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PhD Thesis

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The mighty Bt:
Interactions between pests and pesticidal proteins from *Bacillus thuringiensis*

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Que el Sr. Daniel Pinos Pastor, Graduado en Biología, ha realizado bajo su dirección el trabajo de investigación recogido en esta tesis doctoral que lleva por título "The mighty Bt: Interactions between pests and pesticidal proteins from *Bacillus thuringiensis*", para optar al Grado de Doctor por la Universitat de València.

Y para hacer constancia, de acuerdo con la legislación vigente, firman la presente en Burjassot, a 13 de Diciembre de 2021.

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Resumen extendido

Bacillus thuringiensis, también conocida como "Bt", es una bacteria gram positiva que forma endosporas. Se trata de un organismo ubicuo, aunque se encuentra principalmente en el suelo o en ambientes con alta presencia de insectos. Fue aislada por primera vez en 1901 por el bacteriólogo Shigetane Ishiwata en muestras de intestino de gusanos de seda (*Bombyx mori*) infectados y fue apodada como "Sottokin-Bacillus" ("Bacilo de muerte súbita") por la muerte que causaba cuando era ingerida por larvas de gusanos de seda. Pocos años después, el biólogo Ernst Berliner aisló *B. thuringiensis* de crisálidas de polilla mediterránea de la harina (*Ephestia kuehniella*) infectadas con esta bacteria en la provincia de Thuringia, Alemania, otorgándole el nombre por el que se conoce en la actualidad. Además, Berliner describió la presencia de una especie de cristal dentro de la célula bacteriana, aunque la naturaleza de este elemento era desconocida en ese momento. Fue en 1954 cuando el microbiólogo Thomas A. Angus descubrió que esas inclusiones proteicas con formas de cristal, producidas en la fase de esporulación de Bt, eran responsables de su acción insecticida y las apodó "proteínas Cry" (del inglés crystal). A parte de estas proteínas, se han descrito varios factores de virulencia posteriormente, tal y como la β-exotoxina, la cual puede inhibir ARN polimerasas dependientes de ADN, las hemolisinas, o exoenzimas como las quitinasas. En el año 1996 se descubrió una nueva clase de proteínas, conocidas actualmente como Vip3 (de las siglas en inglés "Proteínas Vegetativas Insecticidas"), producidas durante la fase vegetativa de Bt, las cuales son altamente tóxicas para lepidópteros.

Desde entonces, una amplia variedad de proteínas de Bt tóxicas para diferentes invertebrados (principalmente insectos y nematodos) han sido identificadas a partir de un gran número de cepas aisladas, y han sido clasificadas en función de la similitud de su secuencia proteica. En la actualidad existen 391 secuencias holotípicas de proteínas Bt, y los principales órdenes de insectos que han mostrado susceptibilidad a algún tipo de proteína son Lepidoptera, Diptera, Coleoptera, Hemiptera e Hymenoptera. Esta tesis se centra en el estudio de dos familias de proteínas, las proteínas Cry de tres dominios ("3D-Cry") y

las proteínas Vip3 que afectan a lepidópteros. El modo de acción de estas proteínas, así como el ciclo de vida de Bt tras la infección del insecto constan de una serie de pasos, desarrollados en las siguientes páginas.

El primer paso supone la ingestión de Bt por parte del insecto. Tras ello, los cristales que forman las proteínas Cry se solubilizan por la rotura de los puentes disulfuro, liberando las protoxinas presentes en el cristal. Esta solubilización es dependiente tanto del pH como de condiciones reductoras del intestino. En el caso de las proteínas Vip3, no precisan de este paso de solubilización, ya que son secretadas al exterior de la bacteria en forma soluble. Tanto las protoxinas Cry solubilizadas como las protoxinas Vip, son procesadas por enzimas digestivas como las tripsinas y quimotripsinas presentes en los fluidos intestinales, produciendo una toxina activa que en general es resistente a posteriores procesos proteolíticos. Una vez activadas, las toxinas deben atravesar la membrana peritrófica del intestino medio. Esta membrana es una matriz rica en quitina, cuya función principal es la separación entre el alimento y las células epiteliales. Esta separación impide que el alimento pueda tener un efecto abrasivo sobre el intestino y también sirve como primera barrera contra infecciones de tipo bacteriano, vírico o parasítico. Asimismo, el papel protector que ejerce la membrana peritrófica puede verse comprometido por la acción de las quitinasas endógenas o exógenas de Bt (enzimas capaces de degradar la quitina). Tras superar la barrera de la membrana peritrófica, se produce una interacción entre las proteínas Cry o Vip3 y la membrana de borde en cepillo de las células del intestino medio, consideradas como las células diana de las toxinas. Uno de los retos principales en la investigación de Bt durante las últimas décadas ha sido identificar cuáles son las moléculas que se unen de forma específica con las toxinas Cry o Vip3, así como deducir la relevancia de la función de esta interacción, tanto en el modo de acción de las toxinas como en los mecanismos de resistencia.

Actualmente, existen tres tipos de proteínas de membrana que pueden funcionar como receptores putativos para las proteínas Cry en insectos: las aminopeptidasas N ("APN"), las cadherinas y los

transportadores "ABC" (del inglés "ATP-Binding Cassette"). Aunque inicialmente la fosfatasa alcalina ("ALP") también fue propuesta como posible receptor, al estar asociada con la resistencia, no se dispone de evidencias claras de su implicación en la unión. En cuanto a los transportadores ABC, son los receptores que mayor importancia han adquirido en los últimos años. Son proteínas de membrana con capacidad para transportar distintas moléculas de forma activa gracias a la hidrólisis de ATP. Dentro de esta gran superfamilia, el transportador más caracterizado ha sido el ABCC2, tanto por su implicación como receptor de proteínas Cry1, como por haber sido encontrado alterado en distintas especies de lepidópteros resistentes a proteínas Bt.

En cuanto a las toxinas Vip3, los estudios de interacción han demostrado que los sitios de unión no son compartidos con las toxinas Cry. Existen pocos estudios concluyentes sobre los posibles receptores específicos para las toxinas Vip3. Algunos trabajos indican que tanto la proteína ribosomal S2 como el "scavenger" tipo C pueden actuar como receptores de la toxina Vip3A en varias especies del género *Spodoptera*. Además, este último se encuentra involucrado en la vía endocítica, siendo capaz de mediar la internalización de la toxina.

Existen numerosos estudios que han señalado que la actividad tóxica de las proteínas de Bt se debe principalmente a su habilidad para formar poros en la membrana de las células diana. Este modelo (conocido como modelo de formación de poro) es actualmente el más respaldado por la comunidad científica debido al gran número de trabajos experimentales que lo apoyan tanto para toxinas de tipo Cry como para Vip3. Una vez unidas las toxinas a los receptores específicos, se forma una estructura oligomérica que se inserta en la membrana de las células intestinales constituyendo el poro. De esta forma se pierde la integridad de la membrana, generando un desequilibrio osmótico que provoca un hinchamiento de las células, desembocando en lisis celular. Tras la lisis de las células intestinales se produce una septicemia, causada principalmente por el paso a la hemolinfa no sólo de las propias esporas o formas vegetativas de Bt, sino también de todas aquellas bacterias oportunistas y otros patógenos presentes en el bolo

alimentario. Como consecuencia, el insecto acaba muriendo. Por último, Bt puede aprovechar este nicho para continuar creciendo y esporular, favoreciendo su posterior dispersión en el medio.

Dada su eficacia como insecticida, el ser humano ha utilizado Bt para contener la expansión de distintas plagas. El primer producto basado en Bt se comercializó en 1938, y desde entonces, su uso, ya sea en formato de formulado Bt o plantas con expresión de genes Bt, ha incrementado notablemente. Como ejemplo, las hectáreas de plantas de maíz, algodón o soja Bt aumentaron de 1,1 millones en el año 1996 a 101 millones en 2017. Por ello, no es de extrañar que *B. thuringiensis* sea el agente microbiano más utilizado entre las soluciones biotecnológicas presentes en la actualidad. Aunque los investigadores han trabajado durante muchos años en descifrar cómo se comportan las proteínas de este excepcional organismo en el ambiente intestinal de los insectos, aún queda mucho por entender. El conocimiento de forma detallada del modo de acción de las proteínas insecticidas de Bt es un punto clave para garantizar su uso a largo plazo por varias razones; nos permite entender en cierta medida, cuáles son los mecanismos alterados en el desarrollo de la resistencia, dónde se localizan y por qué se encuentran alterados. Además, gracias a este conocimiento podemos combinar proteínas Bt con diferentes modos de acción con la finalidad de maximizar su efecto. En la presente tesis, hemos profundizado en el estudio de estas interacciones mediante distintas aproximaciones.

En el **primer y segundo capítulo**, nos hemos centrado en estudiar las interacciones que ocurren entre las propias proteínas Cry, así como con el transportador ABCC2 de la rosquilla verde, *Spodoptera exigua*. Dado que esta plaga es de alta relevancia por su impacto en la agricultura, y se encuentra ampliamente distribuida en todo el mundo, conocer cómo las proteínas Cry1 interaccionan con los receptores del intestino es muy importante, ya que los productos basados en Bt y las plantas Bt que contienen proteínas Cry1 son comúnmente utilizadas para controlar esta plaga. En estos dos capítulos, elegimos una aproximación *ex vivo*, ya que el uso de estos sistemas, como el cultivo de células de insecto o las preparaciones de vesículas de membrana intestinales ("BBMV", de sus siglas inglés, *Brush Border Membrane*

Vesicles), nos permiten entender y profundizar en interacciones específicas que pueden pasar desapercibidas durante los ensayos *in vivo*. Para ello, utilizamos una línea de células de insecto que expresa el transportador ABCC2 en su versión completa, denominada "Sf21-FRA". Además, utilizamos proteínas Cry1A marcadas con yodo para realizar ensayos de unión y no marcadas para ensayos de viabilidad celular. Descubrimos que las proteínas Cry1A unen de forma específica y con alta afinidad a células que expresan el transportador, y que la presencia de éste en células es suficiente para causar toxicidad de las proteínas Cry1A probadas. Estos resultados apoyan que el ABCC2 de *S. exigua* actúa como receptor funcional de las proteínas Cry1A, tal y como se ha observado en otras especies plaga. A través de los dos capítulos, la falta de competencia de la proteína Cry1C, así como la ausencia de toxicidad en células HEK que expresan el transportador, descartaron al ABCC2 como receptor para esta proteína. Estos resultados indican que la proteína Cry1C pueda poseer distintos sitios de unión en el intestino medio de *S. exigua*, ya que es altamente efectiva en el control de esta plaga.

Por otra parte, observamos un fenómeno insólito en los ensayos de unión con I^{125}-Cry1Aa, en los que concentraciones bajas de Cry1Aa no marcada y utilizada como competidor, producía una respuesta estimulatoria, causando un incremento de la unión total conseguida por la I^{125}-Cry1Aa a las células expresando el transportador. Dado que a concentraciones altas de competidor, éste competía de una forma habitual, exploramos por qué ocurría este comportamiento bifásico. Pudimos observar también estimulación causada a bajas concentraciones, e incluso más acentuada, al utilizar las proteínas Cry1Ab, Cry1Ac o la proteína híbrida H04 (que contiene los mismos dominios I y II que las proteínas Cry1Ab/c, pero contiene el dominio III de la Cry1C) como competidores contra la I^{125}-Cry1Aa. Sin embargo, no observamos dicho comportamiento bifásico al utilizar la proteína Cry1C o la proteína híbrida H205 (con los mismos dominios I y II que la proteínas Cry1C y mismo dominio III que la Cry1Aa), lo cual indica la importancia de poseer el mismo dominio I, común a estas tres Cry1As, para que se produzca la fase de estimulación de la unión.

Curiosamente, también pudimos observar dicho comportamiento, aunque de forma mucho más discreta, cuando utilizamos I^{125}-Cry1Ac y Cry1Aa como competidor. Para revelar la naturaleza del agente causante de la fase estimulatoria, realizamos electroforesis en geles de poliacrilamida (SDS-PAGE) de la fase insoluble de los ensayos de competencia, así como autoradiografías de la misma. Tanto el tamaño molecular como la intensidad de la banda observada nos permitió determinar que la forma oligomérica de la proteína es la responsable de la respuesta estimulatoria, señalando este hecho la habilidad de las proteínas Cry1A de formar tanto homo- como hetero-oligómeros. Para confirmarlo, utilizamos dos versiones mutantes de Cry1Aa y Cry1Ab, que poseen una sustitución del aminoácido Arg por Glu en la posición 99 del dominio I que impide la oligomerización. La ausencia de fase estimulatoria o banda correspondiente a oligómero en las autoradiografías al usar estos mutantes como competidores, confirman la habilidad de homo- y hetero-oligomerizar de las proteínas en su versión no modificada, a través del dominio I, así como de unir con alta afinidad al transportador ABCC2.

Además, las toxicidades observadas con las proteínas Cry1Ab y Cry1Ac en células expresando el transportador, así como los ensayos de competencia llevados a cabo con I^{125}-Cry1Ac en el primer capítulo, nos permiten concluir que estas dos proteínas comparten un sitio de unión, que tiene poca afinidad por la proteína Cry1Aa. La presencia de este sitio compartido se pudo confirmar con el uso del híbrido H04, lo cual señaló al dominio II de Cry1Ab/c como dominio con mayor afinidad por este sitio. Dado que la proteína Cry1Aa parecía tener un papel menos relevante en la unión a este sitio, pero aún así es tóxica para células expresando el transportador, realizamos experimentos de competencia tanto con I^{125}-Cry1Aa como con I^{125}-Cry1Ac, usando Cry1Aa y el híbrido H205 como competidores. Pudimos concluir que la proteína Cry1Aa debe de tener otro sitio de unión en el transportador, el cual tendría alta afinidad por el dominio III de Cry1Aa. A este sitio de unión, también se podría unir la proteína Cry1Ab con alta afinidad, dado que comparte el mismo dominio III que la Cry1Aa. Por último, a través de distintas combinaciones de proteínas

Cry1Aa en ensayos de toxicidad pudimos observar que, la ventaja obtenida al formar mayores concentraciones de hetero-oligómeros que de homo-oligómeros no sólo también se trasladaba a mayores tasas de unión, sino a mayor toxicidad sobre las células. En resumen, el primer capítulo destaca la importancia del ABCC2 como un receptor multivalente para las proteínas Cry1A y el segundo capítulo demuestra la habilidad de formar hetero-oligómeros por las proteínas Cry1Aa para beneficiarse de la multivalencia del transportador.

Hasta ahora, es evidente que el transportador ABCC2 de *S. exigua* y de otros lepidópteros juega un rol relevante en la unión y toxicidad de al menos las proteínas Cry1A, pero pocos miembros de la familia de este transportador han sido caracterizados. Es muy probable que estemos pasando por alto numerosos transportadores ABC que interaccionen con proteínas insecticidas de *B. thuringiensis*, ya que hasta ahora se han descrito más de 400 transportadores ABC en artrópodos. Por ello, estudiar nuevas interacciones entre transportadores ABC y proteínas insecticidas es un gran reto en la investigación con Bt. A parte del ABCC2, sólo unos pocos transportadores ABC se han relacionado con proteínas Bt, como el ABCB1 del coleóptero *Chrysomela tremulae*, receptor de la proteína Cry3Aa, el ABCA2 de los lepidópteros *Helicoverpa armigera* y *B. mori*, receptor para las proteínas Cry2A, o el ABCC3 de *S. exigua, Spodoptera litura, Plutella xylostella, H. armigera* y *Spodoptera frugiperda*, relacionado con la acción de la proteína Cry1Ac. Curiosamente, ningún otro miembro de la familia ABC (aparte del ABCC2) se ha caracterizado en el gusano bellotero *Heliothis virescens*, plaga en la que se relacionó por primera vez un transportador ABC con las proteínas de Bt, hace ya más de una década.

En el **tercer capítulo** de la tesis, hemos realizado una búsqueda de nuevos transportadores ABC en *H. virescens* para abordar esta falta de conocimiento. Hemos identificado y descrito dos nuevos transportadores, el ABCC3 y ABCC4. El análisis filogenético ha demostrado alta similitud entre el transportador ABCC3 y el ya descrito ABCC2, como se ha observado en otras especies como *H. armigera*. Sin embargo, el transportador ABCC4 es una proteína más distante

atendiendo a su secuencia de aminoácidos. Según los modelos de predicción, ambos transportadores estarían formados por dos dominios transmembrana y seis regiones en la parte exterior que se corresponden con asas extracelulares. Evaluamos la funcionalidad de los nuevos transportadores como posibles receptores para proteínas Bt desarrollando mutantes de deleción para cada uno de los genes *HvABCC3* o *HvABCC4* mediante la técnica de CRISPR/Cas9. Para ello, microinyectamos huevos y cruzamos los adultos resultantes hasta obtener líneas de homozigotos mutantes. Seguidamente, realizamos bioensayos con Cry1Aa, Cry1Ac y Vip3A. Los resultados preliminares no mostraron reducción en la toxicidad para ninguna línea mutante, en comparación con la colonia de *H. virescens* usada como control. Sin embargo, sí se observaron mortalidades ligeramente disminuidas en la concentración más baja de Cry1Ac utilizada, especialmente en la línea de mutantes con deleción en el ABCC3, aunque el grado de significación debe de estudiarse en mayor profundidad dado el bajo número de réplicas en los bioensayos. Además, falta por confirmar la correcta deleción de los transportadores en ambas líneas de mutantes mediante la secuenciación del ARN mensajero de los genes. Dado que los ARN guías utilizados se diseñaron en el primer exón de cada gen, podrían haber pautas de lectura alternativas, por lo que debemos de asegurar que no se estén expresando otras versiones funcionales de los transportadores. Por tanto, la posible implicación de los nuevos transportadores ABC con proteínas Bt tendrá que estudiarse en mayor profundidad. Por ahora, se sabe que el ABCC3 de *S. exigua, H. armigera S. exigua, H. armigera* y *S. frugiperda* comparte redundancia funcional con el ABCC2 en el modo de acción de las proteínas Cry1A y juega un rol menor y secundario en su toxicidad. Para comprender si este fuera el caso en *H. virescens*, son necesarios experimentos adicionales como desarrollar líneas de mutantes con deleción del ABCC2 o dobles mutantes con deleción del ABCC2 y ABCC3, así como la evaluación, mediante ensayos con proteínas marcadas, de las capacidades de unión sobre las líneas de los mutantes. Por último, aparte del ABCC2, ABCC3 y ABCC4, aún queda un gran número de transportadores ABC por describir en *H. virescens*.

Además de estudiar la interacción entre receptores putativos y proteínas Bt y buscar receptores desconocidos, debemos de tratar de entender cómo y por qué ciertas especies plaga consiguen desarrollar resistencia a proteínas Bt, ya que la resistencia es la principal amenaza para el uso continuado de plantas Bt o bioinsecticidas basados en Bt. Está ampliamente aceptado que la alteración de unión al receptor de membrana es la principal causa de resistencia a proteínas Bt en especies plaga, aunque otros mecanismos pueden estar involucrados. Las alteraciones de unión pueden ocurrir por diferentes razones, tales como la reducción en la expresión de un gen que codifique para una proteína de membrana, deleciones en su secuencia, o mutaciones aminoacídicas que impidan la interacción entre la toxina y el receptor. Por ello, es interesante caracterizar la funcionalidad de los receptores que han sido encontrados alterados en diferentes plagas resistentes. A día de hoy, existen numerosos estudios funcionales con las versiones "wild-type" de transportadores ABC usando diferentes técnicas, tales como silenciamiento con ARN de interferencia, deleción mediante CRISPR, o expresión en un sistema *ex vivo*, pero pocos estudios han caracterizado la funcionalidad de las versiones alteradas de los transportadores ABC de cepas resistentes. Además, se propuso que el mecanismo de transporte activo de los transportadores tiene que funcionar correctamente para que pueda actuar como receptor de las proteínas Cry. En el **cuarto capítulo**, caracterizamos la funcionalidad de una versión alterada del ABCC2 de una colonia de *S. exigua* resistente a Xentari™, un bioinsecticida basado en Bt. Este transportador contiene una deleción en el segundo dominio de unión a nucleótidos ("NBD2") que fue genéticamente ligada a la resistencia en un estudio previo. Sin embargo, no se han realizado ensayos de funcionalidad del transportador mutado para dilucidar si esta mutación es la causa de la resistencia. Para ello, utilizamos una línea celular de insecto que expresa la versión truncada del transportador denominada Sf21-XenR, así como la línea celular utilizada en los dos primeros capítulos que expresa la versión original del ABCC2 (Sf21-FRA).

A través del estudio de la secuencia de aminoácidos, identificamos cuatro mutaciones adicionales, en forma de sustitución,

tres de las cuales se encontraban en los NBD intracelulares y una de ellas en una de las asas extracelulares (asa extracelular número 4). Las primeras caracterizaciones mediante Western blot e inmunohistoquímica mostraron la expresión del transportador truncado y su localización en la membrana de las células Sf21-XenR, lo cual indica que las mutaciones presentes en el transportador no impiden su presencia en la membrana. Tras ello, realizamos ensayos de viabilidad celular con las proteínas Cry1Aa, Cry1Ab y Cry1Ac, tanto en células Sf21-XenR como en células Sf21 no transformadas como control. Las tres proteínas ensayadas fueron tóxicas contra células con expresión del transportador truncado, y a un nivel muy similar al encontrado previamente en células que expresan el transportador en su versión original (Capítulo 1). Además, los ensayos de unión con Cry1Ac marcada con I^{125} revelaron unión específica al transportador truncado, así como un valor similar en la constante de disociación (K_d) al observado para células con transportador original en ensayos de competencia. Por otra parte, la proteína Cry1Ab pudo competir parcialmente por los sitios de unión de la Cry1Ac marcada, mientras que la Cry1Aa no compitió. Estos resultados confirman que las sustituciones en la secuencia de aminoácidos y la falta de parte del NBD2 no impide la unión y, consecuentemente toxicidad, producida por las proteínas Cry1A sobre la versión truncada del transportador ABCC2 en *S. exigua*. Por ello, estos resultados señalan que la versión truncada del transportador ABCC2 no sería la causa directa de la resistencia al producto Xentari™ en la colonia de *S. exigua* estudiada en la presente tesis.

Otro lepidóptero plaga que actualmente causa un gran impacto global en la agricultura es *Ostrinia furnacalis*, el taladro del maíz asiático, el cual se alimenta principalmente de cultivos de maíz, pero también de caña de azúcar, algodón, o pimiento. El impacto de las larvas de esta especie es notorio de China a Australia, donde puede causar entre un 10-30% de pérdida en la producción del maíz cada año. Una herramienta prometedora para su control efectivo es el uso de maíz Bt, el cual expresa varias proteínas Bt a las cuales esta especie es susceptible, como Cry1Ab, Cry1Ac y Cry1F. Por lo tanto, es de gran

interés conocer el modo de acción de estas proteínas Bt en *O. furnacalis* y sus posibles mecanismos de resistencia antes de que ocurran en el campo. En el **quinto capítulo** hemos analizado en primer lugar los parámetros de unión de una colonia susceptible de *O. furnacalis* para establecer un modelo preliminar de sitios de unión de las proteínas Cry1 en esta especie. Para este fin, hemos marcado con I^{125} las proteínas Cry1Ab y Cry1Aa hemos realizado ensayos de competencia con Cry1Aa, Cry1Ab, Cry1Ac y Cry1F. De estos ensayos, podemos concluir que existen al menos dos sitios de unión distintos en la membrana de las células de intestino, que pueden ser compartidos principalmente por Cry1Aa y Cry1Ab, pero también en menor medida con Cry1Ac y Cry1F. Este modelo presentaría ciertas similitudes con el modelo previamente propuesto para la especie *Ostrinia nubilalis*, el taladro de maíz europeo, en el hecho de que las proteínas Cry1A pueden unirse a más de un sitio. Seguidamente, analizamos una colonia de laboratorio de *O. furnacalis* que fue seleccionada para desarrollar resistencia a la proteína Cry1Ab. Evaluamos la presencia de resistencia cruzada mediante bioensayos con proteínas Cry1A y Cry1F en ambas colonias. Con una resistencia a Cry1Ab mayor a 700 veces, los análisis de resistencia cruzada mostraron resistencias moderadamente altas a Cry1Aa (unas 180 veces resistente), pero también a Cry1Ac o Cry1F (más de 192 veces a ambas) comparado con los valores de susceptibilidad obtenidos para la colonia susceptible. Teniendo estos datos en cuenta, conocer el mecanismo que produce resistencia a la proteína utilizada en la selección, pero también resistencia cruzada a otras proteínas, es un elemento clave para preservar el uso a largo plazo del maíz Bt. Dado que el principal mecanismo de resistencia es la alteración de receptores en la membrana de células del intestino medio, llevamos a cabo ensayos de unión entre las mismas proteínas utilizadas en bioensayos y BBMV de la colonia resistente. Pudimos observar una reducción parcial en la unión específica de Cry1Ab y Cry1Aa a las BBMV, lo cual indicaría alteración de receptor. La alteración de este sitio en la colonia resistente estaría causando una deficiencia en la unión de, al menos, las proteínas Cry1A probadas aquí y ello sería la causa de la reducción en su toxicidad. En cuanto a la Cry1F, las

pequeñas diferencias observadas son poco concluyentes y sólo podemos especular que otros mecanismos de resistencia estén ocurriendo, por lo que es necesario investigar más para entender por qué se producen estos niveles de resistencia cruzada con Cry1F en esta colonia. Por lo tanto, no podemos concluir de forma categórica que la alteración del sitio de unión observado aquí sea el único responsable de los niveles de resistencia observados, y estudios adicionales serán necesarios para identificar la naturaleza del receptor alterado.

Una estrategia actual para evitar, o al menos retrasar la aparición de la resistencia a proteínas Bt en el campo es el uso de plantas Bt "piramidadas". Esta estrategia se basa en la co-expresión de distintas proteínas Bt con diferentes modos de acción en una misma planta, generalmente combinando proteínas Cry y proteínas Vip3, lo cual obstaculiza el desarrollo de resistencia por parte de las plagas. Esta estrategia se utiliza desde hace algunos años y aunque el modo de acción de las proteínas Vip3 se presupone distinto al modo de acción de las proteínas Cry, aún no está bien caracterizado. No obstante, ya se han conseguido resistencias (en condiciones de laboratorio) a Vip3A en algunos lepidópteros. Estas colonias resistentes sirven para caracterizar los posibles cambios genéticos y bioquímicos que causan la resistencia y también nos permiten acercarnos al modo de acción de estas proteínas Bt. En el **sexto capítulo**, hemos explorado una colonia resistente de *H. virescens* que presenta niveles de resistencia a Vip3A mayores de 2000 veces comparado con la colonia susceptible. Mediante el marcaje con I^{125} de la proteína Vip3Aa, se realizaron ensayos de unión con BBMV, además de ensayos de "ligand blot", tanto con la colonia sensible como con la resistente. En ninguno de los dos casos observamos diferencias significativas en la unión. Estos resultados iniciales indican que la resistencia a Vip3Aa podría deberse a mecanismos distintos a la alteración de sitios de unión. La ausencia de alteración de sitios de unión también ha sido observada en otras colonias resistentes a Vip3Aa de *H. armigera* y *Mythimna separata*. Además, detectamos una gran reducción en la actividad enzimática de la fosfatasa alcalina ("ALP") en los intestinos de la colonia resistente. Mediante ensayos de Western blot y RT-qPCR pudimos confirmar la

reducción en la cantidad de proteína ALP de membrana, así como su reducción a nivel transcripcional. Para saber si la ALP está actuando como receptor de la Vip3Aa, expresamos la ALP en células de insecto (Sf21) y realizamos ensayos de toxicidad con la proteína Vip3Aa. La susceptibilidad de la línea celular que expresaba la ALP no fue significativamente distinta a la línea celular control, lo cual apoya la idea de que la ALP no actúa como receptor funcional para la proteína Vip3Aa en *H. virescens*. Aunque la alteración de la ALP ha sido correlacionada con la resistencia a proteínas Cry1 en diferentes especies como *H. virescens, H. zea, H. armigera, P. xylostella, S. frugiperda* o *S. litura*, actualmente no podemos afirmar que sea receptor funcional o si su alteración se debe a una respuesta fisiológica a la resistencia. Del presente estudio, podemos descartar que la ALP de membrana actúe como receptor para la proteína Vip3Aa, al menos, en *H. virescens*, y podemos reseñar que la aparición de resistencia a proteínas Vip3A puede ser causada por mecanismos alternativos a la alteración de un sitio de unión. Para confirmarlo, necesitaremos más estudios que caractericen otras colonias resistentes a Vip3A y que desentrañen otros mecanismos de resistencia menos caracterizados.

Actualmente, existe una amplia bibliografía en la que se constatan distintos mecanismos de respuesta de los insectos para superar la infección de Bt y sus proteínas, aparte de la alteración de receptores que se encuentran en las células del intestino medio. Sin embargo, existe la necesidad de recabar toda esta información para poder ponerla en valor, ya que parte de ella ha sido pasada por alto. En el **séptimo y último capítulo** de la tesis, hemos llevado a cabo una búsqueda exhaustiva de todos los mecanismos conocidos que puedan contribuir de alguna manera al desarrollo de la resistencia. Para ello, hemos escrito un artículo de revisión que clasifica estos mecanismos de respuesta atendiendo al paso en el que intervienen dentro del modo de acción de *B. thuringiensis* y sus proteínas. Hemos definido ocho categorías: (i) Ingestión, (ii) Solubilización del cristal, (iii) Activación, (iv) Secuestro de toxinas, (v) Reparación del epitelio intestinal, (vi) Rutas de defensa celular intrínsecas al epitelio, (vii) proteínas de respuesta a patógenos (proteínas REPAT) y (viii) Respuestas inmunes,

y las hemos discutido, así como las interacciones entre ellas a lo largo de la revisión. Este trabajo pretende ilustrar todos los eventos conocidos que puedan contribuir a la defensa de los huéspedes contra *B. thuringiensis* y sus proteínas, y resaltar que el estudio de cómo interactúan estos mecanismos entre sí será uno de los principales retos en la investigación con Bt.

Para concluir, los resultados obtenidos en esta tesis permiten tener una mejor comprensión de algunas de las interacciones que se producen entre insectos, *B. thuringiensis* y sus proteínas insecticidas, lo cual puede ser útil para garantizar el uso a largo plazo de este poderoso organismo.

Summary

Bacillus thuringiensis (Bt) is one of the most popular biological pest control alternatives to the use of chemical insecticides. The proteins produced by this entomopathogenic bacterium can be toxic to several insects, mites, and nematode species. Currently, the main insecticidal proteins used in Bt-based products or Bt crops (genetically modified crops expressing Bt proteins) belong to the Cry and Vip3 protein groups. However, field-evolved resistance to Bt proteins can threaten the long-term use of Bt technology in agriculture. It is a key point to understand how Bt proteins produce their toxicity inside the insect, as well as to know how insects can overcome their toxic effect. One of the most important families of functional receptors of Cry proteins are the ATP-Binding Cassette (ABC) transporters located in the membrane of the midgut cells of the insect, which have been characterized in different species over the last decade.

In Chapters 1 and 2 from the present thesis we have investigated the role of the ABCC2 transporter from *Spodoptera exigua*, the beet armyworm, a global polyphagous pest that can feed from more than 200 different crops. By expressing the SeABCC2 in insect cells we have found that its presence enables high specific binding of Cry1A proteins to the transporter, which also rendered susceptibility of the cells to Cry1A proteins. Moreover, our results have pointed out the co-existence of two different binding sites in the SeABCC2 protein, one main site that can bind the domain II from Cry1Ab/c proteins, and a secondary site that can interact with domain III from Cry1Aa/b. We have identified that the main conformation of Cry1A proteins that binds to the SeABCC2 is the oligomer, a state that allows higher levels of binding that the one achieved by the monomer. Also, the ability of Cry1A proteins to hetero-oligomerize (that is, forming an oligomer composed by different Cry1A protein monomers) was observed. These hetero-oligomers formed by Cry1Aa-Ab or Cry1Aa-Ac allowed even higher binding and toxicity levels than the homo-oligomers of the Cry1Aa protein, indicating a possible reason why *B. thuringiensis* can co-express different Cry1 proteins with (apparently) similar binding preferences.

