ha resultado altamente tóxica para el insecto.

Nos propusimos diseñar un nuevo ensayo de toxicidad, basado en la preparación de una pasta de harina mezclada con las cepas de *Bt* esporuladas, para hacer discos completamente homogéneos y replicables y ampliar el rango de búsqueda de cepas de *Bt*, con el objetivo de encontrar alguna que pudiera ser tóxica para esta especie, permitiendo así el estudio del modo de acción en esta especie y por extensión en el orden de los coleópteros.

Los discos utilizados permiten controlar mejor la dosis ingerida por la larva y observar fácilmente el efecto deterrente y el consumo. Se probaron con este procedimiento, en larvas de *T. castaneum*, las toxinas Cry3Aa, Cry23/37 y Cyt2Ca que habían resultado tóxicas o ligeramente tóxicas mediante otro tipo de ensayos, la toxina Cry34/35 tóxica para el coleóptero *D. virgifera virgifera* (Ellis *et al*., 2002) y otras toxinas del grupo Cry3 específicas contra coleópteros (Cry3Ba y Cry3Ca). Nuestros resultados muestran que Cry3Ba y Cry23/37 resultaron tóxicas y, aunque Cry3Aa apenas lo fue, produjo un claro efecto deterrente en las larvas y una deceleración en el desarrollo, como se había descrito previamente (Oppert *et al*., 2011). En nuestras condiciones de ensayo, las cepas productoras de Cry3Ca y Cry34/35 no produjeron toxicidad ni efectos en el desarrollo, y a diferencia de lo observado en un estudio anterior, tampoco fue activa la cepa productora de Cyt2Ca (Rupar *et al*., 2000).

2. Estudio de la respuesta de *T. castaneum* a cepas de *Bt* tóxicas para el insecto

Las cepas de *Bt* cuya toxicidad frente a las larvas de *T. castaneum* hemos demostrado producen Cry3Ba y Cry23/37, toxinas que pertenecen a grupos estructuralmente diferentes de toxinas Cry. La toxina Cry3Ba pertenece al grupo Cry3, específico de coleópteros, y se incluye dentro de la clase de toxinas Cry de tres dominios, grupo sobre el cual existen muchos estudios relativos a su modo de acción en lepidópteros. Las toxinas Cry23/37, también denominadas CryET33/34, están descritas como tóxicas para algunos coleópteros y pertenecen al grupo de toxinas relacionadas con las Mtx2 y Mtx3 producidas por *Bt.*

Con el objetivo de identificar y cuantificar proteínas diferencialmente expresadas en respuesta a los tratamientos con estos dos tipos de toxinas, en un momento temprano en el proceso de intoxicación, realizamos un análisis proteómico de la respuesta de *T. castaneum.*

Se eligió el método iTRAQ como técnica novedosa de análisis proteómico global, que ofrece gran cobertura y ha sido todavía poco utilizada en estudios relacionados con la respuesta a *Bt*, ya que sólo dos estudios la utilizan para comparar los proteomas de dos cepas de lepidópteros resistentes a toxinas Cry (Tiewsiri y Wang, 2011; Zhang *et al*., 2012). Esta técnica utiliza el marcaje diferencial de proteínas o péptidos con isótopos estables y, combinada con espectrometría de masas, permite la identificación y determinación de ratios de concentración de proteínas expresadas en células o tejidos en diferentes condiciones, como por ejemplo, después de un tratamiento. La cuantificación relativa de proteínas se realiza comparando los pares de péptidos marcados con isótopos en los correspondientes espectros de masas (Wiese *et al*., 2007).

Se identificaron 335 proteínas, de las cuales 239 resultaron comunes a ambos tratamientos y correspondieron a una gran variedad de categorías funcionales, poniendo de manifiesto la gran cobertura de la técnica. La categoría mayoritaria fue la de proteínas con actividad catalítica, mayoritariamente oxidorreductasas e hidrolasas, seguida de proteínas de unión, principalmente de unión a cationes y a pequeñas moléculas.

Como resultado de la intoxicación de larvas de *T. castaneum* con una dosis aproximada a la LC_{50} de cepas productoras de Cry3Ba o de Cry23/37, se seleccionaron 7 proteínas que mostraron una abundancia diferencial frente a larvas control no tratadas. Tres de las siete proteínas presentaron un incremento mayor de 1.5 veces en ambos tratamientos: la proteína de unión a odorantes C12, la apolipoforina III y la proteína quimiosensora 18. Las 4 proteínas restantes, que mostraron una disminución frente a larvas control en ambos tratamientos, fueron: el precursor de la apolipoproteína I/II o

lipoforina, la proteína ribosomal L13a, una proteína cuticular y la subunidad Eα de la piruvato deshidrogenasa.

2.1 Proteínas de *T. castaneum* cuya expresión se induce en respuesta al tratamiento con cepas de *Bt*

Las proteínas de *T. castaneum* que se incrementaron tras el tratamiento con la cepas de *Bt* productoras de Cry3Ba o Cry23/37 fueron la proteína de unión a odorantes C12, la apolipoforina III y la proteína quimiosensora 18. La proteína de unión a odorantes y la quimiosensora pertenecen a la familia de las lipocalinas, algunas de ellas presentes en la hemolinfa y relacionadas con la defensa del insecto, y la apolipoforina III ha sido implicada, además de en el transporte de lípidos, en la respuesta inmune.

2.1.1 Proteínas de unión a odorantes y quimiosensoras

En ambos tratamientos, la proteína que presentó una abundancia diferencial mayor respecto a larvas control fue la proteína de unión a odorantes C12 (OBP C12), que aumentó alrededor de 4 veces. Este incremento se correlaciona con un aumento, incluso mayor, a nivel transcripcional. Para analizar el significado de la inducción observada, se comparó con la expresión de dicha proteína en larvas intoxicadas con la cepa productora de Cry1Ac, toxina activa contra lepidópteros que carece de efecto sobre *T. castaneum*. También se produjo aumento de los transcritos de OBP C12, aunque significativamente menor que con los otros dos tratamientos, lo que sugiere que la inducción de esta proteína puede formar parte de la respuesta general del insecto a la intoxicación por *Bt*.

Las proteínas de unión a odorantes (OBP) son importantes componentes del sistema olfatorio de los insectos que se encuentran normalmente en los órganos olfatorios como las antenas o en los palpos maxilares, y pertenecen a la gran superfamilia de proteínas transportadoras lipocalinas. Se localizan en altas concentraciones en la linfa de la sensila, estructura en forma de pelo donde se localizan las dendritas de las neuronas olfatorias. Las hormonas u odorantes que provienen del entorno exterior atraviesan los poros de la sensila y se cree que las OBP facilitan el transporte a través de esta fina capa de linfa sensilar hasta los receptores de las dendritas de las neuronas olfatorias, ya que esta capa acuosa es impenetrable para los componentes hidrofóbicos. De esta forma, cuando las neuronas receptoras reciben la señal olfatoria la transmiten directamente al lóbulo antenal en forma de impulsos electroquímicos, que son procesados en el protocerebro (Leal, 2005).