From these first two chapters, it has become evident that the SeABCC2 from *S. exigua* plays a relevant role in the toxicity of Cry1A proteins. Similarly, the HvABCC2 from the tobacco budworm (*Heliothis virescens*), a relevant pest in North America, was the first ABC transporter related to the mode of action of Cry1 proteins. However, no other ABC transporter from this noctuid has been linked with Bt proteins since then. In Chapter 3 of this thesis, we have explored and characterized other *H. virescens* ABC transporters from the C subfamily. We have identified two novel transporters, HvABCC3 and HvABCC4. The phylogenetic analysis has shown high similarities between HvABCC3 and the already-described HvABCC2, but HvABCC4 has appeared to be a more distant protein, according to its amino acidic sequence. To understand if either of the two new ABCCs are involved in the toxicity of Bt proteins, we have performed independent knock-outs of the *HvABCC3* and *HvABCC4* genes in eggs from *H. virescens*. After achieving the knock-out lines, preliminary results from bioassays with Cry1Aa, Cry1Ac and Vip3Aa proteins show no difference in toxicity levels, pointing out that neither transporters have a marked role in toxicity. The redundancy of the ABCC3 transporter as a functional receptor for the same proteins as the ABCC2 has been proven recently in other insects, but the data so far points to a secondary, less important role of ABCC3 compared to ABCC2. If it is the same case for *H. virescens* remains unknown.

As previously stated, field-evolved resistance to Bt proteins is the main threat over the long-term use of this technology. Understanding why some pests can develop resistance to Bt proteins is therefore highly relevant to propose new strategies in the use of Bt-based products and Bt crops. The main resistance mechanism involves the alteration of receptors for Bt proteins in the insect midgut cells. In the case of *S. exigua*, an alteration in the *SeABCC2* gene was genetically linked to resistance to the Bt-product Xentari™ in a previous study. In Chapter 4 of the thesis we have fully characterized this truncated SeABCC2 transporter and we have explored whether its mutations are affecting the functionality as a receptor for Cry1A proteins. We have found four additional amino acid changes in positions 671, 805, 1200,

and 1314, besides the large deletion found in the Nucleotide Binding Domain II, that went unnoticed in the previous study. By expressing the truncated version of the transporter in insect cells we have observed that the Cry1Ac protein could bind specifically to the truncated SeABCC2, indicating that the mutations would not be affecting binding. Moreover, cell viability assays demonstrated that the Cry1A proteins tested can exert toxicity to the cells after binding to the truncated SeABCC2, demonstrating that the partial lack of the nucleotide binding domain II and the amino acidic changes do not affect the functionality of the transporter as a Cry1A receptor in *Spodoptera exigua*.

Another pest that causes major concern is the Asian corn borer, *Ostrinia furnacalis*, a lepidopteran causing great corn losses from China to Australia. Similarly to *S. exigua* and *H. virescens*, Bt-based technology is a promising tool to control this pest. Although Cry1 proteins have proven to be toxic to *O. furnacalis*, a model for the binding sites of these proteins has not been stablished yet. In Chapter 5 we have analyzed a laboratory-selected colony of *O. furnacalis* resistant to Cry1Ab protein to deepen the knowledge on the mode of action and resistance mechanisms that this pest could develop, before they happen. We have explored cross-resistance with other Cry1 proteins besides Cry1Ab by performing bioassays, finding moderate-to-high levels of resistance to Cry1Aa, Cry1Ac, and Cry1F. Since the alteration of binding sites is usually the main cause of resistance, we performed binding assays, and found that the specific binding of Cry1Ab as well as Cry1Aa was reduced in the resistant colony. By competition assays with a susceptible colony and Cry1Ab-resistant colony, we have stablished a preliminary binding site model with at least two binding sites shared by Cry1A proteins. One of these binding sites is altered in the resistant colony, causing a partial loss in the binding of the Cry1A proteins. These findings can help in assessing which Bt proteins are effective to control this pest in a possible resistance scenario, but further studies are required to fully understand the mode of action of Bt proteins in the midgut of *O. furnacalis*.

The implementation of new Bt proteins from the Vip3 family to co-express with the more traditional Cry protein family in Bt crops helps to delay the appearance of resistance, since the mode of action of Vip3 proteins differs from the Cry mode of action. This combination, known as pyramided strategy, has allowed to prolong the use of Bt crops in the last decade. Although there are no documented cases of field-evolved Vip3A resistance yet, it is important to stay one step ahead and know the possible resistance mechanisms for this protein family. In Chapter 6, we have studied the >2,000-fold resistance to Vip3Aa in a *H. virescens* laboratory-selected colony. Binding and ligand blot assays showed no significant alterations in the resistant colony, indicating an alternative Vip3A-resistance mechanism to the alteration of binding sites. Moreover, a major reduction of the membrane alkaline phosphatase (HvmALP) at the transcriptional level was detected. To explore if it can act as a functional receptor, the HvmALP was expressed in insect cells. Viability assays performed with the Vip3Aa protein did not show increased cell susceptibility, discarding a direct role of mALP as a Vip3A receptor.

Lastly, in Chapter 7, we have gathered all the available research on response mechanisms of insects to overcome the infection of Bt and its proteins, besides the alteration of receptors from midgut cells. This review classifies the response mechanisms in eight categories according to which step of the mode of action is affected: Ingestion, Crystal solubilization, Activation, Toxin sequestration, Gut epithelium healing, Epithelium intrinsic cellular defense pathways, REPAT proteins, and Immune responses. The review illustrates how the complexity of the insect defense should be a matter of study in the Bt research world to ensure its long-term use.

To conclude, the results obtained in this thesis allow a better understanding of some of the interactions that occur between insects, *B. thuringiensis* and its insecticidal proteins, which may be useful to guarantee the long-term use of this powerful organism.

INTRODUCTION

I. What is *Bacillus thuringiensis* ?

Bacillus thuringiensis, also known as Bt, is a Gram-positive endospore-forming bacterium closely-related to *Bacillus cereus, Bacillus anthracis* and *Bacillus mycoides* (Priest et al., 2004). Although mainly found in soil, it is an ubiquitous organism that can be found in leaf surfaces, aquatic environments, animal feces, and specially in insect-rich environments, such as the gut of different kinds of caterpillars (Brock, 2005). Isolated in 1901 from infected silkworms (*Bombyx mori*) by the bacteriologist Shigetane Ishiwata, the bacterium was first nicknamed as "Sottokin-Bacillus" ("Sudden death-Bacillus") for the disease it caused when ingested by silkworm larvae (Ishiwata, 1901, 1905). Almost parallel, the biologist Ernst Berliner isolated Bt from infected chrysalids of the Mediterranean flour moth (*Ephestia kuehniella*) from the province of Thuringe (Germany), later describing the bacterium and naming it as it is currently known, *Bacillus thuringiensis* (Berliner, 1911, 1915). Berliner also reported the presence of a crystal within the bacterial cell, although the nature of this element was unknown at that time.

In 1954, the microbiologist Thomas A. Angus discovered that these crystalline protein inclusions, formed during the sporulation of Bt, were responsible for the insecticidal action and were named as "Cry" proteins (Angus, 1954) (**Figure 1**). Besides these proteins, several broad spectrum virulence factors have been described, such as the β-exotoxin that can inhibit DNA-dependent RNA polymerases (Farkas et al., 1976; Sebesta and Horská, 1970), hemolysins (Weinstein et al., 1988; Honda et al., 1991) or exoenzymes such as chitinases (Sampson and Gooday, 1998). In the year 1996, a new class of proteins were discovered. Currently known as Vip3, (from "Vegetative insecticidal proteins"), they are mainly produced over the vegetative phase of Bt and were found to be highly toxic for lepidopteran insects (Estruch et al., 1996).

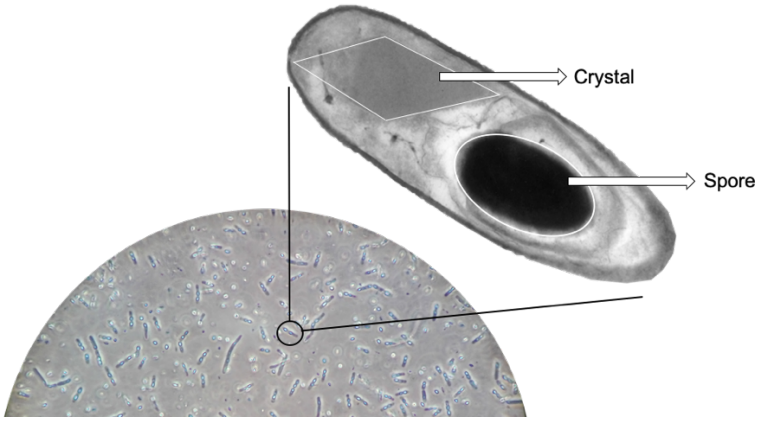

Figure 1. Microscopic view of *Bacillus thuringiensis*. Left: Phase photomicrograph of a sporulated culture). Right: Transmission electron micrograph of a longitudinal section of a sporulating cell, adapted from Sanchis (2011).

II. Bt proteins

II.1 Variety and target spectra

A wide variety of proteins from Bt that are toxic against different invertebrates (mainly insects and nematodes) have been identified from a large number of strain isolates, and have been classified according to protein sequence similarity since 1998 (Crickmore et al., 1998). Nevertheless, the growing list of newly characterized pesticidal proteins from bacteria different to Bt has caused the need to rethink how to name Bt proteins. Crickmore et al. (2020) have recently proposed a new nomenclature that solves this issue, but still maintains the basic principles of the previous version. Thus, in the present thesis, all the protein names have been adapted from and are referred to the new nomenclature. Currently, there are 391 holotype sequences of Bt proteins, and the known host spectrum of these Bt proteins is summarized in **Figure 2**. The main insect orders where susceptibility of any Bt protein has been documented are Lepidoptera, Diptera, Coleoptera, Hemiptera, and Hymenoptera (Palma et al., 2014). The present thesis is focused on two protein families; the three-domain (3-D) Cry proteins and the Vip3 proteins.

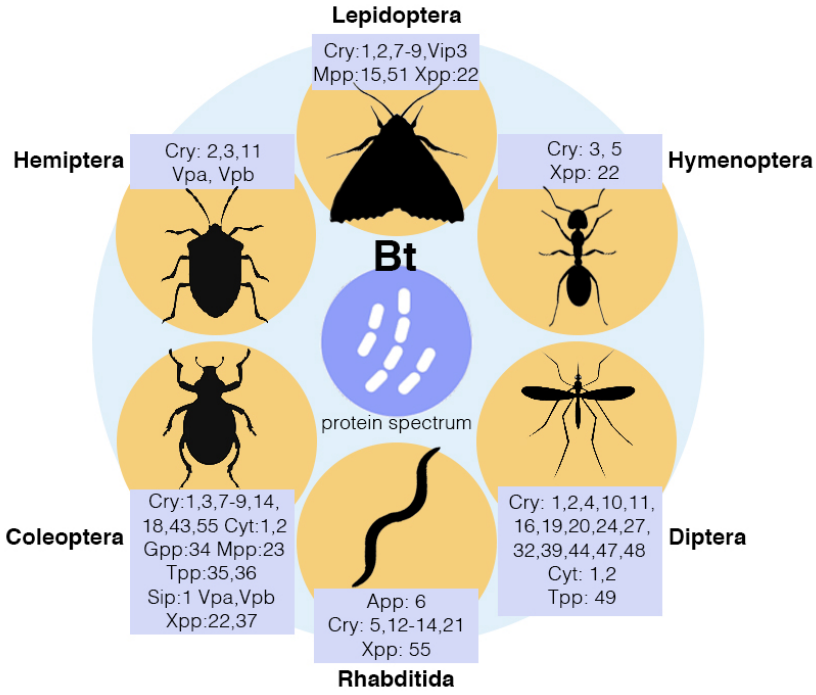

Figure 2. Summary of the host spectrum of Bt proteins, according to invertebrate order. Information on toxicity adapted from Palma et al. (2014). Updated nomenclature of proteins according to Crickmore et al. (2020).

II.2 Structure of 3D-Cry proteins

From the 391 different holotype protein sequences, 271 belong to the "three-domain (3-D) Cry" protein family (Crickmore et al., 2020). This family is by far the largest group of Bt proteins, and are also the best characterized. The development of new techniques in molecular biology, such as X-ray crystallography or cryogenic electron microscopy (Cryo-EM) has allowed elucidation of the structure of some of these Cry proteins, finding that many of them share three different structural domains (as seen in the left panel of **Figure 3**). This is the reason why proteins with this property have been renamed to a dedicated family as "3D-Cry" proteins. The present thesis focuses on the study of Cry1A proteins, which have an approximate protoxin size of 130 kDa, and from 60 to 70 kDa on their activated toxin form (Bravo et al., 2007).

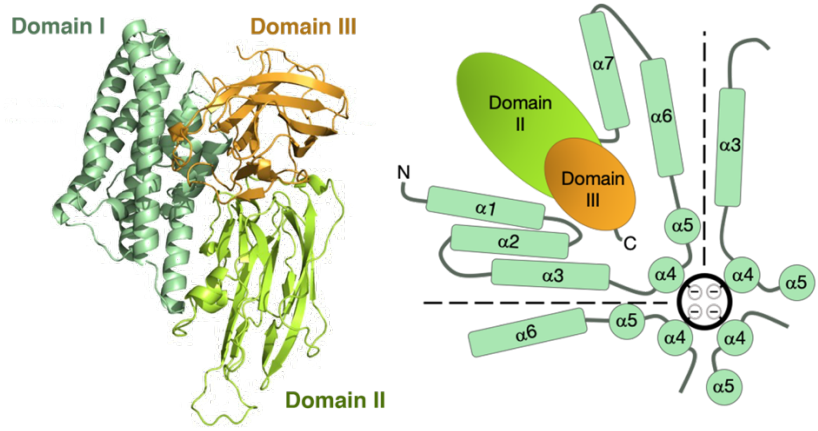

Figure 3. Diagram of the activated Cry1Aa toxin structure. Left: Ribbon diagram of a monomeric Cry1Aa protein (Adapted from Xu et al. (2014)). Right: Schematic representation of a putative tetrameric Cry1Aa ion channel, the four toxin molecules are aligned by the α4 helices facing the hydrophilic lumen of the channel (Adapted from Masson et al. (1999)). Each domain has been colored differently.

In the three domain Cry proteins, domain I is located at the N terminus and is constituted by a seven α-helix cluster that shares structural similarity with the pore-forming domain of the toxin colicin A (Xu et al., 2014). It has been proposed to be involved in membrane insertion and pore formation (de Maagd et al., 2001). Domain II is formed by three antiparallel β-sheets and two short helices, with a hydrophobic core inside. This domain plays a relevant role in toxin-receptor interactions (Jenkins et al., 2000; Xu et al., 2014). Last, domain III consists of two twisted anti-parallel β-sheets that form a sandwich. The inner sheet contacts with domain I on its C-terminal strand, and the outer sheet is exposed to the solvent. This domain, also known as "galactose-binding domain" is also involved in receptor binding (Xu et al., 2014). It is worth to note that, as shown in the right panel of **Figure 3**, Cry1A proteins have the ability to oligomerize through interactions with α4 from domain I (Masson et al., 1999; de Maagd et al., 2001). This feature is further discussed in Section III: Mode of action of Bt.

II.3 Structure of Vip3 proteins

The Vip3 protein family includes 14 holotype proteins out of the 391 holotype protein sequences that have been classified (Crickmore et al., 2020). Their name comes from "Vegetative Insecticidal Protein", since these proteins are produced and secreted outside the Bt cell during its vegetative growth phase. Interest in this family of proteins has grown since their discovery 26 years ago, given their high toxicity against lepidopterans and their distinct mode of action and sequence compared to Cry proteins (Estruch et al., 1996; Gupta et al., 2021).

The genes that code for Vip3 proteins are commonly found among Bt strains, since up to 90% of strains that carry *cry1* or *cry2* genes are also carrying at least one *vip3* gene (Beard et al., 2008; Hernández-Rodríguez et al., 2009). Vip3 proteins consist of ~787 amino acids, and have an average molecular mass of ~89 kDa. The N-terminus of Vip3 proteins contains a signal peptide that allows the protein to translocate across the cell membrane (Estruch et al., 1996). This structure can be further processed by proteases producing a different toxic form with two fragments of *ca*. 60 and 20 kDa (Chakroun et al., 2016; Bel et al., 2017).

In the present thesis, special attention is paid to Vip3A proteins. Recent research on the structure of Vip3A proteins has demonstrated the presence of five different domains, as represented in **Figure 4** (Jiang et al., 2020; Núñez-Ramírez et al., 2021). The first domain, is composed of four α-helices with a protease cleavage site, that remains tightly associated with the core protein by interacting with domain II. The second domain, composed by 5 α-helices, represents the core of the tetramer and maintains the oligomeric structure. The role of the third domain, formed by three antiparallel β-sheets is still not well known. Finally, the fourth and fifth domains are connected through a linker and contain carbohydrate-binding motifs (Núñez-Ramírez et al., 2021; Gupta et al., 2021). It has been observed that they have a strong binding preference towards chitosan and chitin, although

the precise binding substrates needs further examination (Jiang et al., 2020). Among different Vip3A proteins the maximum divergence is usually found at the C-terminus, which is why it has been considered as the area responsible for specificity (Chakroun et al., 2016; Chakrabarty et al., 2020).

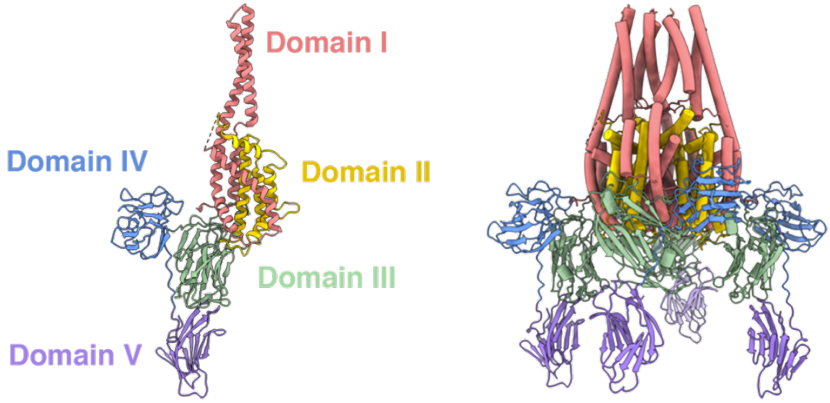

Figure 4. Diagram of the Vip3Aa protein structure. Left: Protoxin monomer. Right: Protoxin tetramer. Each domain has been colored differently. Adapted from Núñez-Ramírez et al. (2020).

III. Mode of action of *B. thuringiensis* and its proteins

In the following section, the life cycle of *B. thuringiensis* after infecting a larvae is explained, along with the mode of action of the pesticidal proteins it can produce, with an especial focus on Cry and Vip3 proteins. The eight main steps of this cycle are described next, and summarized in **Figure 5**.

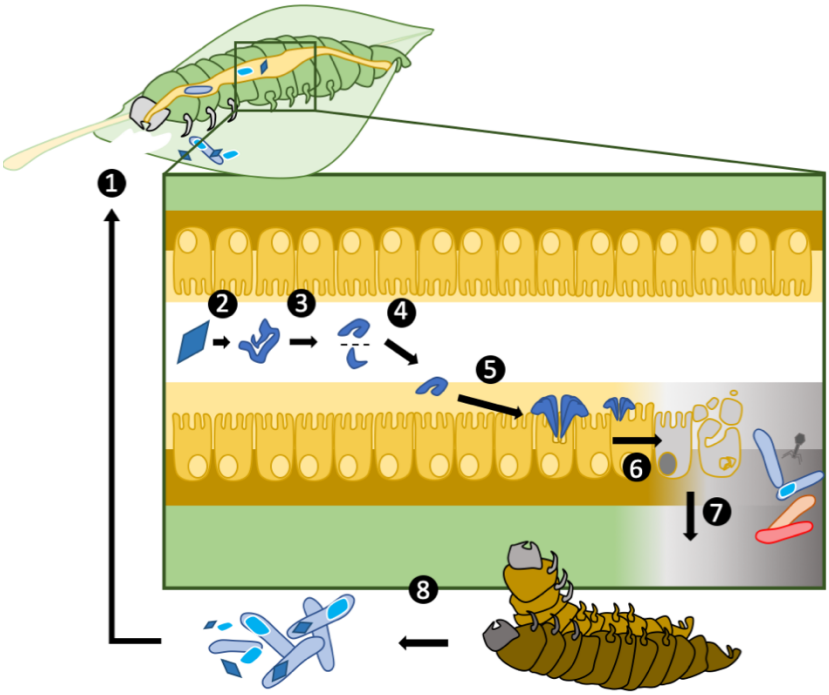

Figure 5. Schematic representation of the general sequential steps in the life cycle of *Bacillus thuringiensis* and the mode of action of its pore-forming toxins. (1) Ingestion, (2) Solubilization, (3) Activation, (4) Crossing the peritrophic matrix, (5) Interaction with the midgut, (6) Oligomerization, pore formation and cell lysis, (7) Septicemia and death, (8) Dispersion. Pinos and Hernández-Martínez (2019).

III.1 Ingestion

The first step in the mode of action entails the ingestion of Bt by the susceptible species (**Figure 5**, step 1). It is worth to note that, differently to other pesticidal treatments, Bt and its proteins (both in formulates and Bt crops) can exclusively act if they are previously ingested by the insect, and never by surface contact.

III.2 Solubilization

The second step after ingestion of Bt spores and crystals (i.e., from Bt-based sprayed crops), at least for Cry1 proteins, is the solubilization of the crystals. This process occurs when disulfide bridges in the crystal structures are broken, causing the liberation of the protoxins present in the crystal. Solubilization is dependent upon both the pH and the reducing conditions of the gut environment (Angus, 1954). For this reason, solubilization is considered to be a relevant factor in Cry1 protein specificity, since it will depend of appropriate conditions in the midgut of insects to obtain an optimal solubilization of crystals to release the protoxins (Jurat-Fuentes and Crickmore, 2017) (**Figure 5**, step 2). In the case of Vip3 proteins or Cry proteins expressed in transgenic plants, they do not require a solubilization step, since they are produced in a soluble form.

III.3 Protoxin processing by digestive enzymes

Both solubilized Cry1 protoxins and Vip3 are processed by endogenous proteases that are present in the midgut fluids, rendering an active toxin that is generally resistant to further proteolytic processes (**Figure 5**, step 3). This process is accomplished mainly by serin proteases: trypsins and chymotrypsins (Andrews et al., 1985; Bietlot et al., 1989; Caccia et al., 2014), the most abundant enzymes in midgut fluids of insects (Terra y Ferreira, 1994).

During the activation process, the majority of Cry1 protoxins (*ca*. 130 kDa) are digested sequentially, producing a resistant protein nucleus with a size of 55 to 65 kDa. Cry1 protein activation is produced by cutting off the N-terminal part (first 25-30 amino acids), and the C-terminal part located over the second third of the protein (500-600 amino acids). In the case of Vip3 protoxins, which have a size of *ca*. 89 kDa, activation renders two protein fragments with a size of 62 and 20 kDa, which remain attached together (Chakroun et al., 2016; Bel et al., 2017). The ability to process Bt protoxins by the insect has also been described as a major factor involved in specificity of these proteins (Jurat-Fuentes and Crickmore, 2017). Several studies have demonstrated that the lack of toxicity, or differences in susceptibility in close species was due to lack of activation capacities (Zalunin et al., 2015).

III.4 Crossing the peritrophic matrix

Once activated, all the toxins must cross the peritrophic matrix of the midgut (**Figure 5**, step 4). This matrix is rich in chitin, the main function of which is to separate the food and the epithelial cells. The peritrophic matrix impedes the food to have an abrasive effect over the midgut, as well as serving as a physical barrier against bacterial, viral or parasite infections (Wang and Granados, 2011).

Consequently, this barrier can reduce the amount of toxins that will later interact with midgut cells, having a direct effect in the susceptibility of the insect (Hayakawa et al., 2004; Rees et al., 2009). Nevertheless, the protective role offered by the peritrophic matrix can be compromised by the action of endogenous or exogenous chitinases (enzymes able to degrade chitin) from Bt (Kramer and Muthukrishnan, 1997).

III.5 Interaction with the midgut

After surpassing the peritrophic matrix, an interaction between Cry1 or Vip3 proteins and the brush border membrane from midgut cells takes place. These cells are considered as the main target cells of the toxins (**Figure 5**, step 5). One of the main challenges in Bt research is identifying which are the molecules that bind in a specific manner to Cry1 or Vip3 toxins, as well as understanding the relevance of this interaction in the mode of action of the toxins and the mechanisms of resistance (Ferré and Van Rie, 2002; Pigott and Ellar, 2007; Bravo et al., 2011; Jurat-Fuentes and Jackson, 2012, Jurat-Fuentes et al., 2021).

III.5.1 Putative Cry1 receptors

Currently, there is experimental evidence confirming that at least three membrane proteins can act as putative receptors for Cry1 proteins in insects; type N aminopeptidases (APN), cadherins and ABC transporters (Knight et al., 1994; Vadlamudi et al., 1995; Gahan et al., 2010). Initially, alkaline phosphatases (ALP) were also proposed as possible receptors, and although evidence of their implication with binding are scarce, ALPs are associated with resistance (Jurat-Fuentes and Adang, 2004).

Aminopeptidase-N. The insect midgut aminopeptidases-N (APN) show sequence similarity to the vertebrate zinc-dependent APN enzymes that cleave amino-acids from the amino terminus of proteins. These APNs are attached to lipids in the epithelial membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor. Midgut APNs were the first proteins to be described as putative Cry1 receptors (Knight et al., 1994, 1995). Binding interactions can occur via GalNAc moieties, at least in *M. sexta* (Masson et al., 1995), *H. virescens* (Gill et al., 1995), *L. dispar* (Jenkins et al., 2000) and *H. armigera* (Sarkar et al., 2009). Nevertheless, other studies have shown that this binding can be produced independent from GalNAc (Banks et al., 2001). Although

APNs can operate as functional receptors for Cry1 proteins, a clear understanding of their role in the mode of action is still needed.

Cadherins or cadherin-like proteins. Cadherin proteins represent a large family of calcium-dependent, transmembrane glycoproteins that maintain the integrity of contact between cells in multicellular organisms. They can interact with other cell junction proteins and with signal transduction pathways via their ectodomain and cytoplasmic domain, respectively (Ruoslahti and Obrink, 1996; Nollet et al., 2000; Angst et al., 2001; Takeichi, 1991). The cadherins that are shown to interact with Cry1A proteins have 12 ectodomains and single transmembrane and cytoplasmic domains. They were first shown to bind with high affinity in *M. sexta* larvae (Vadlamudi et al., 1995) and the same cadherin expressed in cultured insect cells conferred sensitivity to Cry1A toxins (Hua et al., 2004). The functionality as a Cry1A receptor has been reported in other lepidopterans, such as *B. mori* (Nagamatsu et al., 1999), *O. nubilalis* (Flannagan et al., 2005) or *H. virescens* (Jurat-Fuentes and Adang, 2006).

Alkaline phosphatases. Alkaline phosphatases (ALPs) are protein enzymes with the physiological role of dephosphorylating compounds. In the midgut of insects different ALP forms can be found, such as soluble ALPs in the lumen or GPI-anchored ALPs attached to the membrane of the midgut cells. Their interaction with Cry1 proteins was first detected by proteomic analyses, where ALPs from BBMVs from different species, such as *M. sexta* (McNall and Adang, 2003) or *H. virescens* (Krishnamoorthy et al., 2007), were able to bind with Cry1A proteins. In a similar way to APNs, binding between ALPs and Cry1 proteins involve interactions with GalNAc (Jurat-Fuentes et al., 2004; Ning et al., 2010). Through these findings, ALPs were proposed as low affinity receptors for Cry1A proteins that would attract these to the midgut membrane (Jurat-Fuentes et al., 2011). Moreover, it has been demonstrated that binding of Cry1Ab to ALP in *M. sexta* larvae is more relevant that binding to APN to cause toxicity, through RNAi

silencing experiments (Flores-Escobar et al., 2013). Accordingly, membrane-bound forms of ALPs could provide binding sites for Cry1 proteins yielding higher concentrations of the toxins at the epithelial surface, as APNs (Jurat-Fuentes et al., 2021), but their clear role in the mode of action is not yet known.

ABC transporters. The ATP-Binding Cassette (ABC) transporters are a wide and ancient superfamily of membrane proteins. They are formed by two cytosolic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP, and two integral transmembrane domains (TMDs), each consisting of six membrane-spanning helices that change orientation to allow the passage of small molecules across membranes (**Figure 6**, panel A) (Holland et al., 2003; Higgins and Linton, 2004). This mechanism of active transport of allocrites is known as the ATP-switch mechanism (represented in **Figure 6**, panel B), where the transporter changes between a closed and an open conformation of its TMDs, with respect to the outer membrane surface (Linton, 2007). In the closed state, the extracellular loops in the TMDs block the access through the channel (step 1). Binding of internal allocrites with the inner transmembrane helices increase the affinity of NBDs for ATP. Binding brings both NBD closer, dimerizing and consequently the TMDs rotate and open towards the outside (step 2) (Heckel, 2021). Thus, the allocrite is secreted out of the cell. After hydrolysis of ATP, the NBDs move apart while releasing phosphate and ADP (step 3), restoring the transporter to the closed configuration (step 4).

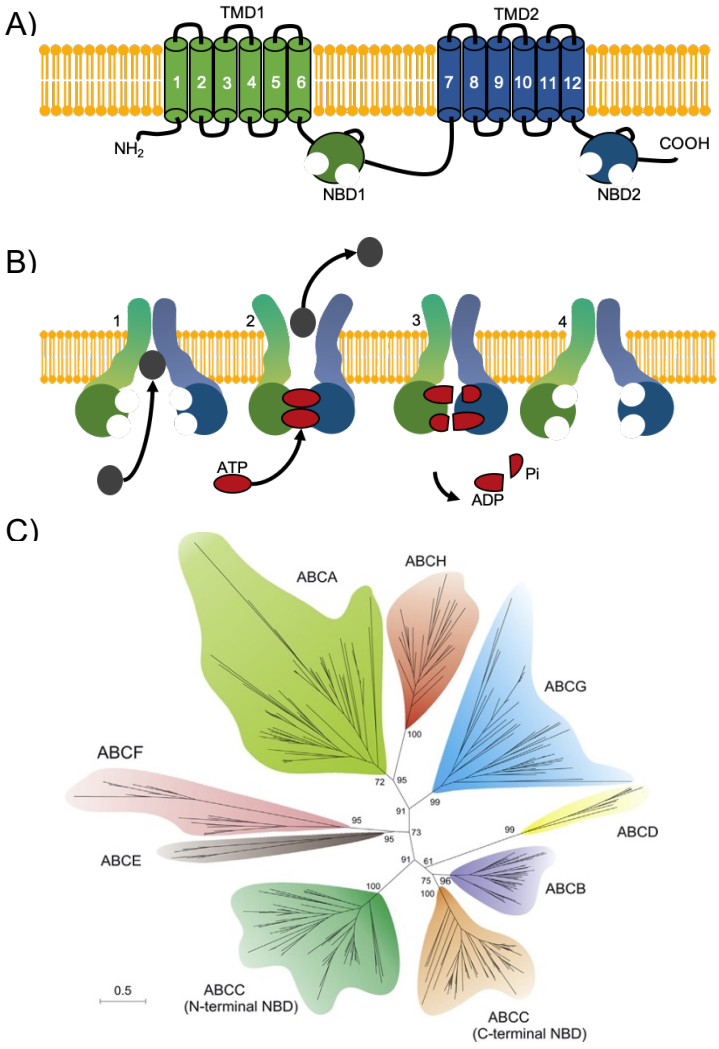

Figure 6. ABC transporters at a glance. (A) Domain structure. (TMD: Trans-Membrane Domain, NBD: Nucleotide-Binding domain). (B) Active transport mechanism (dark grey circle represents an allocrite). (C) Diversity according to sequence similarity (Dermauw and Van Leeuwen, 2014).