Se buscó en las bases de datos proteínas que presentaran similitud con OBP C12 y fue el precursor de 12 kDa de la proteína de hemolinfa b de *T. molitor* (Tm THP12) la que presentó la mayor similitud, con un 64% de identidad de secuencia aminoacídica. Tm THP 12, a diferencia de las demás OBP, está descrita como una proteína de hemolinfa (Graham *et al*., 2001).

También se han encontrado otras OBP en órganos no quimiorreceptores. En *T. molitor*, además de THP12, existen otras 5 isoformas de proteínas de hemolinfa b de 12 kDa y dos proteínas B producidas por las glándulas accesorias de los machos, y también se encontró una OBP en la hemolinfa de *Ceratitis capitata*, y una sericotropina en el cerebro de *G. melonella* (Graham *et al*., 2001; 2003). Todas ellas forman una nueva familia de proteínas con similitud a las OBP pero con una función alternativa a la de transportador de odorantes.

La resolución de la estructura de la proteína THP12 de *T. molitor* puso de manifiesto que contiene 6 α-hélices conectadas con lazos, formando un bolsillo hidrofóbico en su interior capaz de unir pequeñas moléculas hidrofóbicas y feromonas (Rothemund *et al*., 1999).

Entre las proteínas de la base de datos que presentaron homología con OBP C12 encontramos, además de THP12, las demás isoformas de las proteínas de hemolinfa b de 12 kDa de *T. molitor*, las proteínas de hemolinfa de 13 kDa de *T. molitor* y de *T. castaneum*, las proteínas B de las glándulas accesorias de los machos de *T. molitor* y sericotropinas de lepidópteros, que son proteínas OBP con posible función alternativa a la de transportador de odorantes, encontradas en órganos no quimiorreceptores. Entre las 265 proteínas de unión a odorantes generales anotadas en el genoma de *T. castaneum* (Tribolium Genome Sequencing Consortium, 2008), sólo algunas proteínas de hemolinfa de bajo peso molecular se agrupan con OBP C12, reforzando el papel particular de dicha proteína en la hemolinfa.

La posible implicación de OBP C12 en la respuesta inmune general del insecto a los patógenos se comprobó mediante RNAi. Esta técnica se ha utilizado ampliamente en *T. castaneum*, ya que resulta muy útil para el análisis funcional de nuevos genes y este insecto, a diferencia de otros como *D. melanogaster*, presenta una respuesta sistémica al RNA de doble cadena. Hasta la fecha se ha conseguido introducir el RNA de interés mediante inyección en pupas, en el escarabajo adulto y en larvas de último estadio. En cambio, esta técnica no había sido utilizada previamente en larvas de estadios tempranos. En nuestro estudio nos propusimos inyectar larvas más pequeñas, de segundo y tercer estadio, que nos permitiesen realizar los ensayos de toxicidad sin que las larvas llegasen a pupar.

Las larvas silenciadas presentaron una sensibilidad ligeramente mayor a los tratamientos con las cepas productoras de Cry3Ba y de Cry23/37 frente a larvas no silenciadas. En cambio, las larvas silenciadas tratadas con la cepa productora de Cry1Ac no tóxica no mostraron diferencias estadísticamente significativas respecto a las no silenciadas. Estos resultados apoyan que la proteína OBP C12, que es inducible tras tratamientos con cepas de *Bt* tóxicas, tiene una función alternativa a la de transportador de odorantes, por su similitud a Tm THP12 y otras proteínas de hemolinfa y en tal caso, dicha función podría ser la de contribuir en la defensa a estas cepas que resultaron tóxicas.

Existen estudios en los que se observa una inducción de diferentes OBP desencadenadas por infecciones microbianas. Algunos ejemplos son el aumento de Obp99c en *D. melanogaster* y el aumento de OBP4 y OBP7 en *A. gambiae* después de la infección por el hongo *B. bassiana* (Levy *et al*., 2004; Aguilar *et al*., 2005) o el aumento de la proteína de unión 7 de la antena de *B. mori* después de la inyección de *Bacillus megaterium* (Song *et al*., 2006). En estos trabajos se sugiere un posible nexo de unión entre el sistema olfatorio y el sistema inmunitario en invertebrados.

Otra de las proteínas que también se incrementó después del tratamiento de las larvas de *T. castaneum* con las cepas de *Bt* productoras de Cry3Ba y Cry23/37 fue la proteína quimiosensora 18 (CSP 18). Las proteínas quimiosensoras (CSP) constituyen una familia que, al igual que las OBP, pertenecen a la superfamilia de las lipocalinas. Son proteínas de unos 13 kDa, hidrofílicas, formadas por 6 hélices alfa rodeando a un bolsillo hidrofóbico en su interior como las OBP, y que también se han encontrado en la linfa sensilar, pero no comparten similitud de secuencia con éstas. Por ello, han sido involucradas también en la quimiopercepción y el transporte de compuestos químicos hidrofóbicos desde el aire o agua a los receptores olfatorios o gustativos, aunque se han encontrado también algunas CSP en otros tejidos. Se ha propuesto que algunas de ellas puedan tener funciones no olfatorias y uno de los ejemplos más interesantes es el posible papel de la CSP p10 del coleóptero *Periplaneta americana* en la transmisión de la señal química para la regeneración de extremidades, ya que esta proteína se acumuló en las patas del insecto cuando estaban en proceso de regeneración (Nomura *et al*., 1992). También se ha descrito el incremento de CSP antes de la muda en *C. fumiferana* (Wanner *et al*., 2005). Estos hallazgos sugieren que algunos miembros de la familia CSP o OBP, como es el caso de las feroquinas 2 y 3 de *D. melanogaster*, que también se inducen durante la metamorfosis o tras la infección por el virus DCV, están relacionados en el proceso de formación tisular o regeneración. Por todo ello, se podría hipotetizar que el incremento de CSP 18, que comparte un 57% de identidad de secuencia aminoacídica con p10 de *P. americana*, podría deberse al proceso regenerativo

desencadenado en el insecto dirigido a combatir el daño ocasionado por los tratamientos con *Bt*.

2.1.2 Apolipoforina III

La apolipoforina III (apoLpIII) es una proteína involucrada en el transporte de lípidos, pero además se ha visto implicada en el reconocimiento de patógenos y como mediadora de la respuesta inmune en diferentes especies de insectos (Whitten *et al*., 2004).

Esta proteína, que se ha descrito que se incrementa en la hemolinfa de los insectos después de la infección por diversos patógenos (Zdybicka-Barabas y Cytryńska, 2011; Kang *et al*., 2003; Son y Kim, 2011; Gupta *et al*., 2010), también aumenta en *T. castaneum* tras el tratamiento de las larvas de este insecto con las cepas de *Bt* productoras de Cry3Ba y de Cry23/37. A nivel transcripcional, el RNA mensajero de apoLpIII de *T. molitor* aumentó tras la intoxicación con Cry3Aa (Oppert *et al*., 2012), al igual que hemos observado en *T. castaneum* después del tratamiento con Cry3Ba. Además, hemos demostrado que el silenciamiento del gen codificante de apoLpIII produjo una mayor sensibilidad a Cry3Ba que en las larvas sin silenciar, poniendo de manifiesto la importancia de esta proteína en el modo de acción de las toxinas Cry y en la defensa inmunitaria contra su efecto tóxico.