ABC transporters can be found in all kind of organisms, and eight major families are denoted by the letters A-H, a classification according to sequence similarity in the conserved NBDs (**Figure 6**, panel C). Compared to human, bacterial or even nematode ABC transporters, the knowledge about arthropod ABC transporters is still limited. Currently, more than 400 arthropod ABC proteins have been identified (Dermauw and Van Leeuwen, 2014). Nevertheless, the precise function of only a few ABC transporters from several insects, such as *D. melanogaster, A. gambiae*, *A. mellifera*, *B. mori* or *Tribolium castaneum* has been explored (Ewart and Howells, 1998; Roth et al., 2003; Liu et al., 2011; Xie et al., 2012; Broehan et al., 2013).

In regards to their biological function, many of these transporters are involved in the export of xenobiotics, drugs, or endogenous signaling molecules (Sharom, 2011). They have also been associated with the transport, as well as resistance to over 27 different insecticides and acaricides, but also to herbicides, fungicides, antibiotics, and heavy metals (Buss and Callaghan, 2008; Dermauw and Van Leeuwen, 2014). Furthermore, the most recently discovered property of insect ABC proteins is their role in the mode of action of 3D-Cry proteins from Bt (Gahan et al., 2010), which has been recently reviewed in Heckel (2021).

ABC transporters were first related with Cry1 proteins by genetic linkage between an ABCC2 mutation and resistance to Cry1A proteins in a strain of *H. virescens* (Gahan et al., 2010). Shortly after, direct confirmation of the functionality of ABC transporters in Cry1A mode of action was supported by studies between Cry1Ab protein and different insertions in the ABCC2 of *B. mori* (Atsumi et al., 2012), followed by a study expressing the same transporter in insect cell lines (Tanaka et al., 2013). From here onwards, the number of studies that have achieved to relate insect ABC transporters with the mode of action of 3D-Cry proteins has grown significantly over the last years. Most of these studies have been performed through the analysis of different resistant strains of lepidopterans, but also through expression of the transporters in cell lines, as well as silencing or knocking-out in live

insects. A recent review by Sato et al. (2019) gathers the information available on which ABC transporters have been found to act as functional receptors to which 3D-Cry proteins, and the vast majority of interactions observed up to date are between Cry1 or to a lesser extent, with Cry2 proteins. Nonetheless, if there is any relation between ABC transporters and the mode of action of Vip3 proteins remains to be known.

III.5.2 Putative Vip3 receptors

For Vip3 proteins, interaction studies suggest that binding sites are not shared with Cry1 proteins (Chakroun and Ferré, 2014). Few studies are conclusive on which are the specific receptors of Vip3 proteins, and some of them have shown that the ribosomal protein S2, the fibroblast growth factor receptor-like protein, or the scavenger receptor-C, can act as functional receptors for the Vip3A toxin in some *Spodoptera* spp. (Singh et al., 2010; Jiang et al., 2018a; Jiang et al., 2018b).

Ribosomal S2 protein. The precise cellular function of the ribosomal S2 protein (RpS2) is not clearly defined, although it has been proposed to be involved in modulating certain pathways such as methylation or oogenesis control in *Drosophila melanogaster* or suppression of ribosomal protein synthesis to trigger apoptosis (Cramton et al., 1994; Naora et al., 1998; Swiercz et al., 2005). Regarding its involvement with Bt proteins, RpS2 from *Spodoptera litura* (the tobacco cutworm) has been observed to interact with Vip3A, and RNA interference experiments with RpS2 showed reduced Vip3A larval mortality (Singh et al., 2010). To rule out the possibility that these results are anecdotal, further research on the RpS2 is needed.

Fibroblast growth factor. The Fibroblast growth factor receptor-like protein (Sf-FGFR), found in the membrane of ovary-derived *Spodoptera frugiperda* cells (Sf9), has been also described to interact with the Vip3Aa protein. Moreover, a reduction of the

expression levels in Sf9 cells was correlated with decreased sensitivity to Vip3Aa toxicity (Jiang et al., 2018). In mammals, FGFRs can initiate downstream signal transduction, such as activation of MAPK or STAT cascades (Turner et al., 2010). Still, the biological role of this membrane protein in insects has not been characterized, and its implication with Vip3Aa mode of action is not yet known.

Scavenger receptor class C like protein. Scavenger receptors are a large family of structurally and functionally diverse proteins. Little is known about their biological role in insects. In *Drosophila*, it is suggested that they might be involved in host defense (Pearson et al., 1995). In a recent study, the scavenger receptor class C like protein of cultured insect cells derived from *S. frugiperda (*Sf-SR-C) was found to directly interact with Vip3A proteins and cause its internalization via endocytosis (Jiang et al., 2018). In this system, the Sf-SR-C was responsible for allowing Vip3A toxicity.

III.6 Oligomerization, pore formation and cellular lysis

Numerous studies in the scientific literature have pointed out that the toxic activity of Bt proteins is due to the ability to form pores in the membrane of its target cells. This model (known as the pore-forming model) is currently the most supported model by the scientific community, due to a large body of research, especially for Cry1 proteins (Pardo-López et al., 2013; Vachon et al., 2012). It is accepted that, after the binding of Cry1 proteins with specific membrane receptors, an oligomeric structure is formed and then inserted into the membrane of midgut cells, causing a pore. This loss in membrane integrity generates an osmotic imbalance that provokes cell swelling, and consequently the lysis of the cells (**Figure 5**, step 6) (Wolfersberger, 1992).

In the case of Cry1 proteins and lepidopteran larvae, it is generally considered that after low affinity interactions with APNs or ALPs, a high concentration of Cry1 proteins (Pacheco et al., 2009; Masson et al., 1995; Arenas et al., 2010) facilitates the proteolytic cleavage of helix α1

after binding to cadherins and this triggers oligomerization (Masson et al., 1995; Gómez et al., 2002). Subsequent binding of the oligomeric structure to the receptors (APNs, ALPs or ABC transporters) facilitates the insertion into the membrane causing pore formation and cell lysis (Arenas et al., 2010; Pardo-López et al., 2006). Moreover, it was suggested that the ABCC2 might be involved in inducing Cry1A oligomerization and insertion (Heckel, 2012), and a recent study demonstrated it for Cry1Ac and the ABCC2 from *Plutella xylostella* (Ocelotl et al., 2017).

To further explore the oligomerization abilities of Bt proteins, one promising tool is the use of disabled insecticidal proteins (DIP). DIPs are defective proteins that are inhibitory variants of the wild-type version of the protein when both are mixed. They consist in deliberate amino acid point-mutations introduced in the protein sequence. These mutations can inhibit the toxicity in different ways, depending on which feature of the mode of action is affected (Jerga et al., 2019). One strategy, known as "oligomer poisoning" or "Double-negative (DN) mutants", consists in the formation of oligomeric structures including both mutant monomers and wild-type monomers. The oligomeric structure formed cannot be correctly inserted in the membrane and, thus, the toxicity is lost. This strategy has been used to characterize the mode of action and neutralize other bacterial toxins such as *Bacillus anthracis* protective antigen (PA) (Sellman et al., 2001) or *Staphylococcus aureus* leukocidins (Parker and Prince, 2016). In the case of Bt, a study using a DN mutant from Cry1Ab demonstrated that oligomerization, at least in this protein, is a fundamental step in the mode of action (Rodríguez-Almazán et al., 2009). Other strategy is to produce mutants that are unable to oligomerize but can still bind in their monomeric form to the binding sites of the wild-type protein. This strategy can help to better understand the different affinities for binding between monomeric and oligomeric structures (Rainey et al., 2004; Jerga et al., 2019). Still, many questions remain unanswered, such as which of the protein structures are the main agents causing toxicity, since a co-existence of monomers, dimers, trimers, and tetramers has

been found during the oligomerization process of Cry1Aa (Groulx et al., 2011).

In the case of Vip3 proteins, less is known about the steps in the oligomerization process of these proteins, but a tetrameric structure has been shown to form spontaneously in solution (Kunthic et al., 2017; Palma et al., 2017), indicating that oligomerization could be the result from hydrophobic interactions prior to toxin insertion. Furthermore, proteolytically activated Vip3A with lepidopteran gut fluids was shown to form pores by voltage clamp assays, as well as to form stable ion channels in planar lipid bilayers in the absence of receptors (Lee et al., 2003). Recently, the resolved structure of the Vip3A protoxin and the protease-digested tetramer shed light on the mechanism by which this protein produces a pore. Through a conformational change, a needle is formed through a spring-loaded apex in the N-terminal part after protease treatment (Núñez-Ramírez et al., 2021). These results were also observed in the Vip3B protein, which could also be inserted in liposome membranes (Byrne et al., 2021).

III.7 Septicemia, insect death and dispersion

After midgut cell lysis, Bt spreads through the hemolymph, facing both the humoral and cellular immunities of the host. In the first case, as a response to cationic antimicrobial peptides (AMP) produced by the insect, Bt can neutralize the negative charge of its surface (Khattar et al., 2009) or inhibit AMP activity by metalloproteases (Fang et al., 2009). In the second case, Bt can produce an array of cytolysins and toxins that induce necrosis or apoptosis to cultures of insect-immune cells (Tran et al., 2011; Cadot et al., 2010). Consequently, a septicemia is mainly caused by the invasion of spores, vegetative forms of Bt, as well as other opportunistic bacteria and several pathogens that enter the hemolymph (Adang et al., 2014; Caccia et al., 2016) (**Figure 5**, step 7) and the insect dies. Then, Bt can take advantage of this niche to continue dividing and sporulating. It has been suggested that the

extracellular proteases NprA and NprR produced by Bt help to provide nutrients by degradation of the host tissue, allowing the bacterial population to achieve complete sporulation (Perchat et al., 2011, Dubois et al., 2012). Finally, Bt spores are dispersed to start over with the cycle again (**Figure 5**, step 8) (Raymond et al., 2010; Argôlo-Filho and Loguercio, 2014).

III.8 Signaling pathway model

Even though the pore formation model is the most consolidated model, over a decade ago an alternative mode of action was proposed to explain the lack of correlation between pore formation and cytotoxicity for certain Cry proteins. This alternative model was proposed by Zhang et al. (2005) through *in vitro* assays using cell lines expressing the cadherin from *Manduca sexta* and the Cry1Ab protein. Authors proposed that this toxin bound as a monomer to the cadherin, triggering a magnesium-dependent (Mg^{2+}) signal cascade (Zhang et al., 2005) and activating the adenylyl cyclase/protein kinase A pathway (Zhang et al., 2006). These processes would lead to necrosis and consequently cell death. It is worth to note that presently, cytotoxicity would be determined by the action of osmotic lysis (a direct consequence of pore formation), whereas intracellular signaling processes would be related to the insect response to the damage caused by the toxins (Tanaka et al., 2012; Hernández-Martínez et al., 2017).

IV. Biological and biotechnological applications of Bt

Although Bt was originally considered a risk for the silkworm rearing industry, insect pathologists as well as agronomists soon became interested in the entomopathogenic properties of the bacterium, since small amounts of preparations of Bt (containing spores and crystal inclusions) were highly efficient in killing insect larvae (Sanchis 2011). As soon as 1938, the first Bt-based commercial product ("Sporéine") was available (Entwistle et al., 1993) after several successful trials controlling the gypsy moth (*Lymantria dispar*) and the European corn borer (*Ostrinia nubilalis*) in the U.S. and Hungary, respectively. After a 20-year hiatus in adoption of this new biopesticide due to the dominance of chemical pesticides (mainly due to the success of DDT), two Bt-based products emerged, Thuricide® and Bactospéine®, followed by "Entobacterin-3", "Dendrobacilline" and "Biospor" in the 60s. In 1970, two highly effective strains of Bt were serotyped, *kurstaki* and HD-1, which became the basis of new Bt products, such as Dipel ® (de Barjac and Lemille, 1970). In the late 70s, a growing concern started on the use of chemical products, since their continued use severely damaged the environment. Moreover, the appearance of resistance to synthetic pesticides was declared one of the world's most serious environmental problems by the United Nations Environmental Program by 1979. These facts led to a significant increase in the commercial interest of Bt as a pesticide agent.

Still, formulations based on Bt remained rather ineffective against some pests since the sprays coud not reach cryptic insects in stems or roots. In the 1980s, genetic engineered plants carrying *cry* genes from Bt started being developed, being the first one the tobacco plant (Vaeck et al., 1987), and then tomato and cotton plants, by using the Ti plasmid from *Agrobacterium tumefaciens.* In the 1990s and early 2000s, Bt-engineered potato, corn and cotton products were commercially available and approved by the US Environmental Protection Agency (EPA). Thanks to the development of Bt crops, these plant varieties

were protected against different pests such as *Leptinotarsa decemlineata*, *Ostrinia nubilalis*, *Agrotis ipsilon*, *or Spodoptera frugiperda*. The use of these crops since 1996 has led to a significant reduction in the use of pesticides, as well as saving costs for growers. In the same way, the confidence in the benefits of using Bt crops has increased since then throughout the world (Sanchis, 2011).

V. Appearance of resistance and gene pyramiding strategy

Sometime after spray products were stablished as an effective tool to control pest populations, development of Cry protein resistance was found in *Plodia interpunctella* (McGaughey, 1985). Isolated first from Bt-treated grain storage bins, the initially low resistance grew after laboratory selection over 15 generations, achieving 100-fold resistance. In 1993, resistance to Bt-treatments was documented in the lepidopteran pest *Plutella xylostella*, further selection in the laboratory resulted in 1,000-fold resistance to Cry1A proteins (Tabashnik et al., 1993, 1997). In the early 2000s, some populations of *Helicoverpa zea* exposed to Bt-cotton from Arkansas and Mississippi showed 50 to 100-fold resistance to Cry1Ac. Since then, the appearance of resistance to Bt crops and treatments has threatened their effectiveness, becoming a real reason for concern.

The use of gene pyramiding in Bt crops, along planting of non-Bt refuges, has helped to alleviate this issue. Gene pyramiding consists in combining multiple insecticidal Bt proteins in the same plant that have different modes of action, meaning that the binding sites in the membrane of the midgut are not the same. Since 2002, when a new generation of Bt cotton combining the expression of Cry1Ac and Cry2Ab proteins was released, the number of Bt crops benefitting from gene pyramiding has increased notably. Up to date, at least 18 different pyramided Bt corn and cotton varieties are being used in the US (Carrière et al., 2015), allowing to control a wider spectrum of pests.

Currently, there are approximately 101 million hectares planted with Bt crops worldwide, representing more than half of the global cultivated area of genetically modified crops (James, 2017).

However, cases of pest resistance to Bt crops have increased from 3 in the year 2005 to 16 in 2016. This type of pest resistance has been also defined as practical resistance, since it reduces the efficacy of the Bt crop and thus has practical consequences for the control of a certain pest (Tabashnik et al., 2014). Among these 16 cases of practical resistance affecting Bt corn and/or cotton, there are populations of *Busseola fusca*, *Diatraea saccharalis*, *Diabrotica v. virgifera*, *H. zea*, *Pectinophora gossypiella*, *Spodoptera frugiperda*, and *Striacosta albicosta*, with an average onset of resistance of 5.2 years (Tabashnik and Carrière, 2017). Moreover, 3 other populations have been classified as early warnings of resistance, where monitoring data showed a statistically significant decrease in susceptibility although reduced efficacy of the Bt crop has not been reported yet. These 3 cases correspond to populations of *D. saccharalis*, *Helicoverpa armigera*, and *Ostrinia furnacalis*. The proteins to which pests have developed practical resistance are mainly 3D-Cry proteins: Cry1Ab, Cry1Ac, Cry1A.105, Cry1Fa, Cry2Ab, Cry3Bb, mCry3A, and eCry3.1Ab, but also Gpp34Ab1 and Tpp35Ab1. Conversely, no cases of Vip3 practical resistance have been detected yet, since the implementation of this protein (Vip3Aa) in the gene pyramiding strategy is relatively new and the adoption in the field is still low (Tabashnik and Carrière, 2017). In any case, the co-expression of *vip3A* genes, combined with the production of high toxin doses and planting of non-Bt refuges, is helping to reduce the risk of resistance as well as expanding the insect activity range (Jurat-Fuentes et al., 2021).

VI. Resistance mechanisms to Bt proteins

Given the economic relevance of field resistance to Bt proteins, a great effort has been made to study the biological reasons on why a given pest loses susceptibility to the action of Bt and/or its proteins. For

this purpose, one of the main starting tools used in research over recent decades has been the development of laboratory-selected resistant colonies of different pests. These resistant colonies help to validate resistance management practices and provide a means to characterize, from a genetic and biochemical point of view, alterations that might be biologically relevant in resistance evolution (Ferré and Van Rie, 2002, Jurat-Fuentes et al., 2021). To this end, insects are first fed with sub-lethal doses of the desired Bt protein. Then, surviving individuals are reared over several generations to increasing concentrations of the protein, until the lethal concentration causing a 50% mortality (LC_{50}) of the selected population is significantly higher than the control populations not exposed to the Bt product or the insecticidal protein. Further characterizations (genetic, biochemical, etc.) of the selected colony are made with non-selected individuals.

Through studies with both laboratory-selected and field-evolved resistant pest colonies, the most common resistance mechanism found is the alteration of toxin binding to putative receptors, which corresponds to the "interaction with the midgut" step in the mode of action (as shown in **Figure 5**, step 5). Besides this mechanism discussed below, there are other mechanisms such as altered protoxin processing by digestive enzymes, toxin sequestration, immune responses and others. Although they may have gone unnoticed, they have been described as an effective way to overcome Bt toxicity and will be revised and discussed in the objective III of the present thesis.

Alteration of toxin binding

APNs and ALPs. Few cases have associated alterations in APN structure or expression with resistance. In *Spodoptera exigua*, one out of the four analyzed APNs was absent in midguts of a Cry1Ca-resistant colony (Herrero et al., 2005). In *Helicoverpa armigera*, a 22 amino acid deletion of an APN lost binding to Cry1Ac protein in a resistant colony, as well as having a reduced expression of the APN (Zhang et al., 2009).

Moreover, proteomic analysis of a resistant *Trichoplusia ni* colony showed a reduction in APN1 and an upregulation in APN6 expression, which was genetically linked to Cry1Ac resistance (Tiewsiri and Wang, 2011). In the same way, reduced levels of ALP activity have been found in Cry1Ac and/or Cry2Ab-resistant *Heliothis virescens*, Cry1F-resistant *Spodoptera frugiperda*, Cry1Ac-resistant *Helicoverpa armigera* (Jurat-Fuentes et al., 2011), Cry1Ah-resistant *O. furnacalis* (Shabbir et al., 2020), and Cry1Ac-resistant *Plutella xylostella* (Guo et al., 2015). Although there is evidence that both APNs and ALPs can act as functional receptors for Cry proteins (Jurat-Fuentes et al., 2011; Knight et al., 1994), their alteration in protein expression or the presence of mutations appear not to be the only mechanism for resistance in the majority of cases.

Cadherin. In a laboratory-selected Cry1Ac-resistant colony of *H. virescens*, truncation of a cadherin gene was linked to resistance (Gahan et al., 2001). In the same way, laboratory-selected colonies of *Pectinophora gossypiella* resistant to Cry1Ac were found to carry cadherin mutations and lower levels of expression (Fabrick et al., 2021; Morin et al., 2003). Similarly, mutations and downregulation of cadherin were associated with reduced Cry1Ac binding and resistance in *O. furnacalis* (Jin et al., 2014). In *H. armigera*, different mutations in cadherins were linked to Cry1Ac resistance in China and India (Zhang et al., 2012, Nair et al., 2013) and knockouts of cadherin confirmed its importance for Cry1Ac resistance (Wang et al., 2016). Even with the above findings, linkage mapping and mechanistic studies have discarded the involvement of cadherin in Cry1Ac resistance in some resistant colonies, at least for *P. xylostella* and *Trichoplusia ni* (Baxter et al., 2005; Guo et al., 2015; Zhang et al., 2012). As demonstrated for *P. gossypiella*, the fitness cost of carrying these cadherin alleles might be a reason for this contradictory results (Carrière et al., 2018), so that further studies in other species are required to understand the importance of cadherin in Bt resistance.

ABC transporters. A Cry1Ac-resistant *Heliothis virescens* colony and a Cry1Ab-resistant *Bombyx mori* colony were found to have altered versions of the ABCC2 transporter (a deletion in exon 2 and an insertion in the second extracellular loop, respectively) that were correlated with resistance (Gahan et al., 2010; Atsumi et al., 2012). Similarly, different Cry1F-resistant *Spodoptera frugiperda* strains have been correlated with truncation of the ABCC2 (Boaventura et al., 2020; de Bortoli et al., 2019). Genetic mapping of Cry1Ac-resistant *Plutella xylostella* and Cry1C-resistant *Spodoptera exigua* correlated deletions in ABCC2 with resistance (Baxter et al., 2011; Park et al., 2014). Furthermore, it is feasible that the ABCC3 is also involved, since knocking out both ABC transporters yielded higher levels of Cry1Ac resistance in *Helicoverpa armigera* (Wang et al., 2020). Besides Cry1 protein resistance, the alteration of other ABC transporters such as the type A (mainly ABCA2) have also been linked to Cry2Ab resistance in *Helicoverpa armigera* (Tay et al., 2015; Wang et al., 2017), *P. gossypiella* (Mathew et al., 2018), and *T. ni* (Yang et al., 2019). Alterations in the type B (ABCB1) have been linked to Cry3Aa resistance in the coleopteran *Chrysomela tremula* and to Cry3B resistance in *Diabrotica v. virgifera* (Pauchet et al., 2016, Flagel et al., 2015). The increasingly growing amount of evidence demonstrating a linkage between alterations in ABC sequence or expression, has put the spotlight on these membrane receptors as the strongest candidates to be explored in the coming years.

VII. Selected pests

Spodoptera exigua

Spodoptera exigua (Lepidoptera: Noctuidae) (Hübner, 1808), commonly known as the beet armyworm (**Figure 7**), is a widely distributed pest around the globe, located in Africa, Asia, Australia, North America and the south of Europe. It is considered a polyphagous pest, since it can feed on more than 200 different plant species, both crops and weeds (Gho et al., 1993; Takatsuka and Kunimi, 2002). Among the plant crops that are attacked by *S. exigua*, many horticultural crops are found, such as tomato, pepper, eggplant, cucumber, or green beans. The life cycle of this lepidopteran is divided in four different stages: (1) egg, (2) larvae (with five different larval instars), (3) pupa, and (4) the adult moth. In this thesis, the interaction of the ABC transporter C2 from *S. exigua* with Cry1 proteins has been studied. In the same way, the role of an alteration in the ABCC2 from a resistant colony of *S. exigua* has been analyzed.

Heliothis virescens

Heliothis virescens (Lepidoptera: Noctuidae) (Fabricius, 1777), commonly known as the tobacco budworm (**Figure 7**), is a pest found in the North American continent, and certain areas of Central and South America. It is mainly a field crop pest, and it can feed in crops such as alfalfa, clover, cotton, flax, soybean or tobacco (Martin et al., 1976). As for *S. exigua*, its life cycle is divided in the same four stages, with the main difference being that the larvae can have between five and seven instars. In this thesis, the role of ABC transporters from *H. virescens* has been explored. Moreover, a laboratory-selected Vip3A-resistant *H. virescens* colony has been characterized from a genetic and biochemical point of view.

Ostrinia furnacalis

Ostrinia furnacalis (Lepidoptera: Crambidae) (Guenée, 1854), also known as the Asian corn borer (**Figure 7**), is a lepidopteran pest that can be found from China to Australia. Besides mainly feeding from corn, it can also feed from pepper, cotton, hops, or sugarcane among other horticultural crops. Its life cycle shows the same stages as for *S. exigua* and *H. virescens*, presenting the larvae six different instars. In this thesis, a laboratory-selected Cry1Ab-resistant *O. furnacalis* colony has been used to characterize the possible alteration of toxin binding sites, as well as to create a binding site model of this pest for Cry proteins.

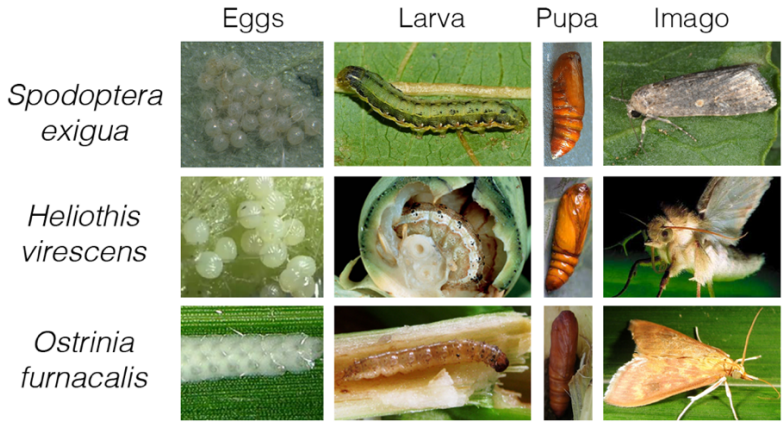

Figure 7. Life stages of the three lepidopteran pests used in this thesis.

OBJECTIVES

The overall aim of the present thesis is to broaden the knowledge of the interactions that occur between *Bacillus thuringiensis* pesticidal proteins and certain membrane proteins that act as receptors in the midgut of different lepidopteran species. Furthermore, this thesis aims to explore the reasons for the appearance of resistance to Bt and its pesticidal proteins by different approaches. In particular, how the ABC transporters from family C relate to Cry1A and Vip3A proteins; their involvement in binding to Bt proteins and with resistance. Also, to deepen the knowledge of Bt resistance by analyzing biochemical and genetic alterations in different Bt-resistant lepidopteran colonies, as well as gathering other possible mechanisms causing resistance beyond receptor binding. For this purpose, the following specific objectives were established:

1. **Study the interactions between the ABCC transporters from pests and Bt proteins**
   - 1.1. To understand the role of the ABCC2 transporter from *S. exigua* in Cry1A toxicity
   - 1.2. To gain a deeper knowledge on how Cry1A proteins interact with the ABCC2 transporter
   - 1.3. To study the role of other ABCC transporters from *H. virescens* in *B. thuringiensis* toxicity

2. **Characterize resistance to Bt proteins**
   - 2.1. To test whether structural mutations in the ABCC2 of *Spodoptera exigua* affect its function as a Cry1A receptor
   - 2.2. To study possible binding alterations of Cry1 proteins in a Cry1Ab-resistant colony of *Ostrinia furnacalis*
   - 2.3. To characterize biochemical and genetic alterations in a Vip3A-resistant colony of *Heliothis virescens*

3. **Review how other response mechanisms beyond receptor binding can counteract the effect of *B. thuringiensis***

CONTRIBUTIONS

Chapter	Concept	Experiments	Analysis	Writing	Review	Funding
Chapter 1	PHM, SH	MMS, DP, HE, LP, PHM	DP, PHM, MMS	PHM, MMS	SH, JF	SH, JF, RS
Chapter 2	PHM	DP, NJ, PHM	DP, PHM	DP, PHM	SH, JF	SH, JF
Chapter 3	PHM	DP, AML, CP	DP, AML, PHM	DP	PHM, JF	JF, OP
Chapter 4	PHM, SH	DP, MMS, PHM	DP, PHM	DP, PHM	SH, JF	SH, JF
Chapter 5	JF	DP, YW	DP, PHM, JF	DP	PHM, JF, KH	JF, KH
Chapter 6	PHM, JF	DP, MC,	DP, AML	DP	PHM, JF, JLJF, DW	JF, JLJF
Chapter 7	PHM	-	-	DP, AAG, PHM	JF	JF

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CHAPTER 1

Role of *Bacillus thuringiensis* Cry1A toxins domains in the binding to the ABCC2 receptor from *Spodoptera exigua*

Adapted from:
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Introduction

Bacillus thuringiensis (Bt) is a bacterium which produces a wide range of insecticidal proteins which are useful in biological control of insect pests (Crickmore, 2006; Schnepf et al., 1998). One of the most studied proteins from Bt are the insecticidal crystal proteins (Cry proteins) which have been used to control insect pests both in formulated sprays or in insect-resistant genetically-modified crops (Bt-crops) (Roh et al., 2007). The extensive use of these proteins in agriculture has led to the emergence of resistance to some Cry proteins in target insects, threatening the long-term use of Bt products (Tabashnik and Carrière, 2017).

The mode of action of Cry proteins has been widely studied, though some aspects still remain unclear. In general, it is accepted that, after the ingestion of the protoxin by the insect, the protein is solubilized and activated by the action of digestive enzymes. The way in that the active forms exert their cytotoxicity is still controversial, though binding to specific receptors in the brush border of the epithelial midgut cells is accepted by either the sequential binding model (Bravo et al., 2007) or the Mg^{2+}-dependent signalling pathway (Zhang et al., 2006, 2005). In the sequential binding model, the activated Cry1A proteins (as monomers) go through a complex sequence of binding events with different midgut Cry-binding proteins, leading to the cleavage of helix α1 of the Domain I and to the oligomerization of monomers. Afterwards, the oligomer binds to other midgut membrane proteins, and finally is irreversibly inserted into the membrane, where it forms pores in the apical membrane of larvae midgut cells which eventually lead to septicemia and insect death (Adang et al., 2014; Bravo et al., 2007; Pardo-López et al., 2013). The second model proposes that the activation of an Mg^{2+}-dependent signalling pathway, after the binding of the Cry protein to a cadherin protein, leads to oncotic cell death (Zhang et al., 2006, 2005).

The specific toxin-receptor interaction has been reported as a crucial step for toxicity (Jurat-Fuentes and Crickmore, 2017). In addition, binding alteration in the insect midgut is the step of the mode of action that has more often been associated with insect resistance to Cry proteins, although other resistance mechanisms have also been reported (Ferré and Van Rie, 2002; Tabashnik and Carrière, 2017). Currently, several molecules have already been proposed as candidate receptors for different Cry proteins: cadherin-like proteins (Nagamatsu et al., 1998; Vadlamudi et al., 1995), GPI-anchored proteins – such as aminopeptidases N (APNs) and alkaline phosphatases (ALPs) – (Knight et al., 1994; Luo et al., 1997; Yaoi et al., 1997) (Arenas et al., 2010; Jurat-Fuentes and Adang, 2004; Ning et al., 2010) and more recently, the ABC transporter subfamily C (ABCC) (Gahan et al., 2010; Tanaka et al., 2013). Interestingly, mutations in these candidate receptors have been reported in several Bt-resistant strains. Mutations in cadherin-like receptors were identified in strains resistant to Cry1A proteins from *Heliothis virescens*, *Pectinophora gossypiella* and *Helicoverpa armigera* (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). Similarly, alterations in apn genes have been associated with resistance to Cry1A proteins in *H. armigera, Diatraea saccharalis* and *Trichoplusia ni* (Tiewsiri et al., 2011; Yang et al., 2010; Zhang et al., 2009).

The ABCC2 transporter was first proposed as a Cry receptor in the YEE strain of *H. virescens* (Gahan et al., 2010). Since then, different genetic studies have revealed the correlation between mutations in ABCC proteins and Bt resistance in different Lepidoptera (Heckel, 2012; Baxter et al., 2011; Atsumi et al., 2012; Xiao et al., 2014; Park et al., 2014). In a laboratory-selected colony of *Spodoptera exigua* (named Xen-R), a mutation in the *ABCC2* locus was described as genetically linked for the resistance of this colony to the Bt commercial bioinsecticide Xentari™ (Park et al., 2014). Interestingly, the mutation found in this resistant colony affects an intracellular domain involved in the ATP binding region. To date, the ABCC2 transporter from different lepidopteran species has been cloned and expressed on

cultured cells to study whether the expression of the transporter correlates with increased susceptibility to Cry proteins (Atsumi et al., 2012; Banerjee et al., 2017; Bretschneider et al., 2016; Endo et al., 2017; Tanaka et al., 2016a, 2013).