En diferentes estudios se ha relacionado la apoLpIII y la activación de la cascada de la proPO, un componente clave en la respuesta humoral de los insectos y que se activa a PO proteolíticamente (Park *et al*., 2005; Mullen y Goldsworthy, 2003). La PO activa conduce a la formación de melanina, que protege frente a los invasores y actúa sellando la herida. Durante el proceso, las especies reactivas de oxígeno generadas resultan tóxicas para los organismos invasores. En *T. castaneum*, hemos demostrado que la actividad PO en la hemolinfa de las larvas tras el tratamiento con Cry3Ba es mayor que en las larvas sin tratar o tratadas con una toxina Cry no tóxica como Cry1Ac, y que las larvas a las que se les silenció el gen codificante de apoLpIII presentaron una actividad PO menor. Por tanto, nuestros datos apoyan la implicación de apoLpIII en la regulación de la cascada proPO también en este insecto.

2.2 Proteínas de *T. castaneum* que disminuyeron en respuesta a los tratamientos con cepas de *Bt*

Las proteínas que, de acuerdo con el estudio proteómico, disminuyeron en larvas de *T. castaneum* tras el tratamiento con las cepas de *Bt* productoras de Cry3Ba o

Cry23/37 fueron: el precursor de la apolipoproteína I/II o lipoforina, la proteína ribosomal L13a, una proteína cuticular y la subunidad Eα de la piruvato deshidrogenasa. Estas 4 proteínas podrían estar relacionadas con el metabolismo y desarrollo del insecto.

2.2.1 Precursor de la apolipoproteína I/II

El precursor de la apolipoproteína I/II es una proteína que sufre un procesado post-traduccional por parte de una furina liberando la apolipoforina I y la apolipoforina II, las dos subunidades que componen la lipoforina del insecto. La lipoforina ejerce un papel importante en el transporte de lípidos de la dieta desde el intestino hasta el cuerpo graso para su almacenaje y del cuerpo graso a los tejidos periféricos. Se ha propuesto la existencia de un equilibrio entre la respuesta al estrés y el mantenimiento de la homeostasis, idea consistente con una posible estrategia para redirigir los recursos utilizados en la biosíntesis de proteínas de almacenaje a combatir el daño o la infección (Lourenço *et al*., 2009). Por ejemplo, en grillos, se demostró el conflicto entre el transporte de lípidos y la función inmune, ya que durante el vuelo intenso, los insectos eran menos capaces de defenderse ante infecciones bacterianas (Adamo *et al*., 2008). Este equilibrio podría explicar que, en situaciones de estrés en las que el sistema inmune está activado, la expresión de moléculas transportadoras de lípidos y de almacenaje, como la lipoforina, se encuentre reprimida.

2.2.2 Proteína ribosomal L13a

Los ribosomas son complejos macromoleculares compuestos de RNA ribosomal y proteínas y son responsables de la síntesis de las cadenas polipeptídicas en todas las células. La actividad catalítica principal del ribosoma la produce el RNA ribosomal, y la gran mayoría de proteínas se encuentran en forma de dominios globulares en la superficie de éste, lejos del sitio de catálisis y se cree que pueden proporcionar estabilidad estructural al ribosoma (Cech, 2000). Numerosos estudios han demostrado que algunas proteínas ribosomales pueden ejercer funciones no relacionadas con la síntesis proteica y se ha detectado una relación estrecha entre la localización de la proteína en la superficie del ribosoma y una función extrarribosomal (Ray *et al*., 2007).

La proteína ribosomal L13a forma parte de la subunidad grande del ribosoma 60S y su homóloga en procariotas está situada en la superficie del ribosoma con un contacto mínimo con el RNA (Ban *et al*., 2000). Además, se ha observado que el interferón gamma, clave en el inicio del proceso inflamatorio, produce la fosforilación de L13a y

su liberación del ribosoma, para ejercer entonces su función extrarribosomal. Se ha descrito que los ribosomas de eucariotas superiores deficientes en esta subunidad son completamente funcionales y ejercen su función en la traducción correctamente, por ello, se sugiere que el ribosoma puede funcionar como un sitio de almacenaje de factores reguladores con una función extrarribosomal (Chaudhuri *et al*., 2007). Sin embargo, la inactivación de los dos homólogos de L13a en levadura resultó en un retardo severo en el crecimiento y muerte celular (Chen y Ioannou, 1999), sugiriendo que el posible papel de L13a ha podido evolucionar desde ser esencial para la función ribosomal en eucariotas inferiores a una nueva función extrarribosomal en eucariotas superiores (Chaudhuri *et al*., 2007).

En humanos, algunas proteínas ribosomales con funciones alternativas ejercen el papel de reguladores de la traducción o la actividad de proteínas relacionadas con el ciclo celular, como por ejemplo: la proteína L26, que regula la traducción del supresor tumoral p53 (Takagi *et al*., 2005; Chen *et al*., 2012); L23, L11 y L5, que regulan la actividad de una proteína que a su vez regula a p53 (Jin *et al*., 2004; Bursać *et al*., 2012); la proteína ribosomal mitocondrial L41, que puede suprimir el crecimiento celular (Yoo *et al*., 2005); o S13, que se le relaciona con la inducción y progresión del ciclo celular de células cancerígenas (Guo *et al*., 2011).

La subunidad L13a libre forma parte de un complejo llamado GAIT que media el silenciamiento a nivel traduccional de un grupo de RNA mensajeros (Mukhopadhyay *et al*., 2009). Algunos de los mensajeros más conocidos regulados por este complejo codifican proteínas relacionadas con la inflamación en monocitos como la ceruloplasmina, quimioquinas y sus receptores y otras moléculas importantes en la respuesta por citoquinas, que han sido inducidas por el interferón gamma. El complejo GAIT, una vez ensamblado, se une a elementos presentes en la región 3' UTR de estos mensajeros, produciendo su silenciamiento traduccional para terminar con el proceso inflamatorio, que puede ser perjudicial si se prolonga de forma crónica (Vyas *et al*., 2009).

Teniendo esto en cuenta, se podría especular que los cambios en los niveles de L13a libre en las larvas después de los tratamientos con cepas de *Bt* productoras de Cry3Ba y de Cry23/37 podrían deberse a la regulación de la expresión de proteínas relacionadas con la inflamación. La disminución de uno de los componentes del complejo GAIT, como la proteína ribosomal L13a, puede ocasionar la disminución de complejos GAIT ensamblados, con la consecuente disminución del silenciamiento de los mensajeros diana. En este caso, se produciría una actividad inflamatoria elevada sostenida en el

tiempo que podría tener como objetivo combatir el daño ocasionado por los tratamientos con toxinas Cry.