In this study, we investigated the role of the *S. exigua* ABCC2 (SeABCC2) transporter in the mode of action of Cry1A proteins and Cry1Ca. To achieve this goal, the SeABCC2 transporter has been expressed in Sf21 insect cells and in HEK293T human cells, first, to characterize their functional role by toxicity cell assays, and second, to assess its ability to bind to these Cry1 proteins. Hybrids were used to identify the critical regions (domains) of Cry1 proteins which are interacting with the transporter.

Material and methods

Cell culture maintenance

Spodoptera frugiperda derived Sf21 cells were cultured at 25 ºC in Gibco® Grace's Medium (1X) (Life Technologies™) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Routine culture was performed in T25 flasks (Nunc) and cells were passaged once a week.

Human embryonic kidney 293T (HEK293T) cells were cultured at 37 °C in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific) supplemented with 10% FBS (BioWest, Nuaillé, France), 4 mM GlutaMAXTM (Thermo Fisher Scientific), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a 5% CO2 atmosphere and 95% relative humidity.

Generation of Sf21 clones expressing SeABCC2-FRA

Total RNA was isolated from midgut tissues of *S. exigua* larvae (FRA colony) (Hernández-Martínez et al., 2010) using the RNAzol RT reagent (Sigma-Aldrich, St. Louis, MO, US) according to the manufacturer's instructions, and used for cDNA synthesis. *Spodoptera exigua ABCC2* full gene (*SeABCC2-FRA*) was obtained by PCR amplification using the corresponding cDNA (KF926100.1 and KF926099.1, respectively) as a template. PCR amplifications were performed using specific primers which added *Sac*I and *Xba*I restriction sites and a FLAG-tag downstream in both *SeABCC2* genes. Primers used for cDNA subcloning are shown in Supplementary Table S1. The amplified fragments were cloned into the *Sac*I and *Xba*I restriction sites of the pIB-eGFP vector (kindly supplied by Els Roode, Wageningen University & Research) by replacement of the *gfp* gene, to generate the pIB-FRA vector. The pIB-eGFP and pIB-FRA vectors were used for stable transfection of Sf21 cells and carried a gene coding for Blasticidin resistance. For this purpose, Sf21 cells were seeded on 6-well plates at about 70% confluency and transfected with 1μg of each plasmid using Cellfectin® II Reagent (Invitrogen) following the

manufacturer's instructions. Monoclonal selection to obtain stable cell lines expressing the SeABCC2-FRA protein was carried out using cloning cylinders as described in Bretschneider et al. (2016). To support cell colony growth, conditioned medium (the supernatant of exponentially growing Sf21 cells, 3–4 days old) supplemented with 10% (v/v) FBS and 50 μg/ml of Blasticidin was used. After the monoclonal selection, only one cell line expressing the *SeABCC2-FRA* gene was obtained and was named Sf21-FRA. The stable cell line was maintained at 27 °C in culture medium containing 10 μg/ml Blasticidin.

Transient expression of the SeABCC2-FRA gene in HEK293T cells

The cDNA of *SeABCC2-FRA* was inserted into the expression vector pcDNA3.1 (Thermo Fisher Scientific) and fused with 3 × FLAG-tag (Sigma-Aldrich) at C-terminus as described previously by Endo et al. (2017).

HEK293T cells were seeded on cover glasses in the bottom of a 6-well plate (TrueLine; Nippon Genetics, Toyama, Japan) and transfected with the constructs using polyethylenimine (PEI Max, Polysciences, Inc., Warrington, PA) in Opti-MEM® (Thermo Fisher Scientific) for 2 h. Then, the media were replaced with fresh Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific) and incubated for 48 h at 37 °C. After incubation, cells were used for further experiments.

RT-qPCR

Expression of the *SeABCC2-FRA* gene was analyzed in the monoclonal Sf21 cell line by RT-qPCR using specific primers (Supplementary Table S1). Total RNA extraction was performed using RNAzol® RT (Sigma Aldrich, St Louis, MO) according to the manufacturer's instructions and it was used for cDNA synthesis using PrimeScript RT Reagent kit (TaKaRa Bio Inc, Otsu Shiga, Japan). RT-qPCR was performed with 5x HOT FIREPOL EvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia) following standard protocols and the DNA synthesis was measured in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To normalize the

gene expression, the *ubiquitin* gene was used as reference (Supplementary Table S1). The resulting slope from dilution series was used to calculate the efficiency and the amplification factor. The *SeABCC2* gene expression was given as copy number per 1000 molecules of the reference gene.

Detection of SeABCC2 proteins by immunostaining

Immunochemistry of Sf21 and HEK293T cells expressing SeABCC2 transporter using monoclonal anti-FLAG® M2 (Sigma) antibody was performed as described in Mang et al. (2016). Briefly, cells were seeded onto cover glass in the bottom of 6-well or 24-well plates and incubated overnight. After 16 h, cells were washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde in PBS and then washed again with PBS. Cells were incubated in TNT buffer (0.1 M Tris-HCl, 150 mM NaCl, 0.01% (v/v) Tween 20) with 0.01% Triton X-100 for 10 min and then blocked using TNT buffer with 1% BSA for 1 h at room temperature (RT). After blocking, cells were incubated with primary antibody (monoclonal anti-FLAG® M2 in TNT buffer at a 1:1000 dilution) for 2 h. After three washes with TNT buffer, cells were incubated with secondary antibody (goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, USA) at a 1:1000 dilution in TNT buffer for 1 h) and washed three times again with TNT buffer. Next, cells were incubated with l µg/ml of 4, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, Schnelldorf, Germany) for 20 min at RT to stain the cell nuclei. After washing with TNT buffer, cover glass were mounted using Dako Fluorescence Mounting Medium (Dako, Glostrup, Denmark), and observed under a confocal microscope (Olympus, FV1000MPE). As negative controls, transfected cells were immunostained with secondary antibody alone and non-transfected Sf21 or HEK293T cells were immunostained by anti-FLAG antibody.

Cry proteins preparation

Cry proteins used in the present work were obtained from different sources. For cell toxicity assays, Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca proteins and the hybrid proteins H04 (a chimera with domains

I and II from Cry1Ab and domain III from 1Ca) and H205 (a chimera with domains I and II from Cry1Ca and domain III from Cry1Ab) (de Maagd et al., 1996) were obtained from recombinant *Escherichia coli* strains kindly supplied by R. A. de Maagd (Wageningen Plant Research, Wageningen University). Inclusion bodies containing Cry proteins were purified, solubilized and trypsin-activated as follows (Herrero et al., 2004). After cell lysis, the pellets were recovered by centrifugation at 40,000 × g for 20 min and then washed five times with washing buffer (20 mM Tris-HCl, pH 8, 5 mM EDTA, 100 mM NaCl). Protoxin solubilization was performed by incubation of inclusion bodies at 37 °C in solubilization buffer (50 mM sodium carbonate, pH 10.5, 150 mM NaCl) containing 10 mM dithiothreitol (DTT). After 2 h, the solubilized protoxin was separated from insoluble debris by centrifugation at 40,000 × *g* for 20 min. The pH of the supernatant was lowered to pH 9 using 1M Tris/HCl (pH 8). The protoxin was activated by adding trypsin at a ratio of 1:10 (trypsin/protoxin, w/w) and incubating for 2h at 37°C.

For oligomer formation assays, the Cry1Ac protein and its mutant Cry1AcE129K were obtained from the *B. thuringiensis* HD73 strain and a recombinant *B. thuringiensis* strain (Portugal et al., 2017), respectively. Both strains were grown in HCT sporulation medium (Muñoz-Garay et al., 2009) at 30 °C until sporulation was completed (3–4 d). The recombinant strain expressing the mutant protein was grown in the sporulation medium supplemented with erythromycin 10 mg/ml. The spores and crystals were recovered by centrifugation at 12,800 × *g* for 10 min, and washed seven times with washing solution (300 mM NaCl, 10 mM EDTA). The three first washes were performed with the washing solution supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). After washing, crystal inclusions were purified by sucrose gradients (Thomas and Ellar, 1983). Protoxin solubilization was performed by incubation of the purified crystals in 100 mM sodium carbonate, 0.2% β-mercaptoethanol (pH 10.5). After solubilization, the pH was adjusted to pH 8.5 by adding an equal volume of 1 M Tris (pH 8.5) and the solubilized protoxins were

activated with trypsin using a ratio of 1:50 (trypsin/protoxin) for 2 h at 37 °C. The proteolytic reaction was stopped by the addition of PMSF at a final concentration of 1 mM. An Amicon ultra-4 centrifugal filter Ultracel-50K (Merck KGaA, Darmstadt) was used to exchange the buffer of the activated toxins to PBS (pH 8.5).

For binding assays, Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Ca were obtained from recombinant *B. thuringiensis* strains EG1273, EG7077, EG11070, EG1081, respectively (from Ecogen Inc., Langhorn, PA). Crystal purification and solubilization, and protoxin activation by trypsin, was performed as described by (Estela et al., 2004). The hybrids H04, H205, and the mutant Cry1AcE129K were prepared as previously described. After trypsin activation, all proteins were further dialyzed in 20 mM Tris-HCl (pH 9) and filtered prior to anion-exchange purification in a HiTrap Q HP column using an ÄKTA explorer 100 chromatography system (GE Healthcare, United Kingdom).

All proteins were analyzed by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (12% SDS-PAGE) and kept at −20 °C until used. The protein concentration was determined by densitometry using TotalLabTM 1D v13.01 software.

Viability assays in insect cells

Viability assays were performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay in Sf21 and Sf21-FRA insect cell lines after exposure to different Cry1 proteins. The MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria (Stockert et al., 2012). This product is impermeable to the cell membranes and therefore it accumulates in healthy or viable cells. Cells were suspended in Grace's medium (without FBS) and plated in 96-well ELISA plates (flat bottom) at *ca*. 70% confluency. A total volume of 100 µl of each cell line was added per well. The 96-well plates were incubated at 25 °C for at least 45 min. Then, 10 µl of the trypsin activated Cry proteins was

added to each well within a range of concentrations from 0 to 150 nM in duplicate on each plate. As a negative and positive controls, 10 µl of carbonate buffer (pH 10.5) and 10 µl of 2% Triton X-100, respectively, were added to the wells. After 3 h incubation at 25 °C, the cell viability was measured using the CellTiter 96® AQueous One Solution Reagent (Promega, Madison WI) following the manufacturer's protocol. Briefly, 20 µl of the reagent was added to each well and then the plate was further incubated for 2 h at 25 °C. After incubation, absorbance was measured at 490 nm (Infinite m200, Tecan, Mannedorf, Switzerland). The percent viability was calculated in relation to cells treated with carbonate buffer (considered as 100% viable), and the cells treated with Triton X-100 (considered as 0% viable).

In order to test whether an excess of Cry1AcE129K has an effect on cell viability exposed to Cry1Ac, a fixed concentration of the latter (1.5nM) was used along with increasing concentrations of Cry1AcE129K (1.5 nM, 15 nM and 150 nM). As controls, both proteins were tested separately using 1.5 and 150nM of Cry1Ac and Cry1AcE129K, respectively.

HEK293T cell swelling assays

HEK293T cell swelling assays were performed as described elsewhere (Endo et al., 2017). Briefly, transfected cells were incubated with Cry protein diluted in Hank's buffered saline solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 1.3 mM CaCl2, 0.5 mM MgCl2 and 0.4 mM MgSO4, pH 7.4) for 1 h at 37 °C. After treatment, cells were observed under phase-contrast microscope to observe the morphological changes and cell swelling.

Binding of ^{125}I-Cry1Ac to Sf21 cells

The Cry1Ac protein (25 µg) was labelled with 0.3 mCi of ^{125}I (PerkinElmer, Boston, MA) using the chloramine T method (Van Rie et al., 1989). The specific activity obtained for the labelled protein was 15 mCi/mg. Prior to binding assays, Sf21 and Sf21-FRA cells were

detached and recovered by centrifugation at 500 × *g* for 5 min at RT, and then washed two times with PBS. The final pellet was resuspended in binding buffer (PBS, 0.1% BSA) to a concentration of 4.6 10^7 cells/ml. The amount of cells was calculated by Countess Automated Cell counter (Invitrogen).

To determine the optimal concentration of cells to use in competition assays, increasing amounts of cells were incubated with 0.1 nM of labelled-Cry1Ac in a final volume of 0.1 ml in binding buffer for 1 h at RT. An excess of unlabelled toxin (150 nM) was used to calculate the nonspecific binding. After incubation, samples were centrifuged at 500 × *g* for 10 min and the pellets were washed with 500 µl of binding buffer. Radioactivity in the pellets was measured in a model 2480 WIZARD^2 gamma counter. Binding experiments were performed at least twice for each cell line.

Competition experiments were performed in binding buffer incubating the Sf21-FRA cells (9.2 10^6 cells/ml) with ^{125}I-Cry1Ac and increasing amounts of different unlabelled toxins in a final volume of 100 µl. After 1 h incubation at RT, samples were centrifuged at 500 × g for 10min, cell pellets washed with 500µl of binding buffer, and radioactivity in the pellets measured. Competition assays were replicated at least three times. The equilibrium dissociation constant (K_d) and concentration of binding sites (R_t) were estimated from the homologous competition experiments using the LIGAND program (Munson and Rodbard, 1980).

Binding of ^{125}I-Cry1Ac to brush border membrane vesicles (BBMV)

BBMV from *S. exigua* were prepared by the differential magnesium precipitation method from dissected midguts of last instar larvae (Wolfersberger et al., 1987) and kept at −80 °C until used. Protein concentration of BBMV preparations was determined by the method of Bradford (1976).

Prior to start, BBMV were centrifuged for 10 min at 16,000 × *g* and resuspended in binding buffer. To determine the optimal

concentration of BBMV for use in competition experiments, increasing amounts of BBMV were incubated with 0.1 nM of labelled-Cry1Ac in a final volume of 0.1 ml of binding buffer for 1 h at RT. An excess of unlabelled toxin (150 nM) was used to calculate the nonspecific binding. Competition experiments were performed in binding buffer incubating BBMV with ^{125}I-Cry1Ac and increasing amounts of unlabelled Cry1Aa, Cry1Ab and Cry1Ac in a final volume of 100 µl. After 1 h incubation at RT, samples were centrifuged at 16,000 × *g* for 10 min, and the pellets were washed with 500µl of cold binding buffer. Radioactivity in the pellets was measured in the gamma counter. Binding assays were performed twice, while competition assays were performed three times.

Oligomerization

The ability to form oligomers of native Cry1Ac and the mutant Cry1AcE129K proteins, when incubated with insect cell lines, was analyzed to see whether the toxicity of Cry1Ac proteins is due to the fact that the SeABCC2 protein is playing a role in the oligomer formation. Oligomerization assays were performed by treating the different insect cells lines with either Cry1Ac or Cry1AcE129K proteins. For this purpose, insect cells were suspended in Grace's medium (without FBS) and 100 µl were plated per well in 96-well plates at *ca*. 70% confluency. Plates were incubated at 25 °C for at least 1 h, and then a final concentration of 500 nM of either Cry1A proteins were added. As a negative control, the same volume of PBS was also added to the wells. After 3 h incubation, the supernatant was removed, and cells were detached and centrifuged for 15 min at 21,000 × *g* at 10°C. Then, cell pellets were resuspended in PBS and washed twice using the same buffer. Final pellets were resuspended in 1X Laemmli sample buffer at 50°C for 5 min. After heating, proteins were separated by 10% SDS-PAGE and transferred into PVDF membrane. Cry1Ac and Cry1AcE129K proteins were detected by Western blot using polyclonal anti-Cry1Ac (1/30,000; 90 min) as a primary antibody, followed by a secondary antibody, a goat anti-rabbit IgG-conjugated horseradish peroxidase (1/30,000; 60 min) and visualized with Super Signal

chemiluminescence substrate (Pierce), using an ImageQuant LAS400 image analyzer. All assays were performed in duplicate. At least three independent biological replicates were performed. The molecular weight (MW) of the oligomer was established by using a pre-stained MW marker (Precision Plus Protein™ Dual Colors Standards, BioRad).

Results

Analysis of the expression of SeABCC2 *genes in Sf21 cells and localization of the expressed proteins*

The *S. exigua* putative receptor ABCC2 was stably expressed in Sf21 cells after transfection and kept with antibiotic selection. The expression of the gene was analyzed by RT-qPCR. (**Supplementary Figure S1**). Expression levels of the *SeABCC2* gene were found significantly higher than the expression of the housekeeping gene in the transfected cell line, whereas no expression was detected for the Sf21 cell line (non-transfected). Immunostaining using an anti-3 × FLAG-tag antibody showed that SeABCC2 transporter was located on the cell membrane (**Figure 8**). For further experiments, two insect cell lines were selected: Sf21 (as a control) and the monoclonal Sf21-FRA.

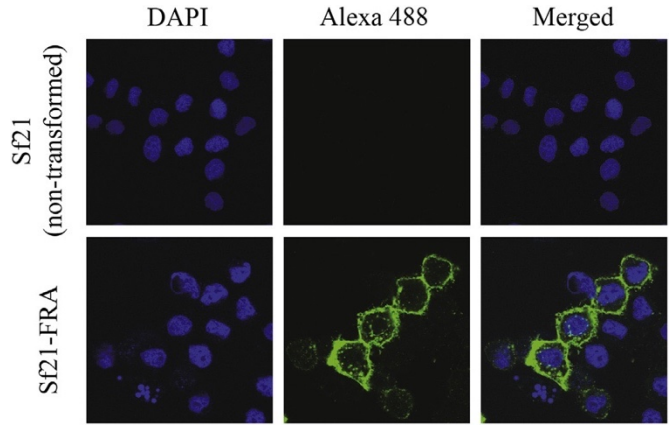

Figure 8. Immunostaining of SeABCC2-FRA transporter expressed in Sf21 cells. Cells were stained with an anti-FLAG tag antibody followed by anti-mouse IgG conjugated to Alexa Fluor 488 (green signal). Cell nuclei were stained with DAPI (blue signal).

Viability assays in insect cell lines

The susceptibility of the two different insect cell lines to four Cry1 proteins (Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Ca) and two Cry1Ab-Cry1C hybrids (H04 and H205) was determined by cell viability assays. None of the Cry1A toxins had any major effect on the viability of non-transfected Sf21 cells, whereas they affected the viability of Sf21-FRA cells in a dose-dependent manner (**Figure 9** and

Supplementary Figure S2). The loss of cell viability was more drastic when treating with Cry1Ab and Cry1Ac than with Cry1Aa (**Figure 9** and **Supplementary Figure S2**).

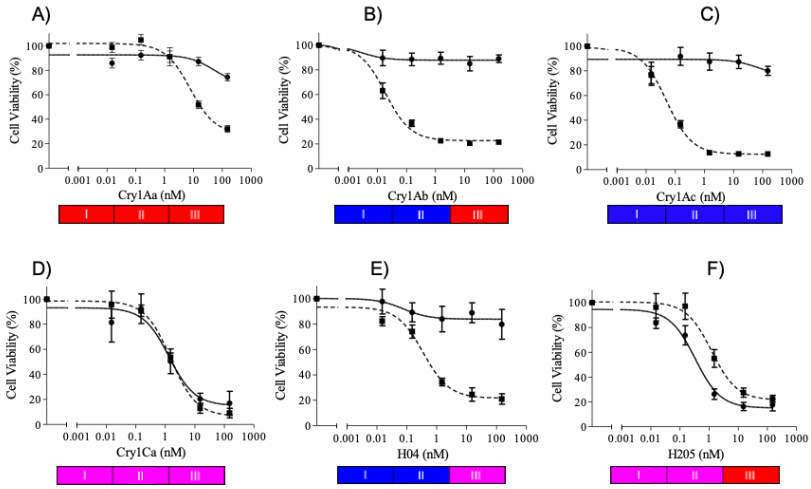

Figure 9. Effect of Cry1A proteins, Cry1Ca and two Cry1A-Cry1C hybrids on the viability of insect cells. Assays were performed using different concentrations of Cry1Aa (A), Cry1Ab (B), Cry1Ac (C), Cry1Ca (D), H04 (E), and H205 (F). Assays were carried out for 3 h with Sf21 (circles) and Sf21-FRA (squares). A schematic representation of the domain composition of the proteins is shown below each graph.

Treatment with Cry1Ca affected cell viability of the two insect cell lines with similar EC_{50} values (values ranging from 0.9 to 1.5nM) (**Figure 9D** and **Supplementary Figure S2**).

The viability pattern of the hybrid H04 was qualitatively similar to that of the Cry1Ab or Cry1Ac proteins (**Figure 9E** and **Supplementary Figure S2**), being active only to those cells expressing the ABCC2 protein. However, its toxicity to the transfected cells was lower than that of the Cry1Ab and Cry1Ac proteins, though higher than that of Cry1Aa (**Figure 9E**). With the hybrid H205 the results were similar to those obtained with Cry1Ca (**Figure 9F** and **Supplementary Figure S2**), suggesting that domains I and II from the Cry1Ca are critical to confer toxicity against Sf21 cells.

HEK293T cell swelling assays

The full-length SeABCC2 protein (SeABCC2-FRA) was transiently expressed in HEK293T cells (**Supplementary Figure S3**). The susceptibility of the two Cry1Ab-Cry1C hybrids (H04 and H205) and their parental proteins was determined by cell swelling assays in the two different HEK293T cell lines. This approach was used to complement the data obtained from the assays performed with the insect cell lines, since HEK293T cells were described as not sensitive to Cry1C (Endo et al., 2017). Non-transfected HEK293T cells were not swollen after 1 h incubation even at the highest concentration of Cry protein tested (1 µM Cry1Ab, 7 µM Cry1C, 2.8 µM H04, and 4.1 µM H205) (**Figure 10**). HEK293T cells expressing the SeABCC2 protein were swollen after 1 h incubation with 100 nM of Cry1Ab, H04 and H205, while no swollen cells were observed when they were treated with Cry1Ca, even at the highest available concentration (7 µM) (**Figure 10**). These results suggest that the SeABCC2 protein mediates the toxicity of Cry1Ab, H04, and H205, but it does not mediate the toxicity of Cry1C. Thus, the toxicity of the H205 protein suggests that domain III from Cry1Aa/b may be crucial in the toxicity of this chimera to HEK293T cells expressing SeABCC2.

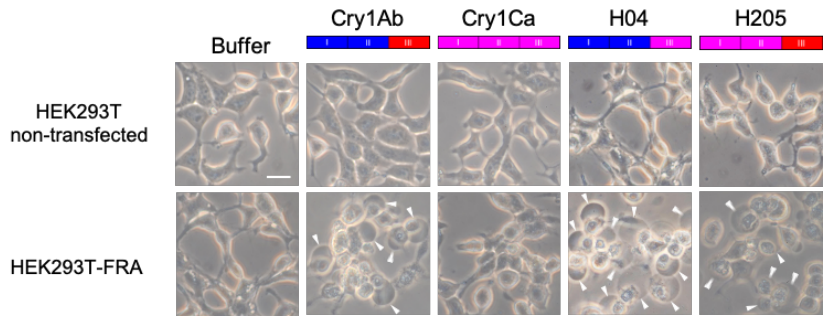

Figure 10. Cell swelling assay of HEK293T cells expressing SeABCC2 transporter against Cry proteins. HEK293T cells were transiently transfected and incubated with 100 nM of Cry1Ab, H04, and H205 proteins, and 7 µM Cry1Ca for 24 h. Images were taken under phase-contrast microscopy. Arrowheads indicate swollen cells with a round shape. Scale bar represents 20 µm. A schematic representation of the domain composition of the proteins is shown.

SeABCC2 transporter mediates binding of Cry1A proteins in Sf21cells

Binding of ^{125}I-labelled Cry1Ac was tested in the two Sf21 insect cell lines. The results showed specific binding of labelled Cry1Ac to increasing concentrations of SeABCC2-expressing insect cells, while no specific binding was found for the control cell line (Sf21) (**Figure 11**).

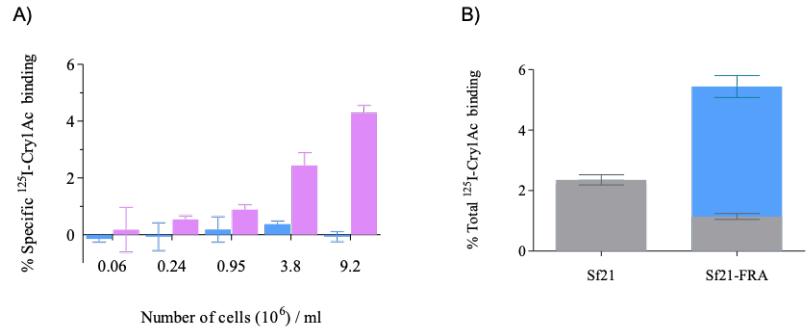

Figure 11. Binding of ^{125}I-Cry1Ac to whole cells from different insect cell lines. (A) Specific binding of ^{125}I-Cry1Ac at increasing concentrations of Sf21 (blue) and Sf21-FRA (purple). (B) Total binding showing the specific (blue) and nonspecific (grey) components at the selected cell concentration (9.2 10^6 cells/ml). Each bar represents the mean of at least three independent experiments (± SEM).

Homologous competition showed that, with Sf21-FRA cells, unlabelled Cry1Ac displaced up to 80% of labelled Cry1Ac (since there is about 20% of nonspecific binding) (**Figures 11B** and **12**). The dissociation constant and concentration of binding sites were estimated from the homologous competition curves, obtaining a K_d = 5.6 ± 1.3 nM (mean ± SEM) and an R_t = 0.030 ± 0.006 pmol/million cells (mean ± SEM). The K_d value indicates that binding of Cry1Ac is of high affinity.

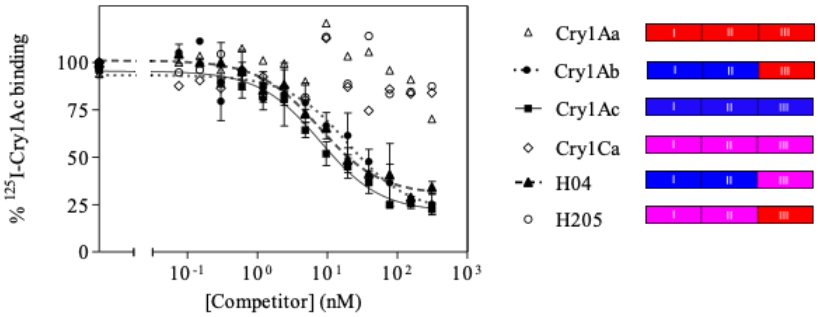

Figure 12. Competition binding assays with 125I-Cry1Ac using Sf21-FRA cells. The reaction was performed in binding buffer (PBS, 0.1% BSA) with a concentration of 9.2 10^6 cells/ml. Curves represent total binding of labelled Cry1Ac protein to increasing concentrations of unlabelled Cry1Aa (open triangles), Cry1Ab (full circles), Cry1Ac (squares) Cry1Ca (diamonds), H04 (full triangles), and H205 (open circles) as competitors. Each competition experiment was replicated at least three times and the error bars represent the standard error of the mean. A schematic representation of the domain composition of the proteins is shown.

Heterologous competition experiments were performed in Sf21-FRA cells using three Cry1 proteins (Cry1Aa, Cry1Ab, and Cry1Ca) and two Cry1Ab-Cry1C hybrids (H04 and H205) to determine whether these proteins shared binding sites with the Cry1Ac protein and, if so, which domains of the protein are responsible. The results showed that Cry1Ab could compete against labelled Cry1Ac, while Cry1Aa did not compete (**Figure 12**). When using Cry1Ca and the hybrid H205 as competitors, no displacement of the labelled protein was observed in Sf21-FRA cells, while the H04 protein was able to compete similarly as Cry1Ab (**Figure 12**). Therefore, only those proteins with domain II of Cry1Ac were able to compete for the Cry1Ac binding.

Binding of ^{125}I-labelled Cry1Ac to S. exigua *BBMV*

To see whether the binding observed in the Sf21 cells expressing the ABCC2 transporter correlated with the binding obtained in classical experiments using BBMV from *S. exigua* (containing all receptors from the midgut epithelial membrane), binding of ^{125}I-

labelled Cry1Ac was tested with *S. exigua* BBMV (**Supplementary Figure S4**). Competition binding assays showed that Cry1Ab competed for all Cry1Ac binding sites, whereas Cry1Aa did not compete (**Figure 13**). The K_d value obtained for BBMV (K_d ± SEM = 1.0 ± 0.4 nM) was not very different from those obtained for Sf21 cells expressing the SeABCC2 transporter. These results suggest that the binding observed with Sf21 cells expressing the ABCC2 transporter represents most, if not all, the binding observed with BBMV.

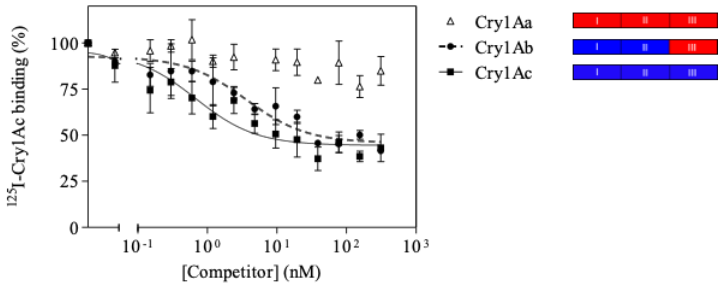

Figure 13. Competition binding assays of ^{125}I-Cry1Ac on S. exigua BBMV using Cry1Aa (open triangles), Cry1Ab (circles) and Cry1Ac (squares) as competitors. Curves represent total binding of labelled Cry1Ac at increasing concentrations of competitors. Each competition was replicated at least three times and the error bars represent the standard error of the mean. A schematic representation of the domain composition of the proteins is shown.

The ABCC2 transporter is not necessary for the oligomerization of Cry1A proteins

To determine the role of the putative receptor SeABCC2 on oligomer formation, incubation of Sf21 and Sf21-FRA with Cry1Ac and its mutant Cry1AcE129K was analyzed. Cry1AcE129K is a mutant which was shown to be unable to insert into the membrane (Portugal et al., 2017). The formation of Cry1Ac oligomers was observed in the cell pellet of both cell lines when treated with Cry1Ac, independently of the expression of the SeABCC2 transporter (**Figure 14**). These results indicate that the presence of the ABCC2 transporter does not mediate the oligomerization of Cry1Ac. In contrast, the oligomeric structure of the Cry1AcE129K mutant was not found in the cell pellets of any of the two cell lines (**Figure 14**).

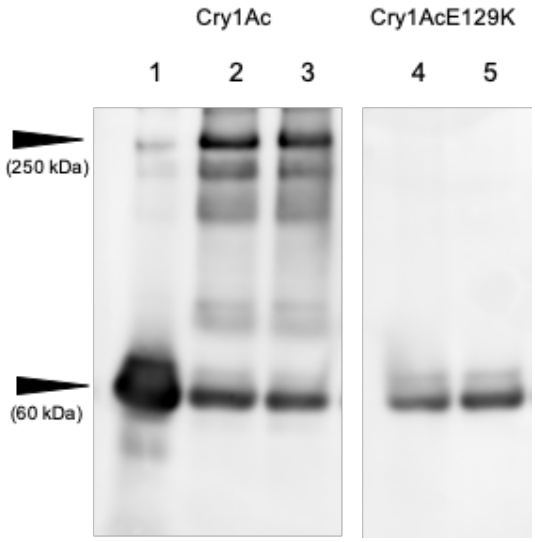

Figure 14. Analysis of the oligomerization of Cry1Ac and the mutant Cry1AcE129K after incubation with insect cells. Cell pellets were loaded onto 10% SDS-PAGE gels, and proteins were detected by Western blot using anti-Cry1Ac antibody and secondary goat-HRP antibody. The oligomeric Cry1Ac structure is observed as a 250 kDa band, whereas the monomeric proteins of both Cry1Ac and mutant Cry1AcE129K are observed as 60 kDa bands. Arrowheads indicate the oligomeric and the monomeric forms. Lane 1: Cry1Ac protein; lanes 2 and 3: protein structures obtained after incubation of Cry1Ac with Sf21, or Sf21-FRA cells, respectively; lanes 4 and 5: protein structures obtained after incubation of Cry1AcE129K with Sf21 or Sf21-FRA cells, respectively.

Effect of Cry1AcE129K on cell viability and binding

The characterization of the Cry1AcE129K mutant was performed in terms of toxic activity and binding ability, to check whether this mutant had altered the ability to interact with the SeABCC2 transporter. The viability results showed that the Cry1AcE129K mutant had no major effect on the viability of any of the cell types (**Figure 15B**). *In vivo* competition assays exposing both insect cell lines with 1.5 nM of Cry1Ac and increasing concentrations of Cry1AcE129K, to test the effect of the excess of the latter on cell susceptibility to Cry1Ac. The percentage of cell viability increased when increasing the concentrations of the mutant protein, suggesting that the

latter is blocking the Cry1Ac binding sites responsible for toxicity (**Figure 15B**). *In vitro* heterologous competition assays, using labelled Cry1Ac and unlabelled Cry1AcE129K as competitor, were performed to check whether the mutant can compete for the binding sites of the wild-type protein. The results showed that the Cry1Ac mutant competes for almost all Cry1Ac binding sites (**Figure 15A**), suggesting that loss of binding was not the reason for the lack of toxicity of this mutant.

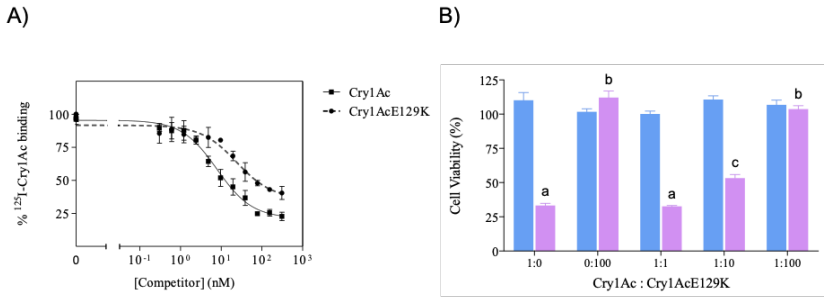

Figure 15. *In vitro* and *in vivo* competition assays with Cry1Ac and Cry1AcE129K using Sf21 and Sf21-FRA insect cells. (A) *In vitro* competition assays with ^{125}I-Cry1Ac using Sf21-FRA insect cells. The reaction was performed in binding buffer (PBS, 0.1% BSA) with a concentration of 9.2 10^6 cells/ ml. Curves represent total binding of labelled protein to increasing concentrations of unlabelled Cry1Ac (squares) or Cry1AcE129K (circles). Each competition experiment was replicated at least three times and the error bars represent the standard error of the mean. (B) *In vivo* competition assays with different Cry1Ac:Cry1AcE129K ratios on Sf21 cells (blue) and Sf21-FRA cells (purple). Cry1Ac (1.5 nM) and Cry1AcE129K (150 nM) were used as a control. Each competition experiment was replicated at least three times and the error bars represent the standard error of the mean. Means were compared by two-way ANOVA, followed by Tukey's multiple comparisons test ($P < 0.0001$). Significant differences between protein ratios on Sf21-FRA cells were indicated by different letters.

Discussion

The role of the ABCC2 transporter as a functional receptor for some Bt proteins has been pointed out in several reports (Atsumi et al., 2012; Banerjee et al., 2017; Baxter et al., 2011; Bretschneider et al., 2016; Gahan et al., 2010; Stevens et al., 2017; Tanaka et al., 2013; Xiao et al., 2014). The aim of the present study was to go deeper into the role of the SeABCC2 transporter in the mode of action of Cry1Ca and Cry1A proteins. For this purpose, functional analyses as well as binding assays were performed using the heterologous expression of SeABCC2 in Sf21 and HEK293T cells.

Results from viability assays showed that the expression of the SeABCC2 transporter in both Sf21 and HEK293T cells conferred susceptibility against Cry1A proteins (**Figures 9** and **10**), supporting the role of the ABCC2 transporter as a functional receptor for these proteins (Banerjee et al., 2017; Gahan et al., 2010; Ren et al., 2016; Tanaka et al., 2013). The fact that HEK293T cells expressing SeABCC2 are not susceptible to Cry1Ca could indicate that the expression of the SeABCC2 transporter in HEK293T cells is not sufficient for toxicity of Cry1Ca, since maybe other molecules which are not present in this system could deem necessary to exert the toxic activity along with the SeABCC2 transporter. Ren et al. (2016) reported that the SeABCC2b transporter is involved in Cry1Ca toxicity using a Sf9 cell line non-susceptible to this toxin. Nevertheless, these authors also found that the expression of *S. exigua* Cadherin (SeCad1b) in Sf9 cells conferred higher susceptibility to Cry1Ca than expression of the SeABCC2b protein, suggesting a secondary role for SeABCC2 in the mode of action of Cry1Ca in these cells. The identification of the main receptor or receptors for Cry1Ca would help to shed light on the mode of action of this protein.

Although some steps in the mode of action of Cry proteins are still unclear, the specific binding step is considered critical for toxicity (Jurat-Fuentes and Crickmore, 2017). To further characterize the role of SeABCC2 in Cry intoxication, binding assays using radiolabelled Cry1Ac were performed. Here, we performed quantitative binding assays with whole cells. The results showed specific binding of Cry1Ac

to the Sf21-FRA cells (**Figure 11**). Thus, the specific binding observed can be directly attributed to the presence of the SeABCC2 transporter on the cells, clearly showing that it acts as a receptor for Cry1A proteins. Binding parameters obtained for the Sf21-FRA insect cell line indicated high affinity binding.

Heterologous competition studies have helped to know whether different Cry proteins share binding sites in different lepidopteran insect pests (Herrero et al., 2016; Jakka et al., 2015). Studies with BBMV from *S. exigua* have shown that Cry1Ca binds to binding sites which are not shared by Cry1A proteins (Luo et al., 1999), whereas Cry1Ab and Cry1Ac share two binding sites, one of them also shared with Cry1Aa (Escriche et al., 1997). Using the heterologous expression of SeABCC2 in Sf21 cells allowed us to determine the relevance of this membrane molecule in the established binding site model. Our results are in agreement with data from BBMV in that binding of Cry1Ac to SeABCC2 was displaced by Cry1Ab, but not by either Cry1Aa or Cry1Ca (**Figure 12**), suggesting that the SeABCC2 protein contains a binding site shared by Cry1Ac and Cry1Ab, which is not recognized by either Cry1Aa or Cry1Ca.

The lack of ability to compete of the Cry1Aa for the Cry1Ac binding site shown in the present study, along with the low efficiency of Cry1Aa competing for Cry1Ab binding sites (Escriche et al., 1997) may shed light on the type of Cry protein domains which can be interacting with the shared site. Several studies on the role of Cry protein domains have evidenced that domain I is involved in the insertion into the epithelial membrane, whereas domains II and III are involved with the interaction with binding sites on the brush border midgut epithelium (Lee et al., 1995; Rang et al., 1999; Gómez et al., 2006; Herrero et al., 2004). Cry1Ab is considered a natural chimera between Cry1Aa (domain III) and Cry1Ac (domains I and II) (de Maagd et al., 2001). Based on this, and according to the results obtained in the present study, we can hypothesize that domain II of Cry1Ab and Cry1Ac is responsible for the interaction with the shared binding site in SeABCC2. Interestingly, domain II of Cry1A proteins has already been related with binding interaction with the BmABCC2 protein

(Adegawa et al., 2017; Tanaka et al., 2016b). Furthermore, since Cry1Aa is also toxic for Sf21 cells expressing SeABCC2, we hypothesize that the SeABCC2 transporter has more than one binding site for Cry1A proteins, one shared by Cry1Ab and Cry1Ac and another for Cry1Aa. In agreement with our hypothesis, a single amino acid insertion in the ABCC2 receptor in *Bombyx mori* disrupted its receptor function for Cry1Ab and Cry1Ac but not for Cry1Aa (Atsumi et al., 2012; Tanaka et al., 2016b).

In order to further study the implication of the Cry protein domains in toxicity and binding processes, two hybrid proteins H04 (a chimera with domains I and II from Cry1Ab/c and domain III from 1Ca) and H205 (a chimera with domains I and II from Cry1Ca and domain III from Cry1Aa/b) were used. A similar pattern of toxicity and binding ability was observed for H04, Cry1Ab, and Cry1Ac in Sf21 cells expressing the SeABCC2 transporter. The three proteins were similarly toxic to cells expressing the SeABCC2 transporter (**Figure 9**), and H04 competed similarly to Cry1Ab for the Cry1Ac binding sites in Sf21-FRA cells (**Figure 12**). Our data are in agreement with a previous study showing that Cry1Ab and H04 competed for the same binding sites in *S. exigua* BBMV (de Maagd et al., 1996). Our results support the relevance of domain II in the interaction with the Cry1Ac/b binding site and suggest that the sole presence of the ABCC2 transporter is responsible for the binding model proposed using BBMV.

Regarding the hybrid protein H205, the viability assays carried out on Sf21 cells showed that it has a similar toxicity pattern than Cry1Ca, suggesting that domains I and II from Cry1Ca are critical to mediate toxicity to non-transfected Sf21cells. Similar results had been previously observed with H205 and Sf9 cells (Kwa et al., 1998). In contrast, when toxicity assays were performed using HEK293T cells, the results revealed a different toxicity pattern between H205 and Cry1Ca. The results showed that Cry1Ca was not toxic against any of the HEK293T cell lines tested, whereas H205 was only active on those cells expressing the SeABCC2 transporter. These results point out that domain III of Cry1Aa/b can interact with the SeABCC2 protein, presumably with the second binding site not shared by Cry1Ac.

Furthermore, it has been reported that Cry1Aa is able to compete binding of labelled Cry1Ab in *S. exigua* BBMV (Escriche et al., 1997). This scenario can only be explained if binding of the Cry1Aa and Cry1Ab proteins to the shared binding site takes place through domain III, which is the only domain that they have in common.

The role of ABCC2 transporters in the mechanism of action of Cry proteins is not well characterized and it has been suggested that it might be involved, in addition to Cry1A toxin binding, in facilitating Cry1A oligomer insertion into the membrane (Heckel, 2012; Ocelotl et al., 2017). To determine if the ABCC2 transporter plays a role in the formation of the oligomer, we analyzed the oligomerization of the Cry1Ac protein and its mutant (Cry1AcE129K) in Sf21 and Sf21-FRA cells. Our results show that the presence of the ABCC2 transporter was not necessary for oligomerization, since Cry1Ac was able to oligomerize independently of the presence of the SeABCC2 transporter. On the other hand, the non-toxic Cry1AcE129K protein (mutant in membrane insertion but which is able to oligomerize) (Portugal et al., 2017) was able to compete with Cry1Ac for binding and block the Cry1Ac toxicity, suggesting that the mutant Cry1AcE129K is interacting with the SeABCC2 receptor. Thus, our results suggest that the oligomer formation, along with the presence of the ABCC2 transporter, are necessary for rendering toxicity. Taking all these data together, we propose that the SeABCC2 transporter could be mediating the insertion of the oligomer into the membrane. In agreement with our data, other authors have proposed that the ABCC2 transporter can be involved in the oligomer insertion process for Cry1A proteins (Bretschneider et al., 2016; Ocelotl et al., 2017; Tanaka et al., 2016a).

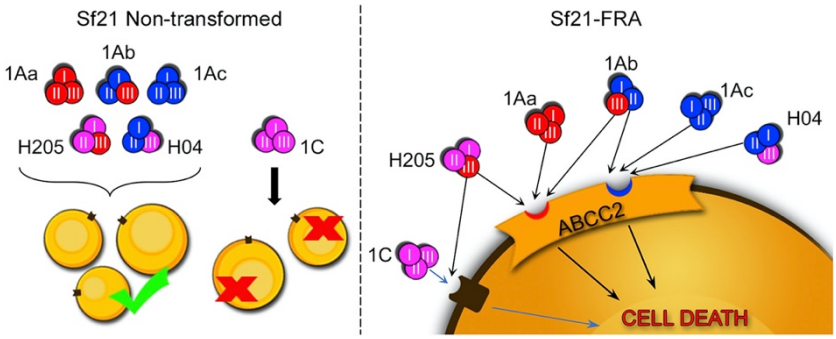

Figure 16. Schematic representation of the main findings of this chapter.

In summary, the results of this study confirm that the SeABCC2 transporter is a functional receptor for Cry1A proteins in *S. exigua*. Moreover, this work elucidates which specific domains of the Cry1A proteins are interacting with the SeABCC2 transporter. According to our results, we propose a model represented in **Figure 16** in which the domain II of Cry1Ac and Cry1Ab would have a common binding site in the SeABCC2, whereas the domain III of Cry1Aa and Cry1Ab would bind to another binding site of the SeABCC2 transporter. This second binding site in the SeABCC2 transporter seems to be less efficient in causing toxicity than the first one, since the Cry1Aa protein is the one with the lowest toxicity to Sf21cells expressing the SeABCC2 among all the Cry1A proteins tested. Although similar mechanisms of action involving multiple binding sites in the same receptor have been proposed by other authors (Arenas et al., 2010; Herrero et al., 2001; Jenkins et al., 2000), further experiments would be necessary to test this hypothesis. In addition, our results also suggest that the role of the SeABCC2 transporter is to facilitate the insertion of the oligomer into the membrane, being a crucial step to render toxicity. This work contributes to a better understanding of the Cry protein specificity based on the receptors which are taking part in the mode of action and, therefore, offers the possibility to improve the strategies to overcome the evolution of insect resistance.

CHAPTER 2

Hetero-oligomerization of *Bacillus thuringiensis* Cry1A proteins enhance binding to the ABCC2 transporter of *Spodoptera exigua*

Adapted from:
Pinos D, Joya N, Herrero S, Ferré J, Hernández-Martínez P. 2021. Hetero-oligomerization of *Bacillus thuringiensis* Cry1A proteins enhance binding to the ABCC2 transporter of *Spodoptera exigua*. Biochemical Journal 478:2589-2600

Introduction

The insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) have been largely used to control different pests in agricultural crops. The most common Bt proteins to control lepidopterans both in Bt crops and in formulated sprays are the Cry1A proteins, being these one of the best characterized group of proteins (Pardo-López et al., 2013; ISAAA 2018). The proposed mode of action of Cry1A proteins starts in the midgut of the larva after the ingestion in the protoxin form. Protoxins are solubilized and partially digested by midgut enzymes to render the active form. Then, the activated proteins, as monomers, undergo a complex sequence of binding events with several midgut proteins that lead to the cleavage of helix α1 (located in Domain I) causing the oligomerization of monomers (Bravo et al., 2007). Next, the oligomer binds to different putative receptors and causes pores in midgut cells that cause the disruption of the membranes, leading to septicemia and insect death (Adang et al., 2014).

The ATP Binding Cassette (ABC) transporters are a family of primary-active transporters with an increasing number of members that have been shown to be used as receptors by Cry proteins, mainly Cry1, Cry2, Cry3 and Cry8 proteins (Sato et al., 2019). The approaches used to characterize the role of these ABC transporters with Cry proteins have been diverse, from expression of the transporters in insect or human cell lines to their silencing or knock-out in live insects (Park et al., 2014; Huang et al., 2020; Chapter 1 in this thesis). In *Spodoptera* spp., the ABCC2 and the ABCC3 transporters have been characterized as putative receptors for certain Cry1A proteins (Park et al., 2014; Huang et al., 2020; Chen et al., 2015; Ren et al., 2016; Banerjee et al., 2017; Chapters 1 and 4 in this thesis). In chapter 1, we investigated the role of SeABCC2 in the mode of action of Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, and two Cry1A-1C hybrids by expressing the receptor in Sf21 cell lines. The binding site model proposed supports that domain II of Cry1Ab/c has a common binding site in the SeABCC2 protein, whereas domain III of Cry1Aa/b binds to a different binding site in the same

SeABCC2 protein. Besides contributing to binding, it has been shown that ABCC2 transporters can also be involved in Cry1A oligomerization as well as insertion (Tanaka et al., 2016; Ocelotl et al., 2017), although a clearer understanding on the involvement of these receptors in Cry oligomerization is still needed.

Here, we have focused on studying the interaction of Cry1A proteins with the SeABCC2 transporter. To study this interaction, we used an insect cell line stably expressing the transporter (Sf21-FRA), and labelled ^{125}I-Cry1Aa. Binding studies reveal that Cry1Aa binds specifically to the receptor as an oligomeric form. *Ex vivo* competition and cell viability assays point to SeABCC2 as a multivalent binding receptor with shared functional binding sites among different Cry1A proteins. Furthermore, our results point out that Cry1Aa can hetero-oligomerize with other Cry1A proteins increasing its binding to the receptor, which is reflected in an increase in cell toxicity.

Material and methods

Cell culture maintenance

Spodoptera frugiperda derived Sf21 cells were cultured at 25 ºC in 25 cm^2 tissue culture flasks (T25 flasks, Nunc) containing 4 ml of Gibco® Grace's Medium (1x) (Life Technologies™, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Sf21 cells expressing the SeABCC2 (Sf21-FRA, previously stablished in Chapter1 were cultured at 27 ºC in the same medium supplemented with 10 µg/ml Blasticidin.

Site-directed mutagenesis

The Cry1AaR99E and Cry1AbR99E mutants were generated by site-directed mutagenesis by the technique of the whole plasmid amplification. Primer design was done according to Zheng et al. (2004). The plasmid amplification reaction was performed with a high fidelity DNA polymerase with strong 3'-5' exonuclease activity and high processivity (KAPAHiFi™ PCR Kit, ref. KK2101, Kapa Biosystems, USA), using the *ca*. 7 kb pBD140 plasmids containing the wild-type *cry1Aa* or *cry1Ab* genes as templates. The PCR reaction was carried out with 20 ng DNA template, 0.6 µM primer pair (forward primer 5' C CAA GCC ATT TCT **GA**A TTA GAA GGA CTA AGC AAT C 3' and reverse primer 5' CC TTC TAA T**TC** AGA AAT GGC TTG GTT CCT AGC 3'), 200 µM dNTPs, 0.5 U of polymerase, and 5 µl of KAPAHiFi buffer in a final volume of 25 µl. The reaction was initiated with a pre-heating step of 3 min at 95 ºC and 16 cycles of denaturation, annealing and extension phases of 98 ºC for 20 s, 58 ºC for 30 s and 72 ºC for 7.5 min, respectively. The reaction was finalized with 15 min of final extension at 72 ºC.

The parental plasmids remaining in the final reaction were digested with FastDigest *Dpn*I (Thermo Scientific, USA) at 37 ºC for 10 min. The enzymatic digestion of the *Dpn*I was stopped at 80 ºC for 5 min and the reaction cooled down on ice. The plasmids carrying the mutant version of both genes were used to transform *Escherichia coli*

DH10β. Plasmid purification was performed on transformed colonies and screened after culture, DNA purification, and Sanger sequencing with the following primers: Forward primer 5' GAG TGA ATT TGT TCC CGG TGC TGG 3' and reverse primer 5' CGG TCC CCA TAC ACG CTC TAA TCC 3'. Plasmids displaying the expected single point mutation were further introduced into *E. coli* WK6 for the production of the Cry1AaR99E and Cry1AbR99E mutants. Again, the sequence was checked after culture, DNA purification and Sanger sequencing before producing the protein.

Cry proteins preparation

For binding assays, Cry proteins were obtained from recombinant *B. thuringiensis* strains EG1273, EG7077, EG11070, and EG1081 expressing Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Ca respectively. For the hybrid proteins, recombinant *E. coli* strains expressing H04 (with domains I and II from Cry1Ab and domain III from Cry1Ca), and H205 (with domains I and II from Cry1Ca and domain III from Cry1Ab) were kindly supplied by R. A. de Maagd (Wageningen Plant Research, Wageningen University). For Cry1AaR99E and Cry1AbR99E, the recombinant *E. coli* strains were obtained as previously explained. Toxins were produced, solubilized, and activated by trypsin as described by Estela et al. (2004). After trypsin activation, proteins were purified by chromatography as described in Chapter 1.

For cell viability assays, *E. coli* strains expressing Cry1Aa, Cry1Ab and Cry1Ac were used, also supplied by R. A. de Maagd. Inclusion bodies containing Cry toxins were purified, solubilized and trypsin-activated as described by Herrero et al. (2004). The hybrid H04 and the mutant Cry1AaR99E were prepared as previously explained. All proteins were checked by 12% SDS-PAGE and kept at −20 ºC until used.

Binding of ^{125}I-Cry1Aa to cells and in vitro *competition assays*

The Cry1Aa and Cry1AaR99E proteins (25 μg) were labelled with 0.3 mCi of ^{125}I (PerkinElmer, Boston, MA) using the chloramine T method (Van Rie et al., 1989). The specific activity obtained for the

labelled proteins was 2.9 mCi/mg and 3.7 mCi/mg, respectively. The optimal concentration of cells to be used in competition assays was previously determined with labelled Cry1Aa, as explained in Chapter 1. Briefly, increasing concentrations of cells (Sf21-FRA) were incubated with 0.1 nM of labelled-Cry1Aa to obtain the total binding. The non-specific binding was calculated by adding an excess of unlabelled Cry1Aa protein (150 nM). The chosen concentration for further assays was 4.6 10^7 cells/ml, as it showed the highest specific binding. Non-transfected Sf21 cells were used as negative control.

In vitro competition experiments were performed in binding buffer (PBS supplemented with 0.1% BSA) incubating the Sf21-FRA cells with labelled proteins and increasing amounts of the different unlabelled proteins (from 0.03 nM to 620 nM) in a final volume of 100 µl. After 1 h incubation at RT, samples were centrifuged, washed, and the radioactivity in the final pellet was measured in a model 2480 WIZARD^2 gamma counter. The final pellets, containing the proteins bound to the cells, were resuspended in 10 µl of DNAse I (10 mg/ml) (Roche Diagnostics, Mannheim, Germany) and incubated for 10 min at RT. After incubation, loading buffer was added and the samples were heated at 50 ºC for 5 min, then 12% SDS-PAGE were performed. After running, gels were dried and revealed by using calcium tungstate screens (Hyperscreen, GE Healthcare, US).

Cell viability assays

Cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at 25 ºC using the CellTiter 96® AQueous One Solution Reagent (Promega, Madison WI, USA) following the manufacturer's protocol. Briefly, Sf21-FRA cells were first suspended in culture medium (without FBS) and plated in flat bottom plates at about 70% confluence. After attaching of the cells, 10 µl of activated toxins were added to each well (concentrations described below, according to the assay performed), in triplicates for each concentration. Carbonate buffer (50 mM, pH 10.5) and 2% Triton X-100 were also added to the wells as negative and positive controls, respectively. After 3 h of incubation, 20 µl of the MTT

reagent was added to each well. The absorbance was measured at 409 nm (Infinite m200, Tecan, Maennedorf, Switzerland) after 2 h incubation. The percentage of viable cells was obtained considering 100% viability in carbonate buffer-treated wells and 0% viability in Triton-X100-treated wells. At least three different biological replicates were carried out. Data of each experiment was compared by one-way ANOVA, followed by Tukey's multiple comparison test.

For *ex vivo* competition assays, fixed concentrations of Cry1Aa (15 nM), Cry1Ab, Cry1Ac and the hybrid H04 protein (1.5 nM) were used along with increasing concentrations of Cry1AaR99E protein (15, 150, 1500, 3000 nM for the Cry1Aa assay, and 1.5, 15, 150, 300 nM for Cry1Ab, Cry1Ac or H04 assays). As controls, each protein was tested separately (concentrations chosen to cause 50% or higher toxicity, as observed in Chapter 1).

For synergism assays, increasing concentrations of Cry1Ab or Cry1Ac (0.02, 0.04, 0.08 and 0.16 nM) were combined with 1.5 nM of Cry1Aa and tested for toxicity. Protein ratios were chosen to facilitate the formation of hetero-oligomers containing Cry1Aa, rather than homo-oligomers of Cry1Ab or Cry1Ac.

Results

The Cry1Aa oligomer binds specifically to Sf21 cells expressing SeABCC2

Binding of ^{125}I-labelled Cry1Aa was tested in Sf21 and Sf21-FRA insect cell lines (control and expressing the SeABCC2, respectively). The results showed specific binding of labelled Cry1Aa to increasing concentrations of Sf21-FRA cells, while no specific binding was found for the Sf21 cells (**Figure 17A**). Therefore, these results clearly indicate that specific binding of Cry1Aa protein is mediated by the SeABCC2 transporter.

Homologous competition assays were performed using ^{125}I-labelled Cry1Aa at increasing concentrations of unlabelled Cry1Aa. Unexpectedly, the curve showed a biphasic response (**Figure 17B**, full circles). Low amounts of competitor elicited a stimulatory response (increase of binding above initial values) to Sf21-FRA cells, whereas high amounts of competitor caused the expected binding site competition. The stimulatory response reached a maximum effect when 9.7 nM of competitor was used. The dissociation constant and concentration of binding sites of Cry1Aa were estimated from the competition curve considering the data from 9.7 nM onwards, obtaining a K_d = 43.9 ± 7.9 nM (mean ± SD) and an R_t = 0.25 ± 0.04 pmol/10^6 cells (mean ± SD). The K_d value indicates a moderate-to-high affinity of Cry1Aa to its putative sites in the SeABCC2 transporter. Autoradiography of the ^{125}I-Cry1Aa bound to cells from the homologous competition assays showed that most, if not all of the binding, is through the oligomeric form of the protein (**Figure 17C**).

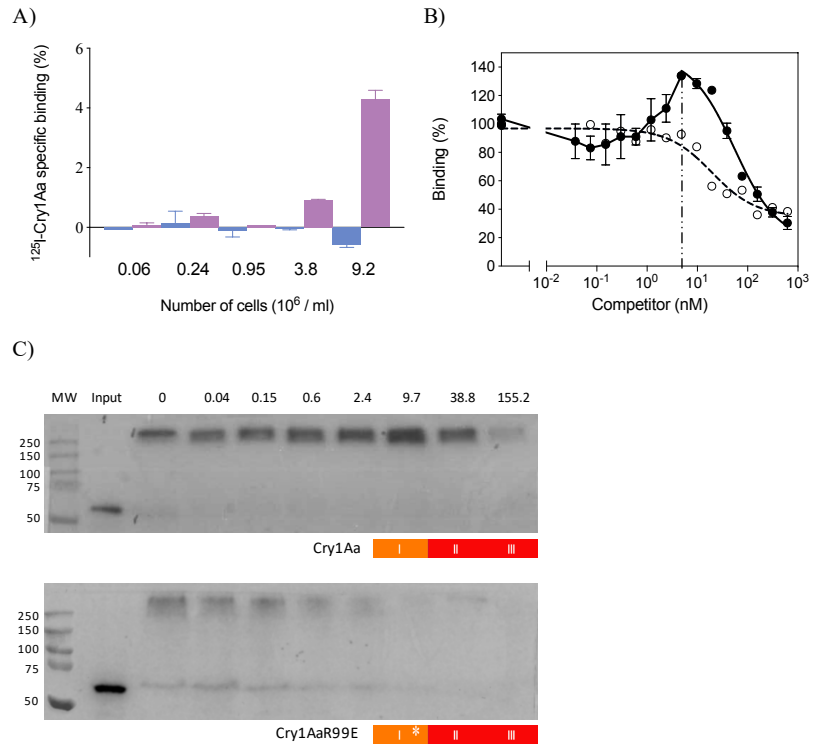

Figure 17. Binding of Cry1Aa and Cry1AaR99E (unable to oligomerize) to Sf21 and Sf21-FRA cells (Sf21 cells expressing the SeABCC2 transporter). (A) Specific binding of ^{125}I-Cry1Aa at increasing concentrations of Sf21 (blue) and Sf21-FRA (purple) cells. (B) Competition binding assays with ^{125}I-Cry1Aa and unlabelled Cry1Aa (full circles) or Cry1AaR99E (open circles) as competitors with Sf21-FRA cells. The dotted line indicates the concentration of competitor where maximum binding was obtained. (C) Autoradiography of pellets from the competition binding assays subjected to SDS-PAGE. The upper gel corresponds to the competition with Cry1Aa; the lower gel corresponds to the competition with Cry1Aa R99E. Lanes 1: molecular weight markers, lanes 2: ^{125}I-Cry1Aa as a control, lanes 3-10: competition at different amounts of competitor (as indicated in nM). A schematic representation of the domain composition of the proteins is shown below the gels. Asterisk represents the point-mutation impeding oligomerization. Each data point represents the mean of at least three independent experiments (± SEM) for panels A and B.

To confirm whether the biphasic response was related to the formation and binding of the oligomer, the mutant protein Cry1AaR99E was used as heterologous competitor, since mutations in domain I changing the arginine in position 99 (R99) in Cry1Ab and Cry1Ac proteins have shown to impede oligomerization without affecting binding (Jiménez-Juárez et al., 2007; Portugal et al., 2017). Using the Cry1AaR99E mutant in *in vitro* competition assays, the biphasic effect was not observed (**Figure 17B**, open circles). Indeed, a classical competition curve representing the displacement of the binding sites of the ^{125}I-Cry1Aa protein by the Cry1AaR99E mutant was observed. This result supports the hypothesis that the increase in binding observed in the homologous competition was due to the formation and binding of the homo-oligomer, with this possessing higher binding ability (**Figure 17B** and **17C**). In the case of the Cry1AaR99E competitor, the autoradiography showed the lack of increase in oligomer formation, thus confirming the inability to form oligomers between the ^{125}I-Cry1Aa and the mutant Cry1Aa protein (**Figure 17C**).

Labelled Cry1AaR99E was used in *in vitro* competition assays (**Supplementary Figure S5**). The results showed that the binding was specific since both unlabelled Cry1AaR99E and Cry1Aa displaced the labelled protein (**Supplementary Figure S5**). In contrast with labelled Cry1Aa competition binding assays, the biphasic effect was not observed with any of the competitors.

Binding of Cry1Aa to the SeABCC2 is enhanced by forming hetero-oligomers with Cry1Ab and Cry1Ac

To see whether the Cry1Aa biphasic response could also be elicited by other Cry1A proteins, Cry1Ab and Cry1Ac were used as heterologous competitors for *in vitro* competition assays. The early stimulatory response previously observed with low concentrations of Cry1Aa was also observed at low concentrations of Cry1Ab and Cry1Ac (**Figure 18A**). The peak of maximum binding was reached at lower concentration of competitor (1.2 to 2.4 nM) and the enhancing effect was much more drastic (over 200%) than when Cry1Aa was used as homologous competitor (140%). The shift of the binding peak to the

left would indicate a higher affinity of the hetero-oligomers for the SeABCC2 transporter, and the larger increase in binding could reflect an increase in sites where the hetero-oligomers are binding with respect to the homo-oligomers. Again, the autoradiography of the bound ^{125}I-Cry1Aa indicated that most of the binding was in the oligomeric form (**Figure 18B**). With concentrations from 2.4 nM of competitor, a classic heterologous competition curve was observed, with Cry1Ab and Cry1Ac competing with the labelled Cry1Aa (**Figure 18A**). The dissociation constant and concentration of binding sites were K_d = 3.9 ± 1.9 nM and an R_t = 0.03 ± 0.01 pmol/10^6 cells for Cry1Ab, and K_d = 2.6 ± 1.2 nM and an R_t = 0.02 ± 0.01 pmol/10^6 cells for Cry1Ac. These K_d values indicate a higher affinity than Cry1Aa to the putative sites in the SeABCC2 transporter.