2.2.3 Piruvato deshidrogenasa subunidad Eα

El complejo de la piruvato deshidrogenasa está constituido por tres enzimas, uno de ellos, la subunidad Eα, se encarga de realizar la descarboxilación del piruvato en la matriz mitocondrial. El complejo enlaza la ruta de la glicolisis con el ciclo del ácido cítrico, ya que transforma el piruvato proveniente de la glicolisis en acetil CoA, que se utiliza en el ciclo del ácido cítrico para llevar a cabo la respiración celular. En insectos, existen diversos estudios en los que se muestra represión de genes que codifican para enzimas metabólicos asociados con glicolisis, ciclo del ácido cítrico y catabolismo de lípidos después de la ingestión de toxinas Cry (Oppert *et al*., 2012; Sayed *et al*., 2010). También en *C. elegans* se observó que se podía adquirir resistencia a toxinas Cry por un estado de hipoxia elevado debido a una mutación en un factor inducible de hipoxia 1, que induce la piruvato deshidrogenasa quinasa, la cual, a su vez, inhibe la piruvato deshidrogenasa y con ello la fosforilación oxidativa (Bellier *et al*., 2009). La presencia del factor inducible de hipoxia 1 ó la disminución de la expresión del complejo piruvato deshidrogenasa y otros genes relacionados con estado metabólico bajo ha resultado efectivo en la supervivencia de muchas especies contra situaciones adversas del entorno, como la desecación, congelación, anoxia, etc. Los insectos deben soportar fluctuaciones predecibles e impredecibles del entorno que no son favorables para la supervivencia, desarrollo y reproducción. Un estado de quiescencia o bajada de la marcha normal metabólica permite al insecto sobrevivir a estos cambios, minimizando así los desequilibrios en procesos celulares que puedan producir el desarrollo de las condiciones patológicas (Hand *et al*., 2011).

En nuestro estudio, hemos observado la disminución de la subunidad Eα de piruvato deshidrogenasa en larvas de *T. castaneum*, en respuesta a los tratamientos con las cepas de *Bt* tóxicas para el insecto. Este resultado está de acuerdo con la represión de genes codificantes para enzimas metabólicos observado en otros organismos al ser intoxicados por toxinas Cry, que podría estar encaminado al mantenimiento de una tasa metabólica baja y a la contención del progreso de la infección.

2.2.4 Proteína cuticular

La cutícula es una estructura dinámica que forma parte de un exoesqueleto multifuncional y una barrera impermeable entre el insecto y su entorno. La cutícula

también es esencial para el mantenimiento de la morfología del cuerpo y su integridad. Las proteínas cuticulares suelen ser proteínas ricas en glicina, y muchas de ellas contienen repeticiones cortas conservadas ricas en este aminoácido (Charles *et al*., 1992). La proteína cuticular de *T. castaneum* que hemos encontrado expresada diferencialmente en nuestro análisis proteómico presenta repeticiones conservadas GXGX contenidas en muchas proteínas cuticulares que pueden formar estructuras flexibles en forma de bucle. Se ha comprobado que, en *L. decemlineata*, estreses ambientales tales como el tratamiento con un insecticida organofosforado o un entorno seco puede inducir cambios en la naturaleza de la cutícula, permitiendo al insecto adaptarse a las condiciones ambientales difíciles (Zhang *et al*., 2008a). También en *L. decemlineata* se observó un descenso de la expresión de diferentes proteínas cuticulares ricas en glicina, cuando el insecto pasaba de tasas metabólicas altas a tasas metabólicas bajas, parando de comer y de moverse (Yocum *et al*., 2009). Este descenso en la tasa metabólica que caracteriza a la diapausa podría, de manera análoga, dar cuenta de la acción deterrente y los efectos en el desarrollo que caracterizan la acción de las cepas de *Bt* tóxicas. Es posible que la deceleración en el desarrollo observada después de los tratamientos con las cepas de *Bt* productoras de Cry3Ba o de Cry23/37 a los que hemos sometido las larvas afecte a los periodos de muda y la duración de los estadios, y con ellos a la expresión de proteínas cuticulares.

3. Receptores implicados en el modo de acción de Cry3Ba en *T. castaneum*

El intestino del organismo susceptible es la diana principal de las toxinas de *Bt* y las proteínas con capacidad de unión a las toxinas, presentes en el mismo, son críticas en la determinación del rango de especies en las que la toxina es activa. La reducción o pérdida de unión de la toxina Cry a su receptor constituye, en muchos casos, un mecanismo importante de resistencia. Así pues, la identificación de moléculas del intestino que confieren susceptibilidad a los organismos diana es esencial para entender el mecanismo molecular de acción de estas toxinas, y a su vez facilitar un diseño más racional de productos basados en *Bt*, para evitar o retrasar el desarrollo de resistencia.

El modo de acción de las toxinas Cry de *Bt* en tres dominios está muy estudiado en los insectos lepidópteros. Las proteínas de la familia de las cadherinas, las aminopeptidasas N, las fosfatasas alcalinas y los glicolípidos han sido las moléculas receptoras caracterizadas y confirmadas como receptores funcionales (Pigot y Ellar, 2007), aunque más recientemente también un transportador ABC ha sido identificado como receptor funcional y responsable de resistencia en lepidópteros (Tanaka *et al*.,

2013; Gahan *et al*., 2010). En dípteros, la cadherina, APN y ALP también se han propuesto como receptores funcionales, sugiriendo un modo de acción similar en los diferentes órdenes de insectos. En coleópteros, aunque en algunos de ellos se han encontrado cadherinas que unen toxinas Cry, tan solo en *T. molitor* se ha confirmado su implicación como receptores funcionales. En este orden, hasta la fecha, no se ha demostrado inequívocamente la función de ninguna otra molécula como receptor de toxinas Cry de *Bt*.

3.1 Proteínas de *T. castaneum* de unión a Cry3Ba

En nuestro trabajo, observamos que la cepa productora de Cry3Ba resultó una de las más tóxicas para *T. castaneum* y los ensayos de unión de la toxina purificada a vesículas del intestino del insecto mostraron que la toxina se unía específica y mayoritariamente a una banda de alrededor de 75 kDa, en la cual se identificó la subunidad A isoforma 2 de la V-ATPasa, una aminopeptidasa N, un simportador de sodio/soluto, el precursor de la apolipoproteína I/II y una cadherina E, entre otras proteínas.

Como se ha mencionado anteriormente, se han identificado glicolípidos capaces de unir toxinas Cry en *M. sexta* y en *C. elegans* (Griffits *et al*., 2005; Griffits *et al*., 2001) y, en este último, se ha demostrado que actúan como receptores funcionales (Griffits *et al*., 2001). Las interacciones entre la toxina y los glicolípidos se han estudiado en el contexto de la membrana, pero también se encuentran glicolípidos similares en transportadores lipídicos como las partículas lipídicas de alta y baja densidad de los vertebrados (HDL y LDL) (Rensen *et al*., 2004) y en las lipoforinas de los insectos. Por tanto, no resulta inesperado que el precursor de la apolipoproteína I/II de *T. castaneum* sea capaz de unir toxinas Cry como Cry3Ba. De hecho, se ha observado que la toxina Cry1Ac forma agregados con la fracción lipídica del plasma de *G. mellonella*, donde se encontraron las apolipoproteínas I y II que forman la lipoforina (Ma *et al*., 2012). A esta lipoforina, además, se le atribuye la capacidad de secuestrar a la toxina en el lumen del intestino y por ello, se la ha implicado en mecanismos de resistencia a las toxinas Cry (Ma *et al*., 2012).