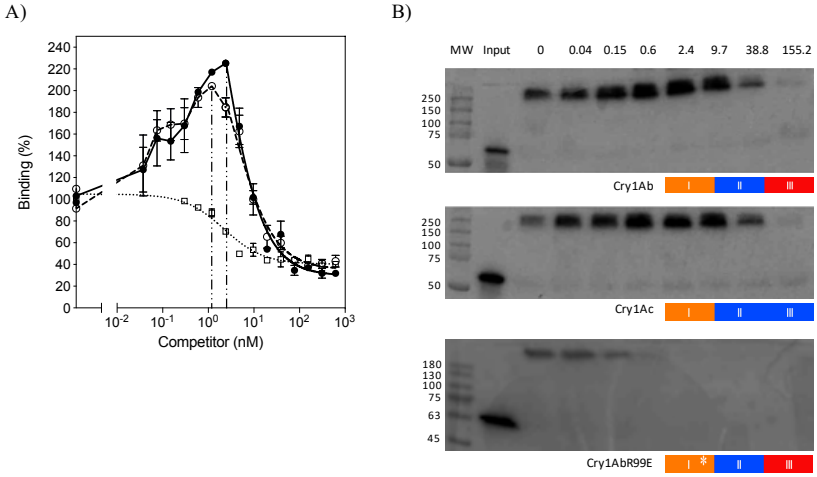

Figure 18. Binding of ^{125}I-Cry1Aa to Sf21-FRA cells in the presence of Cry1A heterologous competitors. (A) Curves represent total binding of ^{125}I-Cry1Aa to increasing concentrations of unlabelled Cry1Ab (full circles), Cry1Ac (open circles) and Cry1AbR99E (open squares) as competitors. The dotted lines indicate the concentration of competitor where maximum binding was obtained. Asterisk represents the point-mutation impeding oligomerization. Each data point represents the mean of at least three independent experiments (± SEM). (B) Autoradiography of pellets from the competition binding assays subjected to SDS-PAGE. Lanes 1: molecular weight markers, lanes 2: ^{125}I-Cry1Aa as a control, lanes 3-10: competition at different amounts of competitor (as indicated in nM). A schematic representation of the domain composition of the proteins is shown below the gels.

A classical competition curve was observed when using the Cry1AbR99E mutant protein (which is unable to oligomerize) (Portugal et al., 2017) as competitor (**Figure 18A**). Accordingly, the autoradiography shows that the Cry1AbR99E mutant competes for the oligomeric form of labelled Cry1Aa (**Figure 18B**). The ability of this mutant to still compete with Cry1Aa but lacking of biphasic effect indicates that Cry1A proteins are able to form hetero-oligomers with Cry1Aa (**Figure 18A**). Taken together, the results suggest that hetero-oligomeric forms present higher affinities and binding abilities to the ABCC2 receptor, compared to the Cry1Aa homo-oligomer.

Hetero-oligomerization is promoted by domains I and II of Cry1A proteins

To better know which domains play a role in the hetero-oligomerization process, two Cry1Ab–Cry1C hybrid proteins (H04 and H205) with different swapped domains, as well as Cry1C, were used for *in vitro* competition assays. The H04 protein is a chimera with domains I and II from Cry1Ab and domain III from Cry1Ca and the H205 protein is the reciprocal chimera (with domains I and II from Cry1Ca and domain III from Cry1Ab). The results showed the biphasic response for hybrid H04 (**Figure 19A**) and the hetero-oligomer formation was confirmed by autoradiography (**Figure 19B**). In contrast, no binding enhancement was observed for either Cry1Ca or hybrid H205, nor the presence of hetero-oligomer by autoradiography (**Figure 19A** and **19B**). The signal observed in the autoradiography remained constant independently of the concentration of Cry1C or H205 used and would correspond to the few homo-oligomers formed by ^{125}I-Cry1Aa that bound to the cells. Furthermore, the H04 hybrid could compete as Cry1Aa for the binding sites, whereas Cry1Ca did not compete and the hybrid H205 could only displace partially some of the specific binding. Since hybrid H205 contains the domain III from Cry1Ab (this domain is practically identical in Cry1Aa and Cry1Ab), the partial competition with ^{125}I-Cry1Aa suggests that Cry1Aa may have two different binding sites in the SeABCC2 transporter. The above results show that Cry1Aa cannot form hetero-oligomers with more

distantly related toxins such as Cry1Ca and that the presence of domain I from Cry1A proteins is critical for the formation of the oligomer.

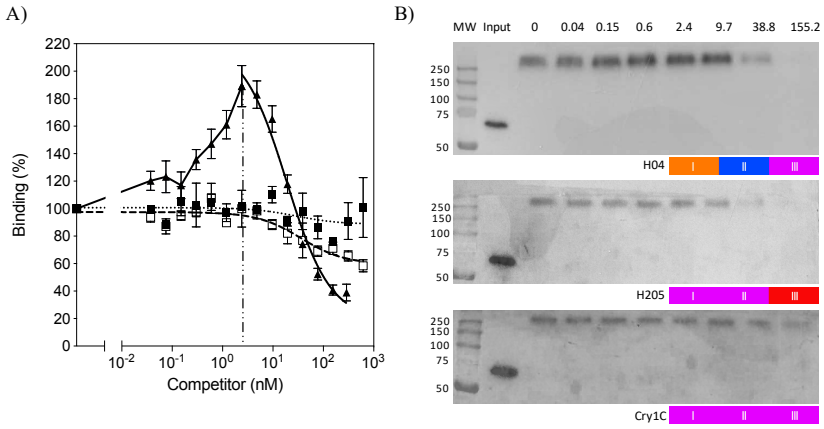

Figure 19. Binding of ^{125}I-Cry1Aa to Sf21-FRA cells in the presence of heterologous competitors. (A) Curves represent total binding of ^{125}I-Cry1Aa to increasing concentrations of unlabelled Cry1C (full squares), H205 (open squares) and H04 (full triangles) as competitors. The dotted line indicates the concentration of competitor where maximum binding was obtained. Each data point represents the mean of at least three independent experiments (± SEM). (B) Autoradiography of pellets from the competition binding assays subjected to SDS-PAGE. Lanes 1: molecular weight markers, lanes 2: ^{125}I-Cry1Aa as a control, lanes 3-10: competition at different amounts of competitor (as indicated in nM). A schematic representation of the domain composition of the proteins is shown below the gels.

Ex vivo *competition assays confirmed shared functional binding sites for Cry1Aa and Cry1Ab/c proteins in SeABCC2*

To see whether the *in vitro* competition among Cry1Aa, Cry1Ab, Cry1Ac, and H04 reflects binding of these proteins to sites involved in their toxic action, *ex vivo* competition assays were performed using the mutant Cry1AaR99E (which is non-toxic to Sf21 FRA cells) as heterologous competitor. As expected, no loss of cell viability was observed when only the Cry1AaR99E was used, demonstrating that the mutant has no toxicity on its own (**Figure 20**). Cry1AaR99E blocks the toxicity to Sf21-FRA cells caused by Cry1Aa when an excess of 10-fold or more of the mutant protein is added. A higher excess of this mutant (≥100-fold) was required to decrease the

cell toxicity caused by Cry1Ab, Cry1Ac and the H04 hybrid (**Figure 20**), confirming that they bind to shared sites. No differences on cell toxicity was observed at stoichiometric concentrations of any protein (**Figure 20**, columns 1:1) indicating that the mutant Cry1AaR99E does not form hetero-oligomers with Cry1Aa, Cry1Ab, Cry1Ac nor the H04 hybrid and the reduction in toxicity observed at higher concentrations is due to the direct interference with the SeABCC2 binding sites.

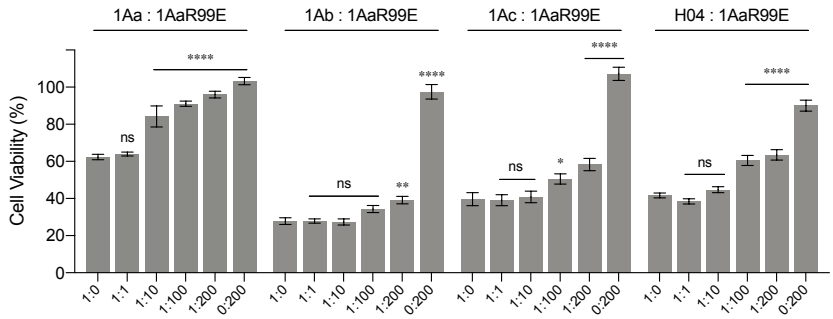

Figure 20. *Ex vivo* competition assays between Cry1Aa, Cry1Ab, Cry1Ac or the hybrid H04 (1.5 nM) and increasing ratios of mutant Cry1AaR99E in Sf21-FRA cells. Each competition experiment was replicated at least three times and the error bars represent the standard error of the mean. Means of each ratio were compared by one-way ANOVA for each assay, followed by Tukey's multiple comparisons test. Significant differences between protein ratios on Sf21-FRA cells and the ratio 1:0 (as control) were indicated by asterisks (****, $P < 0.0001$).

Hetero-oligomerization of Cry1Aa with Cry1Ab/c proteins enhances cell toxicity

To assess if the increased binding ability to the SeABCC2 receptor produced by hetero-oligomerization of Cry1Aa at low concentrations of Cry1Ab or Cry1Ac is also reflected as an increase on cell toxicity, Sf21 FRA cell viability assays were performed using a range of concentrations of Cry1Ab or Cry1Ac, combined with a fixed, effectively non-toxic concentration of Cry1Aa (1.5 nM). For concentrations of 0.02 nM of Cry1Ab or Cry1Ac, no significant effect was observed (**Figure 21A**). For concentrations of 0.04 and 0.08 nM of both Cry1Ab and Cry1Ac, a synergistic effect was observed (**Figure 21,**

panels B and **C**), being the highest at 0.08 nM, with an increase in cell toxicity of about 20% for Cry1Aa-Cry1Ab and 25% for Cry1Aa-Cry1Ac. The synergistic effect observed for these concentrations could be attributed to the formation of hetero-oligomers (formed by monomers of Cry1Aa and Cry1Ab/c) and the subsequent increase in the hetero-oligomer affinity for its receptor and the number of available binding sites (**Figure 18**). At 0.16 nM of Cry1Ab combined with Cry1Aa, synergism can still be observed although with a lower intensity, since this concentration of Cry1Ab is enough to affect cell viability on its own (**Figure 21D**). In a similar way, no significant effect is seen for 0.16 nM of Cry1Ac combined with Cry1Aa over using only 0.16 nM of Cry1Ac, since the latter produces a *ca*. 40% loss in cell viability itself (**Figure 21D**).

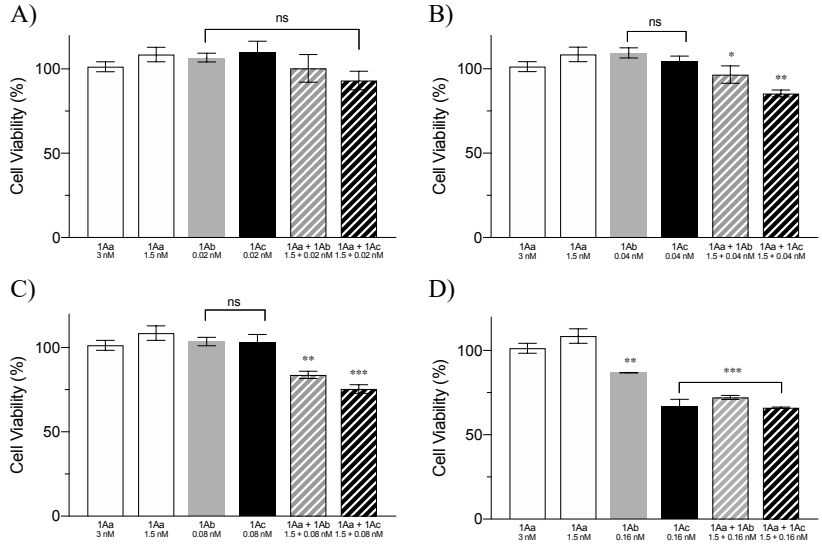

Figure 21. Synergistic effect of combining Cry1Aa (1.5 nM) at different concentrations of Cry1Ab or Cry1Ac on Sf21-FRA cell toxicity. (A) 0.02 nM (B) 0.04 nM (C) 0.08 nM (D) 0.16 nM. Individual proteins for each assay were used as control. Viability assays were performed at least three times (± SEM). Means were compared by one-way ANOVA, followed by Tukey's multiple comparison test. Significant differences between treatments are indicated by asterisks (***, $P < 0.001$).

Discussion

It is well known that the specific interaction that occurs between Cry1A proteins and their receptors is a crucial step for toxicity (Jurat-Fuentes and Crickmore, 2017). Moreover, the most common mechanism of resistance is the lack of toxin binding to larval midgut receptors (Jurat-Fuentes et al., 2021). For this reason, substantial efforts have been performed to identify the putative receptors for each Cry protein and to characterize whether these molecules can bind to one or more Cry proteins. For *S. exigua* we proposed a binding site model with the ABCC2 transporter as a receptor for Cry1A proteins, supporting interaction of Cry1Ab and Cry1Ac proteins through their domain II and Cry1Aa protein likely by its domain III (Chapters 1 and 4 in this thesis). Moreover, we proposed that Cry1Aa had low or even nil affinity to Cry1Ab/c binding sites (Chapter 1). The aim of the present work was to dissect the interaction between Cry1Aa and the SeABCC2 expressed in Sf21 cells, as well as its interaction with Cry1Ab and Cry1Ac proteins in regards to binding, oligomerization and toxicity.

Specific binding for Cry1Aa towards the SeABCC2 was observed, as well as an unusual biphasic response in homologous competition assays. We found that low concentrations of non-labelled Cry1Aa caused a stimulation in binding, while high concentrations of competitor caused a regular binding competition behavior. This phenomenon resembles a hormetic behavior of the protein, a biphasic response produced by stimulation at low doses and inhibition at high doses of the same agent (Mattson and Calabrese, 2010). The enhanced binding ability of the labelled Cry1Aa protein by using determined concentrations of the same unlabelled protein could be explained with the need of a certain stoichiometric concentration of Cry1Aa monomers to increase the formation of labelled oligomers binding to the SeABCC2. Under this hypothesis, monomers would benefit of forming homo-oligomers, being the oligomer the main structure to interact with the SeABCC2 transporter in terms of binding. To test this hypothesis, the non-toxic Cry1AaR99E mutant, which is unable to form oligomers but it is able to compete for the binding sites, was used in *in vitro* competition assays. The results showed a classical binding

competition behavior (no stimulation peak) when used as competitor, suggesting that the binding enhancement observed for the wild-type Cry1Aa protein was related to the oligomer formation and its binding to the receptor. In the same way, no stimulation peak was observed with competition assays with the labelled mutant, supporting oligomerization as the cause of binding stimulation.

The interaction between labelled Cry1Ab or Cry1Ac proteins and the SeABCC2 was studied before in Sf21 cells and no stimulatory phase with the homologous competition was observed (Chapter 1 and unpublished data). However, using Cry1Aa as competitor of labelled Cry1Ac, we observed a small biphasic effect in agreement with our current model (Chapter 1). Also, a stimulatory effect was observed in binding experiments with brush border membrane vesicles (BBMV) from the lepidopteran *Manduca sexta* with labelled Cry1B. In that study, both the homologous and a heterologous competition with unlabelled Cry1Ab produced a binding stimulation at certain doses of competitor that exceeded values of 200% in binding, with a posterior competition when the doses were increased (Hofmann et al., 1988). This phenomenon was shown to be species-specific, since no increase in binding was observed when the same assays were carried out with BBMV from *Pieris brassicae*. No further biological relevance was given to this phenomenon since the concentration of competitor where the peak was observed was relatively high, and was explained as a process produced by aggregation of toxins due to the presence of a factor from *M. sexta* BBMV (Hofmann et al., 1988).

In this chapter, a similar binding behavior of labelled Cry1Aa (initial stimulation and then competition) was observed when Cry1Ab, Cry1Ac and the hybrid H04 (a chimera with domains I and II as Cry1Ab and domain III as Cry1C) were used as competitors. In contrast, no stimulation in binding at low-doses was observed when Cry1Ca and the hybrid H205 (a chimera with domains I and II as Cry1Ca and domain III as Cry1Ab) were used as competitors. These results point out that the hormetic response (most likely due to oligomer formation and binding) can be observed among Cry1Aa and other highly related proteins such as Cry1Ab/c. Likewise, the mutant

Cry1AbR99E behaved as the Cry1AaR99E mutant, and did not show an initial stimulation when used as competitor. Taken together, these results support the relevance of domain I in the oligomerization process and suggest that hetero-oligomerization among different but related *B. thuringiensis* Cry proteins can occur. The formation of homo-tetramers for Cry1Aa proteins was suggested by Schwartz et al. (1997) and reported later by atomic force microscopy (Vié et al., 2001) and single molecule fluorescence experiments (Groulx et al., 2011), where it was described as highly dynamic process that occurs in the membranes. In addition, the homo-oligomer formation was probed to be required in the mode of action of *B. thuringiensis* Cry proteins by using non-toxic Cry1Ab mutants (the so-called Dominant-Negative (DN) mutants) that block the insecticidal activity of the wild-type protein at substoichiometric ratios by forming inactive oligomers (Rodríguez-Almazán et al., 2009). However, few studies have reported the occurrence of hetero-oligomers. Carmona et al. (2011), described that Cry1Ab DN mutant functioned as an antitoxin of Cry1Aa, Cry1Ac and Cry1Fa, suggesting that Cry1Ab can form hetero-oligomers, at least, with these proteins.

Results from the *in vitro* heterologous competition assays showed that Cry1Ab, Cry1Ac and hybrid H04 compete for the Cry1Aa binding sites. Furthermore, the ease in binding hetero-oligomers over homo-oligomers suggests that these proteins bind with more affinity to the transporter than Cry1Aa. In the same line, the results obtained from the *ex vivo* competition assays showed that the mutant Cry1AaR99E was able to partially block the toxicity of Cry1Ab, Cry1Ac, and the hybrid H04 when ratios higher than 100-fold were used, supporting the presence of common binding sites in the SeABCC2 but with lower affinity for Cry1Aa. The higher binding affinity observed for Cry1Ab, Cry1Ac and H04 for the common binding sites would explain the almost lack of competition of Cry1Aa for the binding sites of Cry1Ac reported in Chapter 1 using the same insect cells. In agreement, studies with BBMV from *S. exigua* have shown that Cry1Ab and Cry1Ac share two binding sites, one of them also shared with Cry1Aa (Escriche et al., 1997). On the other hand, binding of Cry1Aa to SeABCC2 was partially

displaced by the hybrid H205, but not by Cry1Ca, suggesting that the transporter has another binding site for the domain III of Cry1Aa. Based on our results, and according to those obtained previously in Chapter 1, we can confirm that the SeABCC2 transporter has more than one binding site for Cry1A proteins, at least one shared by Cry1Aa, Cry1Ab, and Cry1Ac and another that can interact with Cry1Aa (mediated by domain III). Moreover, our results point out that the transporter works as a multivalent binding receptor with different binding affinities depending on the Cry protein that is interacting.

The benefit in binding hetero-oligomers compared to homo-oligomers towards the SeABCC2 was also translated to a synergistic effect in toxicity to cells expressing the transporter. At low concentrations of Cry1Ab or Cry1Ac, combined with a fixed concentration of Cry1Aa, synergism was observed. The maximum synergistic effect could be obtained at 0.08 nM of Cry1Ab or Cry1Ac combined with 1.5 nM of Cry1Aa. At this concentration, a decrease of cell viability of *ca.* 20% and 25% was observed for Cry1Aa-Cry1Ab and Cry1Aa-Cry1Ac, respectively. This result indicates that forming hetero-oligomers can be more advantageous, since these structures are more efficient in terms of binding and consequently in causing toxicity. Interestingly, the synergistic effect was less relevant (or non-existent) when Cry1Aa was combined with higher doses of Cry1Ab or Cry1Ac, since individual toxicities caused the same losses on cell viability as the combination with Cry1Aa. According to our hypothesis, at these concentrations, most of the oligomers formed would be homo-oligomers of Cry1Ab or Cry1Ac and, since these have higher affinity and toxicity than Cry1Aa homo-oligomers, the advantage for binding and toxicity for the Cry1Aa-Cry1Ab/c hetero-oligomers would go unnoticed. Sharma et al. (2010) also reported synergism between Cry1Aa, Cry1Ab and Cry1Ac in *Chilo partellus* larvae, which correlated with an increase in bound toxins to BBMV on *in vitro* experiments. Also, Chakrabarti et al. (1998) hypothesized that the synergism found between Cry1Ac and Cry1F against *Helicoverpa armigera* could be due to the formation of hetero-oligomers. Altogether, this study sheds light on the advantage of forming hetero-oligomeric forms by certain strains

of *B. thuringiensis*, as suggested by Carmona et al. (2011). From an evolutionary point of view, and taking into account the genetic costs of carrying the information to code a wide variety of proteins within a strain, the ability to form hetero-oligomers should be considered. This type of interaction among similar proteins with different binding sites or affinities would benefit the bacteria in achieving the maximum toxic effect towards its target. An example of a *B. thuringiensis* strain that could be taking advantage on this evolutionary success would be *B. thuringiensis* var. *kurstaki* strain BNS3, which contains Cry1Aa, Cry1Ac, and Cry2Aa. The crystals of this strain were found to have higher toxicity against *Ephestia kuehniella* than the toxins used individually or in the combinations tested (Tounsi et al., 2005). Other cases of synergistic effects found in wild-type strains of *B. thuringiensis* that need to be fully understood are those observed between Cry11Aa and Cry4Ba proteins from *B. thuringiensis* subsp. *israelensis* (Bti), usually used to control mosquito populations (Carmona et al., 2011; Fernández-Luna et al., 2010), or *B. thuringiensis* var. *kurstaki* HD-1, which is the main component of Dipel®, a Bt-based product (Hernández-Martínez, 2009). Thus, the study on the interaction between proteins and how they behave is of high interest to explain the evolutionary process that leads to the accumulation of genes from the same *cry* family in a same cell.

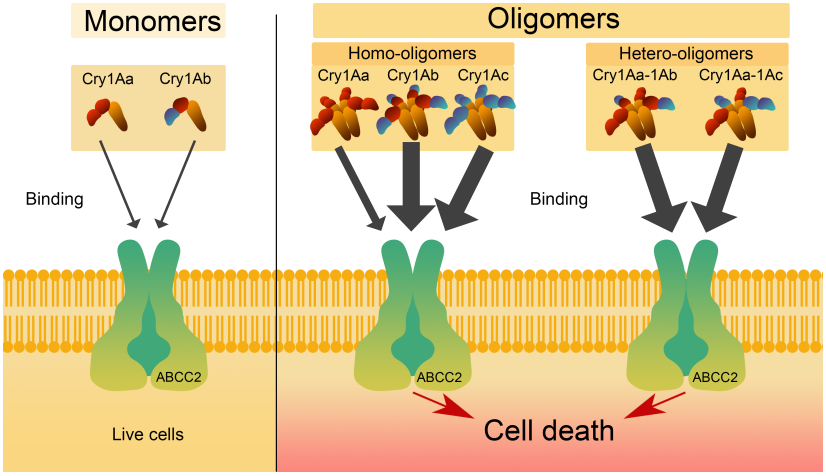

Figure 22. Schematic representation of the main findings in this chapter.

To sum up, as represented in **Figure 22**, the present work illustrates how the ABCC2 from *S. exigua* can act as a multivalent binding receptor for different Cry1A proteins, and highlights the ability of Cry1A proteins to form hetero-oligomeric structures that surpass binding and toxicity of the Cry1Aa homo-oligomer.

CHAPTER 3

Characterization of novel ABC transporters from *Heliothis virescens* and their role in *Bacillus thuringiensis* insecticidal protein toxicity

Introduction

The ATP-Binding cassette transporters are a superfamily of membrane proteins that can be found in all kind of organisms. In arthropods, more than 400 ABC proteins have been identified (Dermauw and Van Leeuwen, 2014). These active transporters are involved in the export of different substances such as xenobiotics or signaling molecules (Sharom, 2011). In regards with pesticidal proteins from *Bacillus thuringiensis*, the ABC transporters were first related with Cry1A proteins, by genetic linkage between a mutation present in the ABCC type 2 transporter of a Cry1Ac-resistant strain of *Heliothis virescens* (Gahan et al., 2010). The functionality of the ortholog transporter as receptor for Cry1A proteins in another insect species, *Bombyx mori*, was confirmed two years later (Atsumi et al., 2012). From this moment, a series of studies related different ABC transporters with the toxicity of Bt proteins, although the main focus was on the relationship between ABCC2 and Cry1 proteins. In the case of *H. virescens*, Bretschneider et al. (2016) confirmed that the ABCC2 is a functional receptor for Cry1A proteins, since the expression of the transporter in an insect cell system conferred susceptibility to Cry1Aa, Cry1Ab and Cry1Ac.

While the ABCC2 has been characterized in several insects as a functional receptor for Cry1 proteins, there are several paralogs inside the *ABCC* gene family that could play a role in the mode of action of several insecticidal proteins from Bt. In *Spodoptera exigua*, the ABCC2 and ABCC3 transporters were reported to play a role in determining susceptibility to Cry1Ac and Cry1Ca (Park et al., 2014), and their expression in human cells conferred susceptibility to Cry1A and to Cry8, but not to Cry1C or Cry1D (Endo et al., 2017). In *Helicoverpa armigera*, the knock-out of both ABCC2 and ABCC3 conferred resistance to Cry1Ac (Wang et al., 2020).

For *H. virescens*, only the ABCC2 transporter and its relation with Cry1 proteins has been explored to date. Here, we have explored the presence of paralogs to the *ABCC2* gene in *H. virescens*, identifying two novel ABC transporters, ABCC3 and ABCC4. Moreover, we have studied their role in toxicity of three different Bt proteins, Cry1Aa, Cry1Ac and Vip3Aa, by developing CRISPR-mediated knock-outs in this insect species.

Materials and Methods

Identification and characterization of new ABC *genes in* H. virescens

To explore novel *ABCC* paralogs from *H. virescens*, *ABCC* genes from a variety of phylogenetically-close lepidopterans were used as templates against transcriptomic studies (from the following SRA studies: ERP021656, SRR2912076, ERP009356, SRP062666, SRP005629, SRP032396) and the genome of *H. virescens* (NWSH00000000.1, from Fritz et al., 2018). Two *in silico* sequences of putative *ABCC* transporters were found and further characterized, which aligned to the *ABCC3* and *ABCC4* of *H. armigera* (MW592373 and LOC110380708, respectively).

To verify the sequences, total RNA from *H. virescens* larvae was isolated using RNAzol reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed to cDNA using random hexamers and oligo (dT) following the instructions provided in the Prime-Script RT Reagent Kit (Perfect Real Time from TaKaRa Bio Inc., Otsu Shiga, Japan). Then, amplification of the fragments of the *HvABCC3* and *HvABCC4* genes was performed, using 5 and 6 pairs of primers for each gene, respectively, previously designed with Geneious software (Supplementary Table S2), according to sequences from assemblies. Then, sequences obtained by Sanger dideoxy sequencing were aligned to obtain the complete *HvABCC3* and *HvABCC4* genes using the same software.

The prediction of transmembrane domains, as well as outer and inner parts of both ABCC3 and ABCC4 proteins were obtained using the TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) which is based in a hidden Markov model.

Phylogenetic analysis of the transporters

The amino acid sequences of ABCC2, ABCC3 and ABCC4 of 32 different transporters available from lepidopterans were aligned using MAFFT software (https://mafft.cbrc.jp/alignment/software/),

and the phylogenetic tree was generated by the Neighbour-Joining method using MEGA X. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. 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 JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 4).

Guide RNA design and ribonucleoprotein complex preparation

The CRISPR RNA (crRNA) used in the experiments for *HvABCC3* and *HvABCC4* genes as well as the evaluation of potential off-target sites in the *H. virescens* genome (NWSH00000000.1) were designed and performed using the 'Find CRISPR sites' tool from Geneious® Software. The best design for each target location (*HvABCC3* or *HvABCC4*) was used to purchase Alt-RT™ CRISPR crRNA from IDT DNA (www.idtdna.com) that could be annealed with Alt-RTM CRISPR-Cas9 tracrRNA to form a functional gRNA for each target, as explained in Perera et al. (2018). Briefly, the crRNAs for each *HvABCC3* and *HvABCC4* genes and tracrRNA were resuspended separately in 10 mM Tris-EDTA to yield a 100 µM final concentration. Each *HvABCC3* and *HvABCC4* crRNA was combined with tracrRNA in nuclease-free tubes to yield a 10 µM final concentration in 1X duplex buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate; IDT DNA). The mixtures were heated to 95 ºC for 5 min on a heat block and slowly cooled to room temperature to anneal the complementary nucleotide sequences in crRNAs and tracrRNAs to generate functional gRNAs. Annealed gRNAs for each target were combined with Alt-RTM *S.p.* Cas9 Nuclease 3NLS in 1X injection buffer (5 mM KCl; 0.1 M sodium phosphate, pH 6.8) to yield final concentrations of 10 µM and incubated on ice for 15 min to facilitate the formation of ribonucleoprotein (RNP) complexes.

Microinjections and insect rearing

Injection needles were prepared by pulling siliconized 10 µl quartz micro-capillaries using a Sutter P2000 CO_2 laser based horizontal micropipette puller (Sutter Instruments, Novato, CA). Eggs were injected with approximately 5 nl of injection mix, by using a Narishige micromanipulator model MMN-333 (Narishige International USA, Inc., Amityville, NY) and an Olympus SZ stereo microscope (Olympus Corporation, Waltham, Massachusetts) equipped with a mechanical stage. All microinjections were completed within 30 min of egg collection so that the eggs would be no more than 60 min old at the time of injection. Coverslips containing injected eggs were placed into 100 mm plastic petri dishes layered with a moist filter paper and covered with a lid. Petri dishes were sealed with plastic tape and placed in a plastic box designated as secondary containment. Eggs were incubated at room temperature for approximately 4 hours prior to transferring to a designated incubator which was set to 26℃ and 80% RH. After hatching, neonates were transferred to diet cups containing artificial diet.

Genotyping of the mutants induced by CRISPR/Cas9

A minimum volume of 5 µl of hemolymph from fourth instar larvae was extracted by clipping the tip of one of the abdominal pseudo-legs. Then, genomic DNA was extracted using MasterPure DNA extraction reagents (Epicenter Technologies, Madison, WI), as described by Perera et al. (2015). Purified DNA from each larvae was re-suspended in 20 µl of 10 mM Tris-HCl, pH 8.0. Forward and reverse primers were designed to anneal within the exon 1 in both *HvABCC3* and *HvABCC4* genes to amplify fragments of 500 and 600 bp on the wild-type version of the genes, respectively (Supplementary Table S2). PCR amplifications were performed on a PTC-200 DNA Engine (BioRad, Hercules, CA) with a thermal cycling profile containing 60 second initial denaturation at 95 °C, followed by 35 cycles of 30 sec denaturation at 95 °C, 15 sec annealing at 57 °C, and 30 sec extension at 72 °C, with a final extension of 120 sec at 72 °C. Amplification products were visualized by electrophoresis on 0.8% agarose gels

(Invitrogen, Carlsbad, CA) using Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0). Amplicons were cleaned by binding to AMPure XP paramagnetic beads (Beckman Coulter, Beverly, MA) at DNA:beads (v/v) ratio of 1:1.8. Nucleotide sequences of the purified amplicons were obtained by direct sequencing with Sanger dideoxy method using the same primers as for PCR amplification (USDA-ARS Genomics and Bioinformatics Research Unit (GBRU), Stoneville, MS). Nucleotide sequence analyses were carried out using Vector NTI Advance v11.5 suite.

Insect husbandry

Insects were reared at 28 ºC, 80% RH, and 14:10 L:D cycles in environmental chambers (Percival Scientific, Perry, IA). Insects with detected mutations (P_0) at the target sites were selected and mated as single pairs with moths of the opposite sex from the SIMRU *H. virescens* laboratory colony. Resulting heterozygotes (F_1) from each single-pair mating were inbred to obtain F_2 progeny. Fourth instar F_2 larvae were genotyped by sequencing the DNA extracted from hemolymph to confirm the homozygous mutations, as previously explained. The F_3 individuals of both ABCC3-KO and ABCC4-KO lines were used in the subsequent bioassays.