Las proteínas de la familia de las cadherinas y las APN, confirmadas como mediadores del proceso tóxico en diversos insectos, también han sido identificadas en este trabajo como moléculas de unión a Cry3Ba en *T. castaneum* y por tanto fueron las primeras moléculas en ser elegidas para el estudio posterior de validación como receptores funcionales mediante RNAi. Es interesante destacar que la cadherina E, que hemos identificado como posible proteína de unión de la toxina Cry3Ba en *T.*

castaneum, es una proteína ortóloga de la cadherina descrita como receptor funcional de Cry3Aa en *T. molitor* (Fabrick *et al*., 2009), con la que comparte un 54% de identidad de secuencia aminoacídica.

La subunidad A de la V-ATPasa (protón ATPasas de tipo vacuolar), junto con la B, constituyen las subunidades catalíticas del enzima. Se ha detectado unión de diferentes subunidades de la V-ATPasa con toxinas Cry en otros insectos (Chen *et al.,* 2010a; Krishnamoorthy *et al*., 2007; Bayyareddy *et al*., 2009), por tanto, es un candidato importante para ser validado como receptor funcional. La relevancia de esta molécula viene además reforzada porque se han relacionado defectos en su función con enfermedades humanas (Marshansky y Futai, 2008), y porque el silenciamiento de la V-ATPasa resulta letal en insectos y en otros organismos (Ferea y Bowman, 1996; Dow *et al*., 1997). Por esta razón, desafortunadamente la validación mediante RNAi no es viable y concretamente en *T. castaneum* el silenciamiento de este gen se ha propuesto que podría ser utilizado para controlar la plaga, por ser un gen esencial en este insecto (Whyard *et al*., 2009).

El simportador de sodio/soluto es una molécula que no ha sido implicada hasta la fecha en el modo de acción de las toxinas de *Bt* y su identificación como molécula de unión a la toxina Cry3Ba en *T. castaneum* resulta especialmente interesante, ya que contiene repeticiones tipo cadherina en su extenso dominio extracelular. Otra molécula transportadora, el transportador ABC de la subfamilia C2, sí ha sido validada como receptor funcional de las toxinas Cry1Ab y Cry1Ac en *B. mori* (Tanaka *et al*., 2013) y está implicada en la resistencia de una cepa de *H. virescens* a Cy1Ac y de *B. mori* a Cry1Ab (Gahan *et al*., 2010; Atsumi *et al*., 2012). Por ello, elegimos el simportador de sodio junto con la cadherina E y la APN para confirmar, mediante RNAi, su función como receptor de la toxina Cry3Ba en *T. castaneum*.

El tratamiento de larvas con la toxina Cry3Ba en las que se silenció mediante RNAi el gen de la cadherina o el gen del simportador de sodio produjo una mortalidad significativamente menor que en larvas control no silenciadas. Este resultado demuestra que ambas moléculas funcionan como mediadores de la toxicidad producida por Cry3Ba. En cambio, cuando el gen silenciado fue APN, no se observó descenso en la mortalidad producida por la toxina Cry3Ba, en relación a la mortalidad causada en larvas control.

3.1.1 Aminopeptidasa N (TcAPN-I)

Las APN han sido identificadas en un gran número de especies de insectos como moléculas de unión a toxinas Cry, pero pocos estudios validan estos receptores como

mediadores funcionales de la toxicidad en lepidópteros (Rajagopal *et al*., 2002; Sivakumar *et al*., 2007; Gill y Ellar, 2002) y en dípteros (Saengwiman *et al*., 2011). Hasta la fecha, en coleópteros, las APN no han sido relacionadas con el modo de acción y en el presente trabajo el silenciamiento de la APN identificada como proteína de unión a Cry3Ba no produjo ningún impacto en la mortalidad de las larvas tratadas.

Las APN son una familia de enzimas que pertenecen a la familia de metaloproteasas dependientes de zinc, con función digestiva y presentes en la membrana de las microvellosidades del intestino de los insectos. Hasta la fecha se han clonado y caracterizado diferentes isoformas de APN de más de 20 insectos lepidópteros, y se han agrupado en clases atendiendo a su secuencia primaria. En algunos lepidópteros se ha identificado la expresión de hasta 7 APN diferentes y se han agrupado en clases, aunque solo hay evidencias de interacción entre miembros de 5 de las clases y toxinas Cry (Pigott y Ellar, 2007; Crava *et al*., 2010). El promedio de identidad de secuencia dentro de cada clase varía entre el 56% y el 67%. Entre clases, la identidad de secuencia es menor y oscila entre el 25 y el 38%. De hecho, algunas APN comparten una identidad de secuencia mayor con APN de especies muy alejadas entre sí que con APN de su misma especie, aunque ambas sean capaces de unir la misma toxina (Pigott y Ellar, 2007).

La APN que hemos identificado en *T. castaneum* como posible receptor de toxinas de Cry3Ba, denominada APN-I, comparte el 47% de identidad con otra APN denominada APN-II y el 41% con APN-III, ambas de *T. castaneum*, y son las dos proteínas más similares a APN-I de las bases de datos. La ausencia de efecto del silenciamiento de APN-I nos llevó a plantear la hipótesis de que diferentes APN del insecto podrían tener una función redundante, uniendo la misma toxina y posiblemente desempeñando la misma función. Por ello, se realizó un silenciamiento múltiple en el que se silenciaron en las larvas las tres APN a la vez. En este caso, tras el tratamiento con la toxina Cry3Ba, tampoco se observó descenso en la mortalidad, aunque ello no excluye la implicación en la acción tóxica de otras APN no silenciadas.

Así pues, los resultados obtenidos indican que la APN, aunque une la toxina Cry3Ba, no actúa como mediadora de la toxicidad. Se han identificado muchas proteínas en la membrana del intestino de los insectos que pueden unir toxinas Cry pero esta capacidad de unión no siempre se relaciona con susceptibilidad, ya que no todas las uniones entre toxina y receptor conducen a la oligomerización de la toxina, formación de poro u otro evento intracelular relacionado con la acción tóxica (Crickmore, 2005; Jenkins *et al.*, 1999).

3.1.2. Cadherina E (TcCad1)

La cadherina E identificada y validada como receptor funcional en *T. castaneum* en este trabajo podría realizar en este insecto un papel equivalente al que se ha descrito para los receptores tipo cadherina en otros insectos lepidópteros y dípteros. En el coleóptero *T. molitor,* susceptible a la toxina Cry3Aa, el silenciamiento de la cadherina E, ortóloga de la de *T. castaneum*, confirió al insecto resistencia a Cry3Aa, y se validó así su función como receptor. Se demostró además, que un fragmento de dicha cadherina fue capaz de promover la oligomerización de Cry3Aa (Fabrick *et al*., 2009).