Bt protein preparation

For bioassays, Cry1Aa and Cry1Ac were obtained from recombinant *B. thuringiensis* strains EG1273 and EG11070 (from Ecogen Inc., Langhorn, PA). Purification and solubilization of the crystals to obtain the protoxin form was performed as previously described by Estela et al. (2004), and resuspended in carbonate buffer (50 mM Na_2CO_3, 100 mM NaCl, pH 10.5). For Vip3Aa, a recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene was expressed and lysed following the conditions described by Abdelkefi-Mesrati et al. (2009). Then, the Vip3Aa protein in the cell lysate was further purified by isoelectric point precipitation (IPP), lowering its pH to 5.5 with acetic acid, as described by Chakroun et al. (2012). All proteins were frozen in liquid nitrogen and lyophilized (Thermo Savant MODULYO

D-230). Prior to use, Cry1Aa and Cry1Ac proteins were solubilised in sterile milliQ-water, and the Vip3Aa protein was solubilised in Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.6). Then, the concentration of proteins was quantified by densitometry after SDS-PAGE electrophoresis using the TotalLab 1D v13.01 software.

Bioassays

To stablish the LC_{50} values (lethal concentration causing a 50% mortality) for the Bt proteins, susceptibility to Cry1Aa, Cry1Ac and Vip3Aa was tested with neonate larvae from the SIMRU *H. virescens* laboratory colony. At least five concentrations of each protein, and controls with Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.6) or distilled water were included. The concentrations were calculated considering the toxicity values previously determined for *H. virescens* to Cry1Aa, Cry1Ac and Vip3Aa (Lemes et al., 2014). Mortality bioassays were conducted by the surface contamination method using 128-well BIO-BA-128 (C-D international Inc., USA) bioassay plates, as previously described by Herrero et al. (2004). Briefly, a volume of 50 µl for each dilution was lied onto the artificial diet per well, spread evenly on the surface and allowed to dry in a laminar flow hood. Once dry, one larva was transferred to each well and at least 64 larvae were used per each concentration. All assays were repeated at least 3 times. Mortality was scored after 7 days. LC_{50} values were estimated from mortality data by Probit analysis (Finney, 1971) using the POLO-PC program (LeOra Software, 1987). For the ABCC3-KO and the ABCC4-KO strains, three different concentrations of each protein were tested. For Cry1Aa, an LC_{25} was used, as well as 10-fold and 100-fold concentrations. For Cry1Ac, a *ca.* LC_{75} concentration was used, and 5-fold and 50-fold concentrations. For Vip3A, an LC_{50} was used, along 10-fold and 50-fold concentrations. A total of 576 neonate larvae of each strain (ABCC3-KO and ABCC4-KO) were tested, with 64 individuals for each protein concentration.

Results

Identification and phylogenetic analysis of ABCC transporters from H. virescens

We identified two new ABC transporters, that showed high similarity with other ABC transporters to phylogenetically-close species of heliothines (*Helicoverpa armigera*, *Helicoverpa zea*, *Helicoverpa punctigera*, or *Heliothis subflexa*) and were named as *HvABCC3* and *HvABCC4* because of their amino acid sequence similarity to the corresponding ABCC3 and ABCC4 clades (**Figure 23**). The sequences of both transporters were deposited in GenBank (OK239703 and OK239704, respectively). Moreover, and according to the analysis, the ABCC3 clade is phylogenetically closer to the ABCC2 clade than to the ABCC4 clade, since they formed different lineages from a common branch, in contrast to the ABCC4 clade. Proteins named as MDRP4 (Multi-drug resistant protein) are non-annotated ABC transporters included in the ABCC4 clade, following the classification performed by Endo et al. (2017).

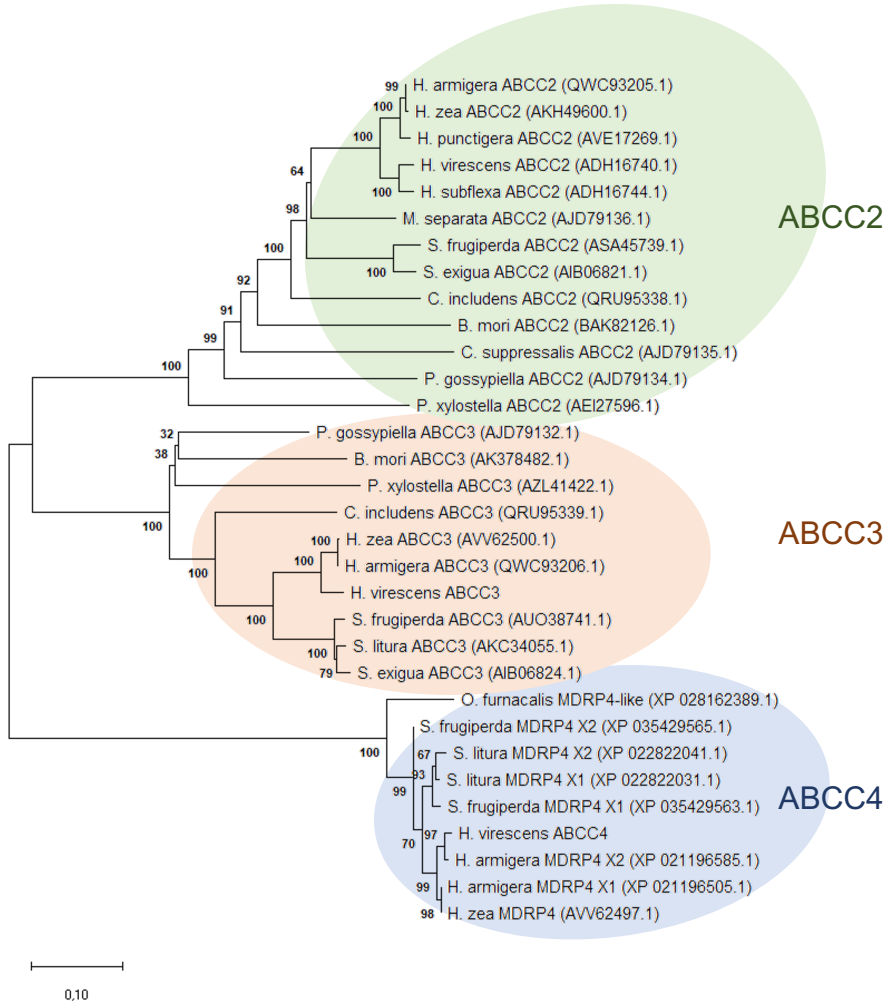

Figure 23. Phylogenetic tree of ABCC transporters of lepidopterans. Entire amino acid sequences of ABCC2, ABCC3 and ABCC4 from lepidopterans are used for the alignment. Genbank accession numbers are shown in parentheses. Colors represent the different clades. MDRP: "Multi-drug resistant protein".

Both *HvABCC3* and *HvABCC4* genes are formed by 25 exons. The amino acidic length of the codifying protein is 1369 aa and 1394 aa, respectively. The prediction of the transmembrane helices for both molecules shows two differentiated transmembrane domains (TMDs), as well as six regions facing the outer part of the membrane, that probably correspond to the usual extracellular loops (ECLs) found in other ABC transporters, as represented in **Figure 24B**.

CRISPR knock-out of HvABCC3 *and* HvABCC4 *genes*

Alignments of sequence data from the P_0 (injected) or F_2 (after inbreeding the progeny) with the wild-type *HvABCC3* and *HvABCC4* versions identified different mutant genotypes with a variety of deletions in the exon 1 target site. Nevertheless, many of the families established from a single P_0 mutant failed to survive after the F_2 generations. The knock-out strains of ABCC3 and ABCC4 established (originally obtained from a single modified P_0 individual crossed as previously explained) were designated as ABCC3-KO and ABCC4-KO. For the ABCC3-KO strain, a 204 bp deletion was produced, located from 52 bp upstream of the start codon of the protein to position 125 from exon 1 (**Figure 24A**). This deletion would produce a frameshift, disabling the correct expression of the ABCC3 transporter. For the ABCC4-KO strain, a 250 bp deletion was originated starting over the gRNA design in exon 1 onwards, causing the same effect as for the ABCC3-KO strain (**Figure 24**).

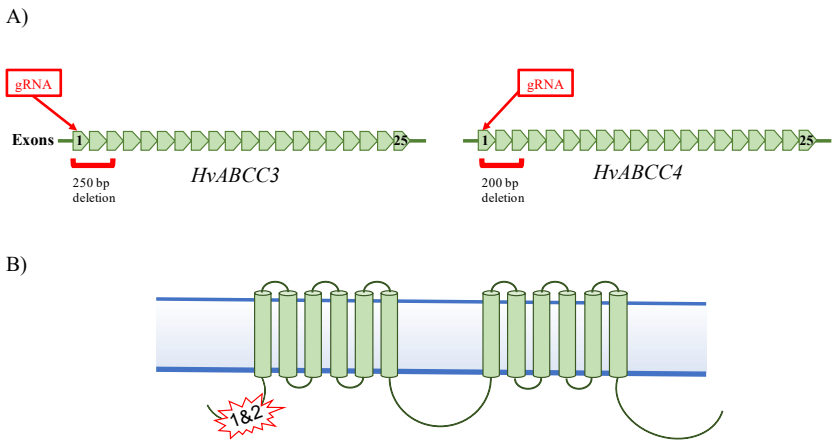

Figure 24. CRISPR/Cas9 knock-out of *HvABCC3* and *HvABCC4*. A) Schematic representation of genomic DNA with the designed gRNAs (arrows) targeting exon 1 of the *HvABCC3* and *HvABCC4* genes. B) Protein structure of both ABCC3 and ABCC4 transporters with the mutations affecting the initial intracellular part of both proteins on gRNA target sites (1&2), producing a frameshift.

Susceptibility to Cry1Aa, Cry1Ac and Vip3Aa proteins in the SIMRU H. virescens *laboratory colony and the ABCC-KO strains*

The results obtained in quantitative bioassays with Cry1Aa, Cry1Ac and Vip3A for the susceptible SIMRU *H. virescens* laboratory colony are shown in Table 1. The three proteins were toxic for *H. virescens*, although Vip3Aa was the least toxic protein, 150 times less than Cry1Aa and 1250 times less than Cry1Ac (Table 1). These results indicate a very high susceptibility for Cry1A proteins and a relatively low toxicity for the Vip3Aa protein for the laboratory colony. Regarding the ABCC-KO strains, no effect on the suppression of the susceptibility to Cry1Aa was observed, since the preliminary data showed a similar mortality compared to the SIMRU *H. virescens* laboratory colony in both ABCC3-KO and ABCC4-KO strains, even at low (*ca.* LC_{25}) concentrations (**Figure 25A**). Moreover, the toxicity of Cry1Ac to the two ABCC-KO strains reached levels similar to the susceptible colony, especially at high concentrations (**Figure 25B**).

However, at the lower concentration of Cry1Ac tested (2.5 ng/cm^2), lower mortalities than the SIMRU susceptible colony were recorded for the two ABCC-KO strains (**Figure 25B**). In the case of Vip3Aa, no significant differences were observed between the susceptibility of both ABCC3-KO and ABCC4-KO strains and the susceptible *H. virescens* laboratory colony (**Figure 25C**).

Table 1. Mortality of the susceptible SIMRU *H. virescens* colony neonate larvae to Bt proteins.

Protein	n	LC_{50} (95% FL[a])	Slope (± SEM)	χ^2
Cry1Aa	712	13.0 (8.6 – 17.1)	2.02 ± 0.19	28.1
Cry1Ac	384	1.66 (0.71 – 3.47)	2.24 ± 0.26	2.91
Vip3Aa	512	2010 (1180 – 3440)	2.19 ± 0.17	33.8

n indicates number of larvae tested for each protein, [a]FL Fiducial limits

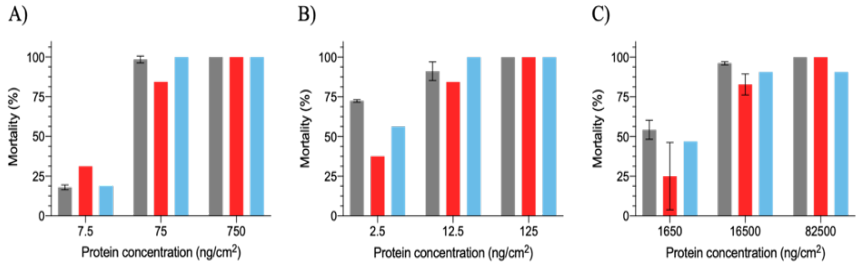

Figure 25. Mortality of the susceptible colony and the ABCC-KO strains to Bt proteins. Bioassays were performed using increasing concentrations of: (A) Cry1Aa, (B) Cry1Ac, and (C) Vip3Aa proteins. Grey bars: *H. virescens* SIMRU colony, red bars: ABCC3-KO strain, blue bars: ABCC4-KO strain.

Discussion

It was more than a decade ago that an ABC transporter was first related with the mode of action of Bt proteins. A mutated version of the ABCC2 protein from a resistant strain of *H. virescens* was genetically linked to Cry1A protein resistance (Gahan et al., 2010). Since then, ABC transporters from many insects, specially lepidopterans, have been related to some extent with the toxicity of Cry proteins, as reviewed by Sato et al. (2019).

Nevertheless, the best characterized ABC transporter by far has been the ABCC type 2, which has been confirmed to act as receptor for Cry1 proteins across many species, and has been often found altered in resistant strains (Sato et al., 2019). The ABC transporter superfamily includes a large number of transporters with high similarities among them that may have hidden roles in the mode of action of Bt proteins. Besides the ABCC2, few ABC transporters have been related to Bt proteins, being one of them ABCA2 with Cry2 in *Helicoverpa armigera*, *Trichoplusia ni*, or *Bombyx mori* (Wang et al., 2017; Yang et al., 2019; Li et al., 2020). Another ABC transporter studied to some extent is ABCC3, which has been related to the toxicity of Cry1Ac. First characterized in a Xentari™-resistant *S. exigua* strain (Park et al., 2014), it was found downregulated also in a Cry1Ac-resistant strain of *P. xylostella* (Guo et al., 2015). The combination of silencing both *PxABCC2* and *PxABCC3* via dsRNA in insects produced a significant reduction in Cry1Ac toxicity (Park et al., 2014; Guo et al., 2015). In the same way, the role of the ABCC3 from *S. litura* in the Cry1Ac mode of action was confirmed in two types of insect cells (Sl-HP and Hi5 cells) expressing *SlABCC3* (Chen et al., 2015). Later, the expression of the *S. exigua ABCC3* in HEK293T cells conferred lower susceptibility to Cry1Aa than the expression of *SeABCC2* (Endo et al., 2017). It was proposed that ABCC3 has a similar but secondary role compared to ABCC2 (Endo et al., 2017).

It is interesting to note that in the first species that the ABCC2 was linked to Cry1A mode of action, *H. virescens*, no other ABC transporter has been related to Bt proteins to date. Here, we have

explored paralog genes from the ABC transporter subfamily C, and we have described the *ABCC3* and *ABCC4* genes for this species. The phylogenetic analysis of both transporters have revealed that the amino acidic sequence of ABCC3 is highly similar to ABCC2, and that ABCC4 is a more distant protein. This sequence similarity between ABCC2 and ABCC3 could also indicate similar functionalities within the mode of action of Bt proteins. In the opposite way, ABCC4 could be involved in the mode of action of other Bt proteins since it differs to a greater extent in its amino acidic sequence to the other transporters. To further characterize these two novel transporters, we have used gRNAs and recombinant Cas9 nuclease to perform knock-outs of *HvABCC3* and *HvABCC4* in *H. virescens* by CRISPR/Cas9 editing. After obtaining two strains with deletions in the exon 1 of both genes, bioassays with Cry1Aa and Cry1Ac as well as Vip3Aa were performed. For the *H. virescens* SIMRU colony, used as control, the most toxic protein tested was Cry1Ac, as observed by Lemes et al. (2014), followed by Cry1Aa, which also presented high toxicity. The least toxic protein was Vip3Aa, with a similar LC_{50} to those found in other studies (Lemes et al., 2014; Pickett et al., 2017). Preliminary data showed that the toxicities among both knock-out strains and the control were highly similar, with the exception of the lowest concentration of Cry1Ac tested (2.5 ng/cm^2), where a reduced mortality was recorded in the ABCC3-KO strain, compared to the SIMRU colony. Although differences were not observed between the ABCC3-KO strain and the SIMRU colony at higher concentrations of Cry1Ac, the reduction in the toxicity could be indicating some type of involvement of the ABCC3 in the mode of action of Cry1Ac.

As for *S. exigua*, it was recently proposed that the ABCC3 of *H. armigera* shared a redundant role with ABCC2, since the knock-outs of each transporter did not yield high levels of resistance, but insects with double knock-outs of presented >15,000-fold resistance to Cry1Ac (Wang et al., 2020). Another study revealed medium levels of resistance to Cry1Ab or Cry1Fa in *SfABCC2* knock-outs of *S. frugiperda*, but low levels of resistance to these proteins when knocking-out the *SfABCC3* (Jin et al., 2021) supporting the hypothesis of the redundant and

secondary role of ABCC3 compared to ABCC2. It is interesting to note that, in that study, the attempt to obtain double knock-outs of the transporters was not viable.

In our case, to understand if the ABCC3 from *H. virescens* is playing a similar redundant role in Cry1Ac toxicity, more bioassays should be performed to corroborate a significant effect at low doses of this protein. Moreover, a knock-out line of the *HvABCC2* gene would be needed, as well as double knock-outs of the *HvABCC2* and *HvABCC3* genes in order to compare the contribution of each of the transporters to the toxicity. In the same way, binding assays between the proteins tested in bioassays and BBMV prepared from extracted midguts of both knock-out strains could be performed to assess if binding interactions are altered. With the previous published data and the results herein, it is plausible that there are alterations in binding that do not translate to a lack of toxicity due to redundance of the transporters. Lastly, a confirmation of valid knock-outs by sequencing the messenger RNA of the ABCC3-KO and ABCC4-KO strains is desirable, since we cannot rule out the possibility that different versions of the transporters are being expressed due to alternative open reading frames on the gene sequence. To conclude, this study is a first glimpse into the diversity of ABC transporters that are present in *H. virescens* and points out the need of studying their functionality as receptors for other Bt proteins.

CHAPTER 4

The *Spodoptera exigua* ABCC2 acts as a Cry1A receptor independently of its nucleotide binding domain II

Adapted from:
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Introduction

Bacillus thuringiensis (Bt) crystal proteins (Cry proteins) have been largely used in biological control as formulated sprays or in genetically-modified crops because of their high and specific toxicity against insect pests (Crickmore, 2006; Roh et al., 2007). Due to the steady increase in the use of these proteins in agriculture, the appearance of resistance has been reported in some insect species, threatening the long-term use of Bt products (Tabashnik and Carrière, 2017). Cry proteins are generally recognized as pore-forming toxins (PFTs), as their main action is to form pores in the membrane of the midgut epithelial cells of susceptible insects (Schnepf et al., 1998; Xu et al., 2014). The mode of action of Cry proteins has been widely studied, especially for Cry1A proteins (Pardo-López et al., 2013; Adang et al., 2014). According with recent models, the mode of action of Cry proteins involves the sequential binding to different membrane receptors (Adang et al., 2014; Vachon et al., 2012). After specific binding events, the protein is inserted in the membrane inducing pore formation of the cells, which eventually leads to septicemia and insect death (Adang et al., 2014).

It is widely accepted that binding alteration is the most important mechanism of insect resistance to Cry toxins, although other mechanisms can also occur (Schnepf et al., 1998; Ferré and Van Rie, 2002). Alterations in the well-characterized midgut receptors for Cry toxins (cadherin-like protein, aminopeptidases N, alkaline phosphatases and ABC transporters) have been reported in different resistant strains (Vadlamudi et al., 1995; Knight et al., 1994; Jurat-Fuentes and Adang, 2004; Gahan et al., 2010). In few cases, the expression of the receptor is altered, leading to the appearance of resistance (Herrero et al., 2005; Tiewsiri and Wang, 2011; Jurat-Fuentes et al., 2011; Jin et al., 2014; Guo et al., 2015). In other cases, the receptors harbor different mutations that can alter the binding ability to Cry toxins (Ferré and Van Rie, 2002; Jurat-Fuentes and Adang, 2004; Baxter et al., 2005; Hernández-Martínez et al., 2012; Banerjee et al., 2017). ABC proteins are primary-active transporters that require the binding and

hydrolysis of ATP to transport substrates across the lipid membrane. A functional ABC transporter consists of two cytosolic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP, and two integral transmembrane domains, which generally consist of 5–6 transmembrane helices and provide substrate specificity (Dermauw and Van Leeuwen, 2014). To date, the number of ABC transporters that are involved in the mode of action of Cry toxins is increasing. The ABCC3 of *Spodoptera exigua* and *Spodoptera litura* and the ABCC4 of *Tribolium castaneum* were shown to be involved in susceptibility to Cry1A and Cry8C, respectively (Chen et al., 2015; Park et al., 2004; Endo et al., 2017). In addition, it has been observed that the ABCC2 acts as a functional receptor for Cry1A proteins for lepidopteran insects (Sato et al., 2019). Interestingly, the ABCC2 from *S. exigua*, *Spodoptera frugiperda*, and *Bombyx mori* and the ABCC3 from *S. exigua* and *B. mori* do not function as functional receptors for Cry1C and Cry1D (Banerjee et al., 2017; Endo et al., 2017). The implication of other ABC transporter subfamilies beyond subfamily C in the mode of action of different Cry proteins such as Cry2 and Cry3 has also been reported (Guo et al., 2015; Tay et al., 2015; Pauchet et al., 2016; Mathew et al., 2018).

From the analysis of resistant strains to Bt proteins, different mutations in the *ABCC2* gene were reported to be associated with Cry1A resistance (Gahan et al., 2010; Baxter et al., 2011; Atsumi et al., 2012). Furthermore, expression or silencing of the transporter using different systems correlates with altered susceptibility to Cry1 proteins (Banerjee et al., 2017; Chen et al., 2015; Park et al., 2004; Endo et al., 2017; Atsumi et al., 2012; Bretschneider et al., 2016; Tanaka et al., 2013; Tanaka et al., 2016; Stevens et al., 2017), supporting the role of this family of transporters in the mode of action of Bt toxins. In agreement with that, binding assays have shown specific binding of Cry1A proteins to ABCC2 transporters from different insect species (Bretschneider et al., 2016; Adegawa et al., 2017; Chapter 1 in this thesis). Additionally, it has been shown that ABCC2 transporters may also be involved in Cry1A oligomerization and/or insertion (Tanaka et

al., 2016; Ocelotl et al., 2017; Chapter 1 in this thesis). An early model for the binding of Cry1A toxins to the ABCC2 proteins suggested that the ABC transporter may interact with extended helices of the pre-pore Bt-toxin structure in its opened state and that, when it closes, an irreversible insertion is formed (Heckel, 2012). However, it was observed in a recent work in *B. mori* that lack of the ATPase activity (by generating NBD-deleted mutants) of the BmABCC2 transporter did not affect the functionality of the receptor variants (Tanaka et al., 2017).

In a laboratory-selected colony of *S. exigua* (Xen-R) resistant to the commercial Bt-product Xentari™ (Hernández-Martínez et al., 2010), a mutation in the *SeABCC2* gene is described as genetically linked to the resistance (Park et al., 2004). In contrast to other mutations in *ABC* genes conferring resistance to Cry proteins, the mutation in *SeABCC2* affects an intracellular domain involved in ATP binding (NBDII). To determine whether this mutation affects both the ABCC2 function as a transporter and the Cry binding ability, the truncated form of the gene from the resistant strain (*SeABCC2-XenR*) was expressed in Sf21 cells. The functional role as receptor was tested by viability cell assays and the ability to bind Cry1A proteins was assessed.

Materials and Methods

Cell Culture Maintenance

Spodoptera frugiperda derived Sf21 cells were cultured at 25°C in 25 cm^2 tissue culture flasks (T25 flasks, Nunc) containing 4 mL of Gibco® Grace's Medium (1×) (Life Technologies™, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Sf21 cells stably expressing the wild-type *SeABCC2* gene, designated as Sf21-FRA in Chapter 1, were maintained at 27°C in the same culture medium supplemented with 10 µg/mL Blasticidin.

SeABCC2-XenR Structural Characterization

The amino acid sequence of the truncated form of *S. exigua* ABCC2 from the Xen-R colony (Acc. number AIB06822.1) was aligned with its wild-type form (Acc. number AIB06821.1) using Geneious software (v10.2.6). The prediction of transmembrane domains, as well as the outer and inner parts of both forms of the SeABCC2 molecule were obtained using TMHMM server v 2.0 (**http://www.cbs.dtu.dk/services/TMHMM/**). The three-dimensional structure of SeABCC2-FRA was predicted using Phyre2 software (**http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index**) with reference to the 3D structure of ATP-binding cassette sub-family C member 8 isoform X2 as a template.

Generation of Sf21 Clones Expressing SeABCC2-XenR

RNAzol RT reagent (Sigma-Aldrich, St. Louis, MO, USA) was used to isolate total RNA from midgut tissues of Xen-R colony larvae (Hernández-Martínez et al., 2010). Then, total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed to cDNA using specific primers (Supplementary Table S1), which added *Sac*I and *Xba*I restriction sites as well as a FLAG-tag downstream in the gene. The cDNA encoding the truncated SeABCC2 (KF926100.1) form was cloned into the pIB-eGFP vector (kindly supplied by Monique Van Oers, Wageningen, The Netherlands) to generate the pIB-XenR vector. Sf21 cells were transfected with either pIB-eGFP or pIB-XenR vectors

using the transfection reagent Cellfectin®II Reagent (Invitrogen). Selection was started 72 h post-transfection and the selective medium was replaced every 3–4 days until the cells reached confluence. Transfected cells were seeded in a T25 flask and maintained in Grace's medium containing 10% FBS and 50 µg/mL Blasticidin (Invitrogen). The resulting polyclonal cell lines were named Sf21-eGFP and Sf21-XenR according to the vector used for transfection. Stable cell lines were maintained at 27ºC in Grace's medium containing 10% FBS and 10 µg/mL Blasticidin.

RT-qPCR

The expression level of the *SeABCC2-XenR* gene in Sf21 cell lines was measured by RT-qPCR using specific primers (Supplementary Table S1). After total RNA extraction from each cell line, cDNA was synthesized using PrimeScript RT Reagent kit (TaKaRa Bio Inc, Otsu Shiga, Japan). RT-qPCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following standard protocols. Primers used in this study were previously described and their efficiency tested in Chapter 1. The gene expression levels were normalized using the *ubiquitin* gene as a reference.

Detection of the SeABCC-XenR Protein

For the detection of the SeABCC2-XenR protein, Western blot was performed using monoclonal anti-FLAG®M2 (Sigma-Aldrich, St. Louis, MO, USA). First, membrane vesicle samples from both cell lines were prepared as described by Van de Wetering et al. (2009). After SDS-PAGE, the samples were transferred to nitrocellulose membranes. Membranes were blocked with 3% of membrane blocking agent in PBST buffer (phosphate buffered saline with 0.1% Tween 20), and, after washing three times with PBST, membranes were incubated with monoclonal anti-FLAG®M2 (1:5000 dilution) in PBST supplemented with 1% blocking agent (PBST-B) for 1 h at RT. Then, membranes were washed three times with PBST and incubated with anti-mouse IgG-conjugated horseradish peroxidase (1:20,000 dilution) in PBST-B for 1

h at RT. Finally, bands were visualized with a chemiluminescence detection kit (RPN2209; GE Healthcare, Little Chalfont, UK) using an ImageQuant LAS4000 image analyzer (GE Healthcare).

For localization of the truncated transporter, immunostaining was performed as described in Chapter 1. Briefly, after overnight growing in 24-well glass chambers, cells were washed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde solution (PFA), permeabilized with 0.01% Triton X-100 at room temperature (RT), and blocked TNT buffer with 1% bovine serum albumin (BSA) for 1 h at RT. Cells were then incubated with monoclonal anti-FLAG®M2 (1:1000 dilution) for 2 h. After washes, cells were incubated with goat anti-mouse IgG conjugated to Alexa Fluor 488 (Abcam, Cambridge, UK) at a 1:1000 dilution for 1 h. Additionally, two negative controls were performed: (1) transfected cells were incubated with the secondary antibody alone; and (2) non-transfected Sf21 cells were incubated with the primary antibody alone. To stain the cell nuclei, 1 μg/mL of 4,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, Schnelldorf, Germany) was used. The stained samples on glass slides were observed under a confocal microscope (Olympus, FV1000MPE, Japan, Tokyo).

Cry Proteins Preparation

Cry1Aa, Cry1Ab and Cry1Ac toxins used in cell viability assays or binding assays were obtained from recombinant *Escherichia coli* or recombinant *B. thuringiensis* strains (from Ecogen Inc., Langhorn, PA, USA), respectively. The recombinant *E. coli* strains were kindly supplied by R. A. de Maagd. Purification of the inclusion bodies, Cry toxin solubilization and trypsin-activation was performed as described in Chapter 1. The Cry1Aa (EG1273), Cry1Ab (EG7077), and Cry1Ac (EG11070) toxins' expression, solubilization, and trypsin-activation were performed as previously described by Estela et al. (2004). Trypsin-activated proteins were dialyzed in 20 mM Tris-HCl (pH 9) and filtered prior to anion-exchange chromatography using an ÄKTA system (GE Healthcare). The purity of all proteins was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (12% SDS-PAGE). All proteins were kept at −20°C until used.

Viability Assays

Viability was determined after exposure of both cell lines (Sf21 and Sf21-XenR) to Cry1Aa, Cry1Ab and Cry1Ac toxins, using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Prior to viability assays, cells were suspended in culture medium (without FBS) and plated (100 µL) in ELISA plates (flat bottom) at about 70% confluence. Plates were further incubated at 25ºC for at least 45 min to allow cells attach to the bottom. Then, 10 µL of activated toxins was added to each well within a range of increasing concentrations (from 0 to 150 nM) in duplicate on each plate. The same volume of 50 mM carbonate buffer (pH 10.5) and 2% Triton X-100 was also added to the wells as negative and positive controls, respectively. Cell viability was measured after 3 h incubation at 25ºC using the CellTiter 96® AQueous One Solution Reagent (Promega, Madison WI, USA) following the manufacturer's protocol. After 2 h incubation, the absorbance was measured at 490 nm (Infinite m200, Tecan, Maennedorf, Switzerland). The percentage of viable cells was obtained as described in Chapter 1. For statistical analysis, means were compared by two-way ANOVA, followed by Bonferroni's comparison test ($p < 0.001$).

Binding of ^{125}I-Cry1Ac to Sf21 Cells

The purified and trypsin-activated Cry1Ac toxin was labelled using the chloramine-T method (Van Rie et al., 1989). Briefly, Cry1Ac (25 µg) was mixed with 0.3 mCi of ^{125}I (PerkinElmer, Boston, MA, USA), and 6 mM of chloramine T for 45 seconds at RT. After incubation, the reaction was stopped by adding sodium metabisulfite followed by sodium iodide (NaI). The specific activity obtained for the labelled Cry1Ac toxin was 15 µCi/µg.

All binding assays were conducted at RT in a final volume of 0.1 mL in binding buffer. Firstly, both cells lines (Sf21 and Sf21-XenR) were recovered by centrifugation (500 × *g* for 5 min at RT), and washed two times with PBS. The cell pellet was resuspended in binding buffer (PBS supplemented with 0.1% BSA). to a concentration of 4.6×10^7 cells/mL. The optimal concentration of cells to be used for the binding

assays was calculated with increasing amounts of cells incubated with 0.1 nM of labelled-Cry1Ac in binding buffer. An excess of unlabelled protein (150 nM) was added to the reaction mixture to determine the nonspecific binding. After incubation, samples were centrifuged (500× *g* for 10 min) and the pellets were washed with binding buffer. Final radioactivity was measured in a gamma counter (2480 WIZARD^2). Binding experiments were performed at least twice for each cell line.

Competition experiments were performed incubating the Sf21-XenR cells (9.2×10^6 cells/mL) with ^{125}I-Cry1Ac and increasing amounts of different unlabelled Cry1Aa, Cry1Ab or Cry1Ac proteins. After incubation, samples were centrifuged, washed, and radioactivity in the final pellets measured. Competition assays were replicated at least three times. The equilibrium dissociation constant (K_d) and concentration of binding sites (R_t) were obtained from the homologous competition experiments using the LIGAND software (Munson and Rodbard, 1980).