Las cadherinas E de *T. castaneum* y *T. molitor,* receptores de Cry3Ba y Cry3Aa respectivamente, comparten una estructura muy similar que incluye un dominio extracelular con 12 repeticiones tipo cadherina en tándem y un péptido señal, un dominio transmembrana y un dominio citosólico. Se postula que la toxina Cry3Aa se une a la cadherina E de *T. molitor* por la región proximal a la membrana (Fabrick *et al*., 2009), como también se ha observado en otras toxinas Cry que unen cadherinas de lepidópteros (Pigott y Ellar, 2007). En el caso de *T. castaneum* se ha sugerido que la presencia de tres residuos de lisina en esta región podría dificultar la unión de la toxina Cry3Aa, lo que explicaría su baja toxicidad (Fabrick *et al*., 2009).

En el coleóptero *D. virgifera virgifera,* se ha descrito que un péptido que comprende una región de la cadherina correspondiente a las repeticiones de cadherina 9 a 11 en *T. molitor,* fuera de la región proximal a la membrana, fue capaz de unir las toxinas Cry3Aa y Cry3Bb, tóxicas para este insecto y de aumentar la toxicidad de estas toxinas en otros coleópteros (Park *et al*., 2009). Estos resultados sugirieren que, en escarabajos, las cadherinas pueden tener varios sitios de unión a toxinas Cry3. En lepidópteros, también existen evidencias de unión de las toxinas Cry1A a la cadherina de *M. sexta* por la repetición de cadherina 7, que tampoco se localiza en la región proximal a la membrana (Gómez *et al*., 2001).

3.1.3 Simportador de sodio-soluto (TcSSS)

En este trabajo hemos demostrado que el silenciamiento del simportador de sodio-soluto confirió resistencia a Cry3Ba en las larvas de *T. castaneum,* demostrando que también actúa como receptor en este insecto. La región de secuencia más similar entre la cadherina E y el simportador (proteínas que hemos validado como receptores funcionales), se localizó en la repetición de cadherina 8 en la cadherina E, fuera de la región proximal a la membrana y en una región adyacente a una de las tres repeticiones de cadherina identificadas, en el extenso dominio extracelular del simportador. En ambas proteínas, en esta región se encontró una secuencia con elevada

homología a los epítopos de unión a toxinas Cry presentes en cadherinas de *T. molitor* y *M. sexta,* situada en la región proximal a la membrana. Se ha descrito que péptidos que contenían estos dos epítopos administrados en combinación con toxinas activas contra los insectos aumentaron la toxicidad de las mismas en diversos coleópteros y en *M. sexta* (Fabrick *et al*., 2009; Chen *et al*., 2007; Gao *et al*., 2011). En el presente trabajo, se demuestra que un péptido del simportador que incluye el homólogo de los epítopos de unión de las cadherinas de *M. sexta* y *T. castaneum* también sinergiza la toxicidad de Cry3Ba en *T. castaneum,* aumentando hasta el doble la mortalidad de las larvas.

3.1.4 Moléculas transportadoras en el modo de acción de *Bt*

De entre las moléculas identificadas en *T. castaneum* como posibles receptores de la toxina Cry3Ba, el simportador de sodio, el transportador ABC y la V-ATPasa son moléculas transportadoras. Las dos primeras han sido validadas como receptores funcionales, el simportador por primera vez en este trabajo, y la V-ATPasa aún no ha podido ser validada mediante RNAi por ser una proteína esencial en diversos organismos.

Los tres transportadores son proteínas de membrana estructuralmente relacionadas. Los transportadores ABC presentan 12 hélices alfa transmembrana. La V-ATPasa presenta un dominio periférico llamado V_1 responsable de la hidrólisis del ATP y un dominio anclado a la membrana llamado V_0 donde se encuentran varias subunidades compuestas por hélices transmembrana formando un anillo responsable de la translocación del protón (Beyenbach y Wieczorek, 2006; Murata *et al*., 2005). En el caso de los simportadores de sodio, suelen presentar de 11 a 15 dominios transmembrana en conformación de hélice alfa (Turk y Wright, 1997). El modelo estructural propuesto en esta tesis para el simportador de sodio de *T. castaneum* identificado presenta 11 hélices transmembrana, con el extremo amino-terminal en el exterior celular y el carboxi-terminal en el citoplasma. Además, entre las dos últimas hélices existe un gran dominio extracelular que no aparece en la mayoría de simportadores de sodio y, en esta región, es interesante destacar que se han encontrado tres repeticiones tipo cadherina.

Las V-ATPasas y tranportadores ABC son transportadores primarios, que utilizan la energía de la hidrólisis del ATP para bombear activamente sustratos a través de la membrana. Las V-ATPasas transportan protones generalmente dentro de orgánulos intracelulares como los endosomas o los lisosomas, acidificándolos y son esenciales en el tráfico de vesículas en las rutas de endocitosis y exocitosis. También existen V-ATPasas situadas en la membrana apical de las células goblet del intestino de los

insectos y se encargan de crear el gradiente de iones necesario para el transporte secundario o para la regulación de la acidificación del lumen (Wieczorek *et al*., 2000; Beyenbach y Wieczorek, 2006). Los transportadores ABC exportan compuestos desde el citoplasma hacia el exterior celular o dentro de compartimentos intracelulares como las vacuolas y han sido muy estudiados por su implicación en resistencia a la quimioterapia y su papel en excreción de drogas, insecticidas, herbicidas, fungicidas, antibióticos, metales pesados y otros xenobióticos (Heckel, 2012). A diferencia de los otros dos transportadores mencionados, el simportador de sodio/soluto es un transportador secundario, ya que utiliza el gradiente electroquímico del ión sodio creado por otras bombas primarias para dirigir solutos acumulados en una parte de la membrana hacia la otra, en contra de su gradiente de concentración (Jung, 2002). Algunos de los solutos transportados son azúcares, aminoácidos, urea, vitaminas, iones o agua.

Se desconoce el papel que pueden ejercer estos transportadores en el modo de acción de las toxinas Cry. Gahan *et al*. (2010) han propuesto que el transportador ABC podría ayudar a la inserción del oligómero de la toxina en la membrana. Según el modelo propuesto, el oligómero, o incluso también la toxina monomérica, se uniría a la configuración abierta del transportador por la parte citoplasmática. La unión del ATP a sus dominios de unión en el transportador, también por la parte citoplasmática, produciría la dimerización de estos dominios y un gran cambio conformacional, pasando al estado abierto hacia afuera y liberando la molécula transportada a la otra parte de la membrana. Aprovechando el cambio de configuración del transportador de "abierta hacia adentro" a "abierta hacia afuera", se facilitaría la inserción de la toxina en la membrana.

Los simportadores de sodio/soluto también presentan cambios conformacionales que permiten el transporte. En este caso, el ión sodio se une al transportador por la parte citoplásmica, induciendo un cambio conformacional que permite la unión del soluto. Esta segunda unión también induce un cambio conformacional que expone el sodio y el soluto hacia la otra parte de la membrana, donde ambos son liberados (Wright *et al*., 1998; Jung, 2002). Así pues, el simportador de sodio podría también estar implicado en la inserción de la estructura oligomérica o de la toxina Cry3Ba monomérica en la membrana.

Una hipótesis alternativa plantea la posibilidad de que el transportador ABC podría también estar participando en el proceso de oligomerización de la toxina (Heckel, 2012), ya que toxinas modificadas capaces de oligomerizar en ausencia de cadherina (receptor que se ha propuesto que es esencial para la oligomerización de la

toxina) resultaron tóxicas para cepas resistentes de lepidópteros que presentaban mutaciones en el transportador ABCC2 (Tabashnik *et al*., 2011). En el caso del simportador de sodio, que actuaría como receptor de Cry3Ba en *T. castaneum*, esta hipótesis resulta especialmente interesante puesto que el simportador presenta tres repeticiones de tipo cadherina en el dominio extracelular, accesible a las moléculas situadas en el lumen del intestino del insecto, que podrían también realizar la misma función que los receptores de tipo cadherina.

Los resultados de RNAi muestran que la cadherina y el simportador, al ser silenciados independientemente, reducen drásticamente la mortalidad de las larvas tratadas. Este resultado no sería esperado si ambas proteínas fueran completamente redundantes en su participación en la acción tóxica de Cry3Ba, más bien sugiere una función complementaria de ambas moléculas en el modo de acción de la toxina.

Así pues, el simportador podría participar tanto en la inserción de la estructura oligomérica como contribuyendo a mantener, a acelerar su formación o a concentrar la estructura oligomérica en la membrana, función que podría ser relevante en un insecto en el que el receptor tipo APN no parece ejercer una función de mediador de la toxicidad.

4. Estudio de los receptores de las toxinas Cry en *L. decemlineata*

La comprensión del modo de acción de las toxinas Cry de *Bt* en *L. decemlineata* resulta de especial interés ya que este insecto constituye la plaga más destructora de la planta de patata en el mundo. Por ello, la identificación de receptores funcionales de toxinas Cry en este insecto y la generación de toxinas más efectivas que permitan eludir o minimizar el desarrollo de resistencia por parte del insecto ha constituido uno de los objetivos de esta tesis. El escaso número de genes del escarabajo de la patata de los que se conoce su secuencia (el genoma de este insecto todavía no ha sido secuenciado) es uno de los problemas que dificulta enormemente la identificación de moléculas capaces de interaccionar con toxinas Cry. A pesar de ello, se han sugerido algunos candidatos, avalados por evidencias experimentales que muestran su participación en el mecanismo de acción de las toxinas de Bt en este coleóptero. Nuestro grupo de investigación ha propuesto que la toxina Cry3Aa se une a una peptidasa de membrana que posee dominios metaloproteasa y disintegrina (metaloproteasa de la familia ADAM) y está presente en el epitelio intestinal de larvas de *L. decemlineata* (Rausell *et al*., 2007; Ochoa-Campuzano *et al*., 2007). En el lazo 1 del dominio II de la toxina se ha identificado un motivo de reconocimiento por el que interacciona con la metaloproteasa ADAM y como resultado de esta interacción se

produce la proteólisis de la toxina, probablemente en el dominio III de la misma, demostrándose que este procesado es esencial para la acción tóxica de Cry3Aa en *L. decemlineata* (Ochoa-Campuzano *et al.*, 2007; García-Robles *et al*., 2012).

En los últimos años, hemos acumulado evidencias que apoyan la participación de múltiples proteínas en el mecanismo de acción de la toxina Cry3Aa en el escarabajo de la patata y revelan una complejidad mayor de la inicialmente anticipada. Además de la metaloproteasa ADAM, entre las distintas proteínas de membrana del epitelio del intestino medio de la larva capaces de unir toxinas de *Bt*, parece ser funcionalmente relevante la proteína prohibitina, identificada, mediante ligand blot, por nuestro grupo como proteína de unión a la toxina Cry3Aa. Hemos demostrado que esta proteína es esencial para la viabilidad de las larvas de *L. decemlineata* y que en larvas en las que se silenció la expresión del correspondiente gen se incrementó la mortalidad producida por el tratamiento con la toxina Cry3Aa.

4.1 Ensayos de proteólisis e interacción entre la metaloproteasa ADAM y las toxinas Cry3

El mecanismo de resistencia a las toxinas Cry más frecuentemente desarrollado por los insectos es la pérdida de interacción de la toxina Cry con su receptor, bien por ausencia del receptor o de alguno de los mediadores responsables de la acción tóxica, o bien por la degradación o falta de activación de la toxina. La segunda causa involucra a proteasas presentes en el intestino del insecto susceptible, pudiendo ser tanto proteínas solubles como de membrana.

Se ha relacionado una alta actividad de proteasas solubles en el intestino de los insectos con una diminución de la susceptibilidad a las toxinas Cry (Forcada *et al*., 1996; Keller *et al*., 1996; Oppert *et al*., 1997). Sin embargo, el papel de las proteasas de membrana, como la metaloproteasa ADAM, está poco estudiado.

En trabajos previos se observó que la incubación de la toxina Cry3Aa con vesículas de membrana del intestino medio del escarabajo de la patata generaba fragmentos de proteólisis de la toxina, y se identificó la metaloproteasa ADAM como una de las proteasas responsables (Ochoa-Campuzano *et al.*, 2007). Con el objetivo de profundizar en el estudio de la proteólisis de las toxinas y su relación en el modo de acción, en el presente trabajo se han analizado los patrones proteolíticos de las toxinas Cry3Aa, Cry3Ba y Cry3Ca, específicas contra coleópteros y activas frenta a las larvas del escarabajo de la patata. Mi contribución en este trabajo se ha centrado en la producción y purificación de la toxina Cry3Ba y en la realización del análisis proteolítico de la misma, tras la interacción con las proteasas de membrana del intestino del insecto. Se

detectaron diferencias en los patrones proteolíticos de las tres toxinas, lo que llevó a formular la hipótesis de que la metaloproteasa ADAM podría estar interaccionando de forma diferente con ellas.

Se postula que Cry3Aa interacciona con la metaloproteasa ADAM por una región situada en uno de los lazos del dominio II (Ochoa-Campuzano *et al.,* 2007). Los dominios II y III de las toxinas Cry han sido implicado en el reconocimiento del receptor en otros insectos, ya que el intercambio de dichos dominios entre diferentes toxinas demostraron su contribución en la especificidad (de Maagd *et al*., 1996; Schnepf *et al*., 1998). Asimismo, la mutagénesis dirigida de los lazos del dominio II afectó a la afinidad de la toxina por la unión a su receptor y a su toxicidad (Dean *et al*., 1996).

Para investigar la importancia de la interacción entre las diferentes toxinas Cry3 y la metaloproteasa a través de la región de interacción situada en el dominio II, generamos mutaciones en el gen de la toxina Cry3Aa que condujeron a la sustitución de aminoácidos de la región de interacción por los correspondientes aminoácidos de las toxinas Cry3Ba y Cry3Ca. Se analizaron los patrones de proteólisis de dichos mutantes, la toxicidad y la accesibilidad que presentan a la proteasa y los resultados obtenidos han reforzado la importancia de esta región de interacción, ya que las proteínas mutantes interaccionaron de forma distinta con la metaloproteasa y su toxicidad frente al insecto disminuyó.

4.2. Proteínas de unión a Cry3Aa: prohibitina

El mecanismo de acción de las toxinas Cry en los insectos diana es un proceso complejo y presumiblemente estas toxinas interaccionan con diversas proteínas que pueden asistir a la toxina en su inserción en la membrana o desempeñar otras funciones no directamente relacionadas con la permeabilización de la membrana (Crickmore, 2005). Además de la metaloprotreasa ADAM, hemos identificado otros potenciales receptores de Cry3Aa en *L. decemlineata* presentes en la membrana epitelial del intestino de las larvas del insecto capaces de unir dicha toxina. Entre ellas, se han encontrado V-ATPasa, actina y la prohibitina 1, que también han sido identificadas en otros insectos como proteínas de unión a toxinas Cry (McNall y Adang, 2003; Bayyareddy *et al*., 2009; Krishnamoorthy *et al*., 2007; Chen *et al*., 2010a). Se ha descrito que la prohibitina actúa como proteína de unión a Cry4B también en células de díptero (Kuadkitkan *et al.,* 2012) y se postula que además actúa como receptor del virus del dengue en estas células (Kuadkitkan *et al*., 2010).

Para analizar la significación funcional de la prohibitina en el modo de acción de toxina Cry3Aa en el escarabajo de la patata, procedimos al silenciamento de la

expresión de dicha proteína utilizando RNAi en larvas de *L. decemlineata*. No se ha demostrado si *L. decemlineata* presenta una respuesta sistémica y eficiente al RNAi (como sí ocurre en el coleóptero *T. castaneum*), pero se ha observado en estudios previos que tanto la inyección como la ingesta de RNA de doble cadena por parte de las larvas del escarabajo de la patata produce en éstas el silenciamiento efectivo de genes. Algunos ejemplos son los genes codificantes de acetilcolinesterasas (Revuelta *et al*., 2011), actina, proteínas relacionadas en el transporte de vesículas del retículo y las V-ATPasas A, B y E (Zhu *et al*., 2011; Baum *et al*., 2007). Algunos de estos genes, como por ejemplo las diferentes subunidades de la V-ATPasa, son esenciales en este insecto y el silenciamiento de los mismos produce la muerte de gran parte de las larvas, al igual que ocurre al silenciar estos mismos genes en los coleópteros *T. castaneum*, *D. virgifera virgifera* o *Diabrotica undecimpunctata howardii* (Baum *et al*., 2007; Whyard *et al*., 2009), por lo que se propone que esta técnica puede ser utilizada para el control de plagas por medio de la contaminación de la dieta con RNAi (Zhu *et al*., 2011; Baum *et al*., 2007) o mediante el uso de cultivos transgénicos productores de RNAi específicos de genes esenciales para los insectos diana (Mao *et al*., 2011; Baum *et al*., 2007).

Aprovechando la experiencia adquirida en la técnica de silenciamiento génico mediante RNAi en *T. casteneum*, se aplicó la técnica en *L. decemlineata* para el silenciamiento de la prohibitina mediante la ingestión de RNA de doble cadena en larvas de *L. decemlineata*, y se obtuvieron elevados niveles de mortalidad en las larvas. Este resultado indica que esta proteína debe ser esencial en este insecto y abre la posibilidad de poder utilizar el silenciamiento de este gen para el control de esta plaga, como se ha propuesto para los genes codificantes de las subunidades de la V-ATPasa u otros genes esenciales (Baum *et al*., 2007). Sin embargo, la cinética lenta propia de la acción del dsRNA puede resultar una limitación para su uso en el campo.

El silenciamiento de la prohibitina combinado con el tratamiento con Cry3Aa produjo un 100% de mortalidad en los bioensayos, tan solo cinco días después del tratamiento, con una dosis de toxina que sin el tratamiento con RNAi no supera el 30% de mortalidad. Incluso una dosis muy baja de toxina que no produjo mortalidad a los cinco días, alcanzó una mortalidad del 80% al ser combinada con RNAi. El sinergismo de dos tratamientos insecticidas que afectan a dianas diferentes de un mismo insecto constituye una estrategia prometedora para controlar las plagas, ya que la probabilidad de que se generen individuos resistentes que consigan eludir ambos mecanismos de toxicidad es menor. El RNAi presenta un modo de acción único que puede complementar la estrategia actual de expresar los genes de las toxinas de *Bt* en diferentes cultivos (Baum *et al*., 2007).

Conclusiones

1. Las larvas de *T. castaneum* son susceptibles a las cepas de *Bt* productoras de las toxinas Cry3Ba y Cry23/37.

2. El tratamiento de las larvas de *T. castaneum* con mezclas de esporas y cristales de las cepas de *Bt* productoras de las toxinas Cry3Ba y Cry23/37 produjo la expresión diferencial de siete proteínas, en relación a las larvas no intoxicadas: la proteína de unión a odorantes C12, la apolipoforina III, la proteína quimiosensora 18, el precursor de la apolipoproteína I/II, la proteína ribosomal L13a, una proteína cuticular y la subunidad Eα de la piruvato deshidrogenasa. Las tres primeras proteínas aumentaron su expresión y podrían estar relacionadas con la defensa del insecto en respuesta al tratamiento, mientras que las cuatro restantes disminuyeron su expresión y podrían estar implicadas en el control del metabolismo y desarrollo de la larva.

3. La actuación en defensa inmune de la proteína apolipoforina III se produce vía activación de la cascada de la profenoloxidasa.

4. La E-cadherina y el simportador de sodio-soluto son receptores funcionales de la toxina Cry3Ba en el intestino de las larvas de *T. castaneum*.

5. El fragmento del simportador de sodio-soluto correspondiente a los aminoácidos 1110-1138 ejerce un efecto sinérgico sobre la actividad insecticida de la toxina Cry3Ba en larvas de *T. castaneum*.

6. En *L. decemlineata*, mediante mutagénesis dirigida en la región correspondiente al motivo de reconocimiento de la toxina Cry3Aa por la metaloproteasa ADAM, se confirmó la relevancia de dicho motivo, tanto en la proteólisis de la toxina asociada a la membrana intestinal como en su actividad insecticida.

7. La prohibitina 1 de *L. decemlineata* es una proteína esencial para las larvas de este insecto y el tratamiento combinado de la toxina Cry3Aa de *Bt* junto con el silenciamiento del gen codificante de la prohibitina 1 produce un efecto tóxico sinérgico que puede ser utilizado para el control de esta plaga.

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