The contribution of reversible and irreversible binding to the observed specific binding in those cells expressing either the full-length or the truncated form of the SeABCC2 molecule was determined as described by Park et al. (Park et al., 2004). Briefly, three reaction mixtures were prepared with 0.1 nM of ^{125}I-Cry1Ac and 9.2×10^6 cells/mL of either Sf21-FRA or Sf21-XenR cell lines. The first sample was used to determine the total binding. In the second sample, an excess of unlabelled Cry1Ac toxin (150 nM) was added to this mixture at the beginning of the incubation to determine the non-specific binding. Finally, the third sample was used to measure the irreversible binding. To that purpose, after one hour of incubation, an excess of unlabelled Cry1Ac toxin was added and the reaction was allowed to proceed one more hour. All samples were incubated at RT for two hours. The specific and the irreversible binding were calculated by subtracting the non-specific binding (radioactivity in the pellet of the second sample) from the total binding (radioactivity in the first sample) or from that in the third sample, respectively. The reversible component was calculated by subtracting the irreversible binding from the specific binding. Experiments were performed three times.

Results and Discussion

Characterization and 3D Prediction of the Structure of the Truncated SeABCC2 Gene

Park et al. (2004) showed that the mutation in the *SeABCC2* gene linked to resistance in the Xen-R colony was lacking part of NBDII, apparently not affecting the extracellular regions of the membrane protein. Compared with the wild-type protein from the susceptible *S. exigua* colony (FRA colony) (Hernández-Martínez et al., 2010), the truncated transporter carries four additional single amino acid changes at positions 671, 805, 1200, and 1314 (**Figure 26**, panels **A** and **B**). Based on the data obtained by the TMHMM server (which predicts transmembrane helices based on a hidden Markov model), one of these mutations (K805T) is located in the extracellular loop 4 (ECL4) of SeABCC2, whereas the other three are located in the intracellular part of the transporter (**Figure 26B**). Interestingly, the ECL4 region has been previously described as an important area for Cry1A toxin binding in BmABCC2 from *B. mori* (Tanaka et al., 2016, 2017).

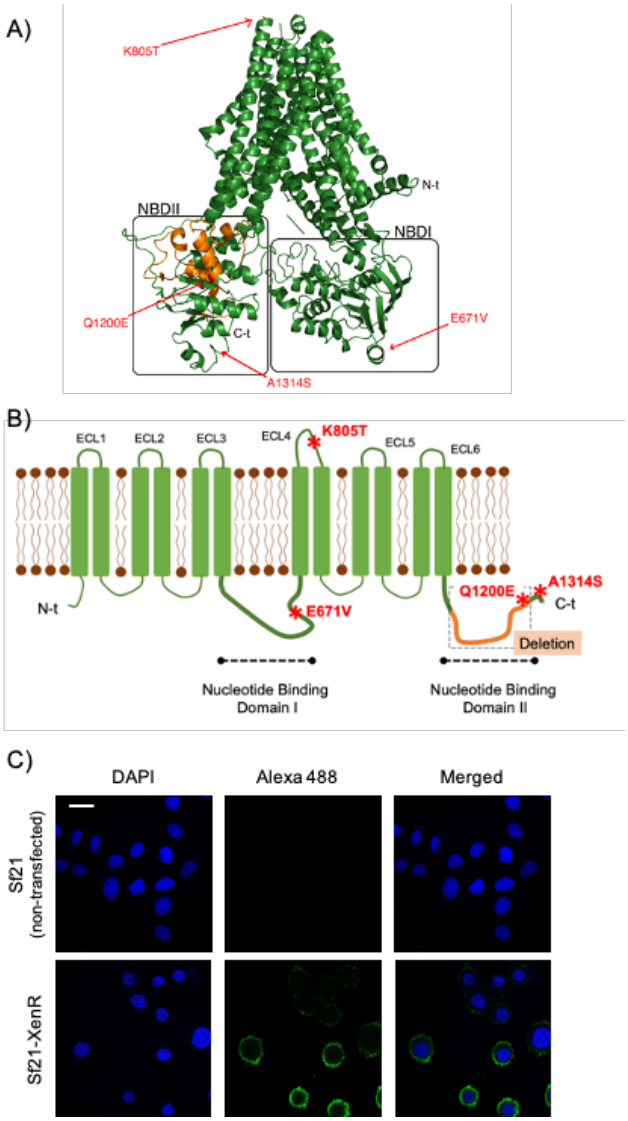

Figure 26. Predicted structure and expression analysis of the truncated SeABCC2. (**A**) Schematic illustration of the 3D structure of SeABCC2-FRA predicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) with reference to the 3D structure of ATP-binding cassette sub-family C member 8 isoform X2. (**B**) 2-D schematic structure of the truncated SeABCC2 showing the ECL and NBD regions. The 1121–1199 amino acid deletion in the NBDII is shown in orange, and amino acid positions that differ in both proteins are shown in red. (**C**) Immunostaining of the SeABCC2-XenR transporter expressed in Sf21 cells. Cells were stained with an anti-FLAG tag antibody followed by anti-mouse IgG conjugated to Alexa Fluor 488 (green signal). Cell nuclei were stained with DAPI (blue signal). Scale bar represents 20 μm.

The Truncated SeABCC2-XenR is Located in the Membrane of the Sf21 Cells

To examine whether single amino acid changes, along with the deleted NBDII region, are relevant for the interaction of Cry1A proteins and the SeABCC2 transporter, the truncated form was expressed in Sf21 cells. Expression of the *SeABCC2-XenR* gene was analyzed by RT-qPCR to determine whether the gene was stably expressed after transfection and selection. The expression levels were found significantly higher than the expression of the housekeeping gene, whereas essentially no expression was detected for the Sf21 cell line (non-transfected) (**Supplementary Figure S6a**). Prior to test whether the truncated SeABCC2 transporter is a functional receptor for Cry1A proteins, its expression was determined by Western blot (**Supplementary Figure S6b**). Moreover, the results from immunostaining showed that the truncated SeABCC2 transporter was located on the cell membrane of Sf21-XenR cells (**Figure 26C**). Therefore, despite harboring the deletion affecting the NBDII, the transporter was located in the Sf21 cell membranes, as reported previously in Chapter 1 for Sf21 cells expressing the full-length form of the transporter.

The Truncated SeABCC2 Still Acts as a Functional Receptor for Cry1A Toxins

Insertion/deletion (INDEL) mutations in the orthologous ABCC2 transporters have been reported to be associated with Cry1A resistance in different insect species (Gahan et al., 2010; Tay et al., 2015; Baxter et al., 2011; Atsumi et al., 2012; Heckel, 2012). However, direct evidence that these alterations on the ABC transporters are involved in Bt resistance by performing functional assays is scarce. Here, the functionality of the truncated ABCC2 molecule was studied. For this purpose, its role in toxicity was assessed in Sf21 cells expressing the SeABCC2-XenR.

The susceptibility of the two cell lines (Sf21 and Sf21-XenR) to three Cry1A proteins (Cry1Aa, Cry1Ab, and Cry1Ac) was determined by the MTT method. Regarding the Sf21 cells, none of the Cry1A toxins

had any major effect on their viability, as shown in Chapter 1 and by Bretschneider et al. (2016). In contrast, the toxins affected the viability of Sf21-XenR cells in a dose-dependent manner. In addition, the loss of cell viability was drastic with Cry1Ab and Cry1Ac. For Cry1Aa, only significant differences were found at the highest concentration used (**Figure 27**). Similar results were found in Chapter 1 on Sf21-FRA cells, which express the full-length form of the transporter. These results demonstrate that the ABCC2 transporter is necessary to render susceptibility to Cry1A toxins in Sf21 cells, independently of the presence of the second ATP binding domain. Thus, our results point out that the deletion on this domain is not directly causing resistance to the Cry1A type toxins in the Xentari^{TM}-resistant *S. exigua* colony. Similarly, Tanaka et al. (2017) found that cells expressing mutants of *B. mori* ABCC2 which lacked substrate-excreting activity still retained receptor activity for Cry1A toxins. Interestingly, cells expressing mutants with a deletion in the NBDII were susceptible to Cry1Aa and Cry1Ab but not to Cry1Ac. For *Helicoverpa armigera*, mis-splicing of the *ABCC2* gene was linked to Cry1Ac resistance (Xiao et al., 2014). The mis-splicing caused a 73 bp insertion that generated a premature stop codon, which expected to yield a truncated ABCC2 protein without the NBDII. However, based on our findings, functional analysis using the truncated HaABCC2 would be required to test if the truncation is causing the resistance to Cry1Ac in this strain or it is only partially contributing to resistance. More recently, a field-evolved resistance to Bt corn expressing Cry1Fa has been closely linked to a mutation in the *S. frugiperda ABCC2* gene (Banerjee et al., 2017). The authors confirmed, by functional assays, that the full-length of the SfABCC2 acts as a functional receptor for Cry1Fa. In contrast, the mutated version of the SfABCC2 lacking the whole second transmembrane domain (consequently including the NBDII) was not functional.

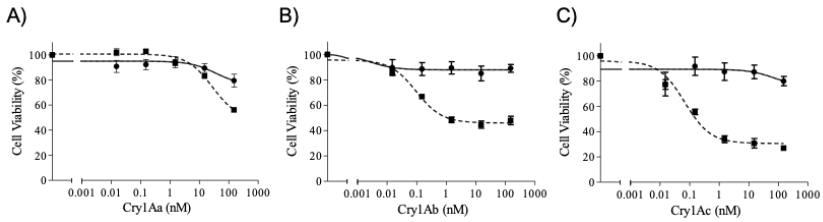

Figure 27. Effect of Cry1A proteins on the viability of Sf21 cells. Assays were performed using increasing concentrations of: Cry1Aa (**A**); Cry1Ab (**B**); and Cry1Ac (**C**). Assays were carried out for 3 h with Sf21 cells (circles) and Sf21-XenR cells (squares). Each value represents the mean of at least three independent assays (± SEM). Means were compared by two-way ANOVA, followed by Bonferroni's comparison test.

The Truncated SeABCC2 Still Mediates Binding to Cry1A Toxins

Binding assays using the ^{125}I-labelled Cry1Ac protein were performed to verify the interaction of Cry1A proteins with the truncated transporter. The results showed specific binding of labelled Cry1Ac to increasing concentrations of the Sf21-XenR cells, while no specific binding was found for the control cell line (Sf21) at any given concentration (**Figure 28A**). Homologous competition assays showed that the competitor could completely displace the specific binding of labelled Cry1Ac to the Sf21-XenR cells (since there is almost 50% of nonspecific binding). Dissociation constant (K_d) and concentration of binding sites (R_t) were estimated from the homologous competition curve (**Figure 28B**), obtaining K_d = 2.4 ± 1.4 nM (mean ± SEM) and *R*t = 0.006 ± 0.003 pmol/million cells (mean ± SEM). The K_d value indicates that binding of Cry1Ac to Sf21-XenR is of high affinity. The equilibrium binding parameter obtained in the present study did not differ significantly with the one obtained in Chapter 1, when labelled Cry1Ac and cells expressing the full-length transporter were used. Interestingly, larger differences were found in the concentration of binding sites. Differences in the R_t values might be attributed to the difference in the expression levels of the transporter in each cell line. Therefore, the specific binding found for Sf21-XenR compared to the Sf21 cell line points out the fact that the intracellular truncation of the transporter does not affect the ability of the transporter to interact with the Cry1A toxins.

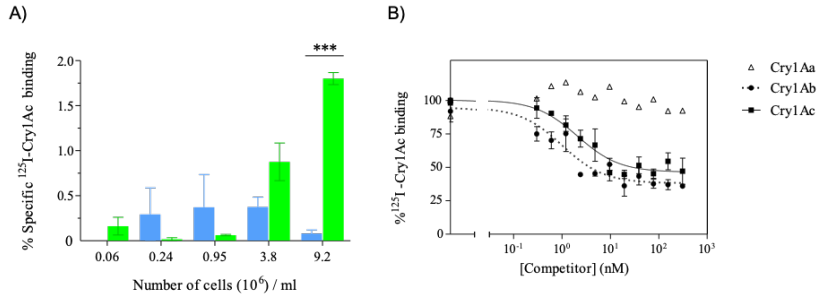

Figure 28. Binding of ^{125}I-Cry1Ac to Sf21 cells expressing the SeABCC2-XenR. (**A**) Specific binding of ^{125}ICry1Ac at increasing concentrations of Sf21 (blue) and Sf21-XenR (green) cells. Each bar represents the mean of at least three independent experiments (± SEM). Means were compared by two-way ANOVA, followed by Bonferroni's comparison test (p <0.001). Significant differences between both cell lines are indicated by asterisks. (**B**) Competition binding assays with ^{125}I-Cry1Ac using Sf21-XenR cells. Curves represent total binding of labelled Cry1Ac protein to increasing concentrations of unlabelled Cry1Aa (open triangles), Cry1Ab (full circles), and Cry1Ac (squares) as competitors. Each competition experiment was replicated at least three times and the error bars represent the standard error of the mean.

Different extracellular loops of ABCC transporters have been proposed as candidate Cry1A binding sites in different insect species (Atsumi et al., 2012; Tanaka et al., 2016, 2017; Liu et al., 2018). In *B. mori*, it was reported that a single tyrosine insertion in the extracellular loop 2 (ECL2) causes resistance to Cry1Ab. Later, the same group showed that the amino acidic length of the ECL2 of the BmABCC2 is more important than the residues forming part of it. Furthermore, the authors stated that an increase in the length of the ECL2 disrupts the receptor function for Cry1Ab/c but not for Cry1Aa in the same species (Tanaka et al., 2016). Here, we reported a mutation located in the ECL4 (position 805) of the truncated SeABCC2 transporter. This mutation, along with the other changes observed, was not affecting the role of the truncated transporter. Interestingly, it was recently reported in *B. mori* that the amino acid sequence from 770 to 773 of the ECL4 is a putative Cry1A toxin-binding site (Tanaka et al., 2017). Liu et al. (2018) reported that ABCC2 amino acid Q125 from SfABCC2 or E125 from SlABCC2 was a key factor for the differential Cry1Ac toxicity to Hi5 cells

expressing these receptors. Interestingly, the authors claimed that, as these residues (Q125 or E125) are located in the ECL1 region of the ABCC2 transporter, this loop could be important for Cry1Ac binding.

A deletion in the ABCC2 transporter of a Cry1Ac-resistant strain of *Plutella xylostella* (NO-QA strain) was genetically linked to resistance by Baxter et al. (2011). In this case, the deletion was predicted to remove the 12th transmembrane domain and aberrantly position the carboxyl-terminal outside the cell. Assuming that the gene is translated and inserted into the midgut membrane, the second ATP binding site was expected to be nonfunctional. Later, Hernández-Martínez et al. (2012) demonstrated lack of Cry1Fa binding in the same *P. xylostella* strain but not to Cry1Aa. Lack of binding of Cry1Fa toxin was also observed on Sf9 cells expressing a mutated SfABCC2 transporter from a Cry1Fa-resistant strain of *S. frugiperda* (Banerjee et al., 2017). The authors concluded that, based on the currently proposed model (Heckel, 2012), the lack of Cry1Fa binding could be due to the loss of the ATP-switch mechanism in the mutated transporter. However, it is important to highlight that the whole second transmembrane domain was absent in the mutated transporter including several ECL regions and the NBDII.

In the present study, heterologous competition experiments were performed in Sf21-XenR cells using Cry1Aa and Cry1Ab to determine whether these proteins shared binding sites with the Cry1Ac protein. The results showed that Cry1Ab competes against labelled Cry1Ac, while Cry1Aa does not compete (**Figure 28B**). Our results are in agreement with previous studies using *S. exigua* BBMV (Luo et al., 1999; Escriche et al., 1997) and the Sf21-FRA cells (Chapter 1). Again, the results point out that the truncated SeABCC2 remains active as a functional receptor for the Cry1Ac and Cry1Ab toxins.

The Irreversible Cry1Ac Binding Component is not Altered in the Truncated SeABCC2

The specific binding is considered as a critical step for the toxicity of Bt proteins (Jurat-Fuentes and Crickmore, 2017). Moreover, it is well established that specific binding of Cry toxins to their membrane receptors consists of a reversible and an irreversible binding component. The latter one has been associated with toxin insertion into the membrane (Liang et al., 1995). It has been suggested that ABC transporters could be mediating the latter component (Sato et al., 2019; Heckel, 2012). Here, both components of the specific binding of Cry1Ac were determined in the Sf21-FRA and the Sf21-XenR cell lines to test whether the modifications found in the truncated transporter are affecting the irreversible component in toxin insertion. The results showed that for both cell lines the predominant component of the specific binding was the irreversible binding (**Figure 29**). For Sf21-FRA cells, from 80% of total specific binding, 64% was irreversible and 16% was reversible. For Sf21-XenR cells, from 50% of total specific binding, 47% was irreversible and 3% was reversible. To test if the differences observed were significant, the proportion of irreversible binding on Sf21-FRA was compared to that in Sf21-XenR, as well as the proportion of reversible binding, finding no significant differences between the two receptors for the irreversible or the reversible binding ($p = 0.1237$, one-way ANOVA). Therefore, these findings, along with the viability assays, suggest that the Cry1Ac toxin can be inserted into the membrane despite the lack of the second ATP binding domain along with the other mutations found in the ABCC2. Recently, it was observed that pre-formed oligomers associate less efficiently with BBMV from the *P. xylostella* strain NO–QA (resistance linked to ABCC2) than with BBMV from a susceptible strain (Ocelotl et al., 2017). Interestingly, it is reported that a single tyrosine insertion in the ECL2 of the ABCC2 of *B. mori* causes resistance to Cry1Ab, although it can bind. Therefore, the authors suggested that the tyrosine insertion in the ECL2 may be affecting post-binding events (Tanaka et al., 2013). Lastly, Park et al. (2004) showed a significant decrease in the irreversible component of the Cry1Ca specific binding in *S. exigua* resistant to Xentari™. This

product is based on a *B. thuringiensis* subsp. *aizawai* (Valent Biosciences), containing Cry1Aa, Cry1Ab, Cry1C, Cry1D and Cry2Ab proteins. Since Cry1Ca is one of the most potent Cry toxins to *Spodoptera* spp. (Herrero et al., 2016), this decrease in membrane insertion could contribute to the observed resistance against Xentari™.

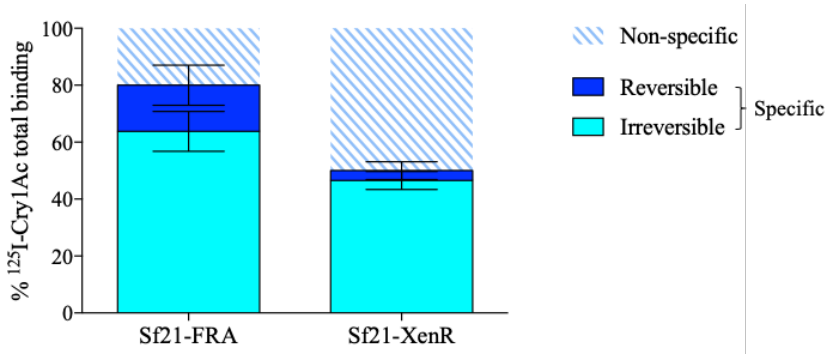

Figure 29. Dissection of total Cry1Ac binding into the non-specific and the specific binding (reversible and irreversible components) in Sf21 cells expressing the full-length (Sf21-FRA) or the truncated form (Sf21-XenR) of the SeABCC2 transporter.

To conclude, as summarized in **Figure 30**, the results from this study endorse that the truncated SeABCC2 (SeABCC2-XenR) transporter that lacks part of the NBDII is a functional receptor for Cry1A proteins in *S. exigua*. In addition, the four additional single amino acid changes (positions 671, 805, 1200, and 1314) described here do not affect the functionality of the truncated receptor. Therefore, our data support that the ATP-switch mechanism of the transporter is not necessary to act as a functional receptor to Cry1A toxins.

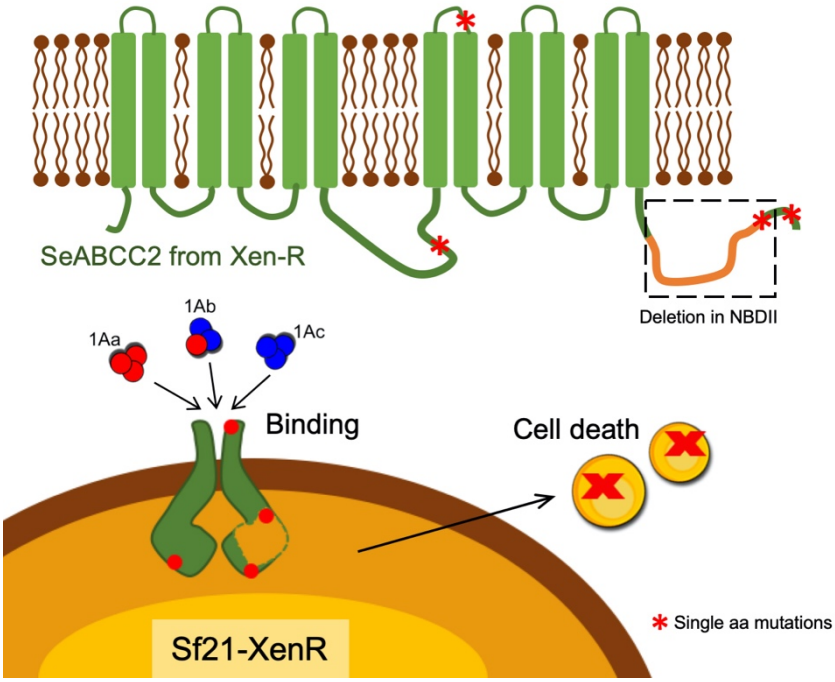

Figure 30. Schematic representation of the main findings in this chapter.

CHAPTER 5

Alteration of a Cry1A shared binding site in a Cry1Ab-selected colony of *Ostrinia furnacalis*

Adapted from:
Pinos D, Wang Y, Hernández-Martínez P, He K, Ferré J. 2022. Alteration of a Cry1A shared binding site in a Cry1Ab-selected colony of *Ostrinia furnacalis*. *Toxins* (14) 32

Introduction

Bacillus thuringiensis (Bt) is known to produce many insecticidal proteins that, either as Bt-based pesticides or expressed in genetically modified crops, can effectively control different insect pests (Raymond et al., 2010). One highly effective tool to control stem borers is Bt corn, which co-expresses different Bt proteins, mainly from the Cry1 family (Schnepf et al., 1998). The adoption of Bt corn expressing Cry1Ab has quickly expanded globally since it has demonstrated to control the European corn borer, *Ostrinia nubilalis*, and other pests (ISAAA, 2017). Bt corn is also considered a promising technology to control the Asian corn borer, *Ostrinia furnacalis*, a highly damaging insect that affects mainly this crop (He et al., 2003; Li et al., 2021).

The mode of action of Bt insecticidal proteins involves, among other steps, binding to membrane molecules in the midgut (referred to as "receptors"). This step is not only responsible for the specificity of the toxic action, but it is also the main responsible for developing high levels of resistance by alteration of the membrane receptor (Jurat-Fuentes et al., 2021). Competition binding studies, in which the inhibition of binding of a labelled protein by different proteins is determined, have provided models showing whether Bt proteins bind to more than one site and whether different proteins share binding sites. These binding site models have been useful to predict and understand the basis of cross-resistance to Bt proteins since the alteration of a shared binding site can confer resistance to more than one Bt protein (Ballester et al., 1999; Hernández-Rodríguez et al., 2013). This approach, along with the use of laboratory-selected resistant colonies, allows us to be one step ahead of the onset of resistance in the field, understanding possible mechanisms of resistance before they even take place. In the case of its sibling species *O. nubilalis*, the binding site model proposes the presence of three shared binding sites for Cry1Ab and Cry1Ac. In that model, the Cry1F protein could bind to two of the binding sites, and Cry1Aa could only bind to one (Jakka et al., 2015). Although a study performed with ligand blots revealed certain similarities in binding patterns between *O. nubilalis* and *O.*

furnacalis (Tan et al., 2013), a binding site model for Cry1A proteins in *O. furnacalis* is still needed. The characterization of the binding sites of this pest, along with the analysis of cross-resistance patterns in a laboratory-selected resistant colony to the Cry proteins expressed in Bt crops, can provide valuable knowledge to decide which of the available alternatives is the best to efficiently control this pest.

In the present study, we have analyzed the binding properties, as well as the insecticidal potency, of Cry1A proteins and Cry1F in two *O. furnacalis* colonies, one susceptible (ACB-BtS) and one laboratory-selected colony resistant to Cry1Ab (ACB-AbR). The ACB-AbR colony developed cross-resistance to Cry1Aa, Cry1Ac and Cry1F. Moreover, the results have shown that Cry1A proteins share binding sites in this insect species and that at least one of these sites has been altered in the resistant insects.

Materials and Methods

Insect colonies

The *O. furnacalis* Bt susceptible colony (ACB-BtS) was originally collected from Huxian, Shaan'xi province (China) and maintained in laboratory conditions using a semi-artificial diet as previously described (Zhou et al., 1998). The *O. furnacalis* Cry1Ab resistant colony (ACB-AbR) was selected from a sample of the Bt susceptible colony by exposure to trypsin-activated Cry1Ab. After an initial exposure (2.5 ng of Cry1Ab/g diet), the protein concentration was increased to target 40-70% mortality. After 51 generations, larvae were reared on diet containing 400 ng of toxin/g diet. This colony achieved >40-fold resistance to the protoxin form of Cry1Ab after 71 generations (Xu et al., 2010). From generation 124, the protein concentration was increased to 2.0 μg/g. Thereafter, the colony has been maintained at this concentration. Larvae used for dissecting midguts in this study were from generation 208. Bioassays were carried out at generation 211 to determine the LC_{50}s of Cry1Aa, Cry1Ab, Cry1Ac and Cry1F.

Bt proteins preparation

Bacillus thuringiensis Cry proteins were obtained from recombinant strains EG1273, EG7077, EG11070, EG11069 expressing Cry1Aa, Cry1Ab, Cry1Ac and Cry1Fa, respectively (from Ecogen Inc., Langhorn, PA). For bioassays, Cry proteins were purified and solubilized as previously described (Estela et al., 2004), stored lyophilized and resuspended in the appropriate buffer before use. For binding assays, Cry proteins were activated by trypsin and then dialyzed in 20 mM Tris-HCl (pH 9) and filtered. Then, they were purified by anion-exchange chromatography in a HiTrap Q HP column using an ÄKTA explorer 100 chromatography system (GE Healthcare, United Kingdom). The Cry1Ab protein used for iodine labelling was further purified by size-exclusion chromatography with a Superdex 200 column (GE Healthcare, United Kingdom) using the same system. The purity of all proteins was analyzed by 12% sodium dodecylsulfate

polyacrylamide gel electrophoresis (SDS-PAGE). All proteins were kept at −20°C until used.

Diet Bioassays

Larval susceptibility of *O. furnacalis* susceptible and resistant insects was evaluated by diet incorporation assays in agar-free semi-artificial diet, as previously described (He et al., 2005). Briefly, neonates were individualized in 48-well trays containing the diet with different concentrations of proteins. Trays were held at 27 ± 1 °C, 80% RH and a 16:8 h photoperiod. Survivors and their weights were recorded after seven days. When the mortality was calculated, the larvae were considered dead if they died (visually motionless while poked with a fine brush) or weighed ≤ 0.1 mg. Bioassay data were subjected to Probit analysis with the PoloPlus software (LeOra Software) to obtain the LC_{50} values for each protein (Finney, 1971).

BBMV preparation

BBMV were prepared from dissected midguts obtained from fifth instar larvae of both ACB-BtS and ACB-AbR colonies by the differential magnesium precipitation method (Wolfersberger et al., 1987) and kept at −80°C until used. Protein concentration of BBMV preparations was determined by the method of Bradford (1976).

Binding assays with ^{125}I-labelled Cry1Ab and Cry1Aa

The trypsin-activated and chromatography purified Cry1Ab and Cry1Aa proteins were labelled using the chloramine-T method (Van Rie et al., 1989). Twenty-five micrograms were mixed with 0.3 mCi of ^{125}I (Perkin Elmer, Boston, MA, USA), and 6 mM of chloramine-T for 45 seconds at room temperature (RT). After incubation, the reaction was stopped by adding sodium metabisulfite (5.75 mM) followed by sodium iodide (250 mM). The specific activity obtained for the labelled Cry1Ab was 1.1 mCi/mg and 3.8 mCi/mg for Cry1Aa.

Prior to use, BBMV from susceptible and resistant colonies were centrifuged for 10 min at 16000x *g* and resuspended in binding buffer (phosphate buffered saline, (PBS), 0.1% BSA). To determine the

optimal concentration of BBMV for use in competition experiments, increasing amounts of BBMV were incubated with 1.22 nM of labelled Cry1Ab, in a final volume of 0.1 ml of binding buffer for 1 h at RT. An excess of the same unlabelled protein (>2000 nM) was used to estimate the non-specific binding. The specific binding was calculated as the subtraction of the total binding minus the non-specific binding. Homologous (using the unlabelled same protein as competitor) and heterologous (using other proteins as competitors) competition experiments were performed in binding buffer incubating 0.2 mg/ml of BBMV with ^{125}I-labelled proteins and increasing amounts of unlabelled proteins in a final volume of 0.1 ml for 1 h at RT. After incubation, samples were centrifuged at 16000x *g* for 10 min. Then, pellets were washed with 0.5 ml of binding buffer and centrifuged again. Radioactivity in the pellets was measured in a model 2480 WIZARD^2 gamma counter. The equilibrium dissociation constant (K_d) and concentration of binding sites (R_t) were estimated from the homologous competition experiments for each colony using the LIGAND program (Munson and Rodbard, 1980).

Results

Susceptibility of O. furnacalis *colonies ACB-BtS and ACB-AbR to Cry1 proteins*

There were significant differences in susceptibility to Cry1Aa, Cry1Ab, Cry1Ac and Cry1F between the ACB-BtS colony and the ACB-AbR colony (Table 2). The ACB-BtS colony was highly susceptible to all tested Cry1 proteins with LC_{50} values lower than 1 μg/g in all cases. Based on the LC_{50} values, the ACB-AbR colony evolved >714-fold resistance to Cry1Ab compared to the ACB-BtS colony. Regarding the other Cry proteins, the ACB-AbR colony showed high levels of cross-resistance: 178-fold to Cry1Aa, and >192-fold to both Cry1Ac and Cry1F.

Table 2. Toxicity of *Bacillus thuringiensis* proteins to neonate larvae of *Ostrinia furnacalis* after 7 days of exposure.

Protein tested	Colony	n[a]	LC_{50} (95% FL) [b] (μg protein/g diet)	Slope (±SE)	χ^2	df (χ^2)	RR[c] (95% CI)
Cry1Ab	ACB-BtS	768	0.28 (0.20-0.36)	1.55 ± 0.13	5.2	14	-
	ACB-AbR	864	>200 [d]	-	-	-	>714
Cry1Aa	ACB-BtS	768	0.18 (0.15-0.22)	1.90 ± 0.13	8.6	14	-
	ACB-AbR	864	32 (25-40)	1.62 ± 0.15	7.6	-	178 (131-237)
Cry1Ac	ACB-BtS	768	0.26 (0.19-0.34)	1.36 ± 0.11	9.5	16	-
	ACB-AbR	768	>50 [d]	-	-	-	>192
Cry1F	ACB-BtS	864	0.52 (0.36-0.70)	1.33 ± 0.11	6.4	14	-
	ACB-AbR	672	>100 [d]	-	-	16	>192

[a] Number of larvae tested in bioassays. [b] Concentration of protein killing 50% of larvae and its 95% fiducial limits.

[c] Resistance ratio and its 95% confidence interval compared with the susceptible colony at LC_{50}.

[d] Mortalities [(mean ± SD) %] were 39.6 ± 2.1, 40.6±1.0 and 35.4±2.1 at the highest concentrations of Cry1Ab (200 μg/g), Cry1Ac (50 μg/g) and Cry1F (100 μg/g), respectively. Due to the limitation in the amount of protein available, we were unable to increase the concentrations.

Binding of ^{125}I-labelled Cry1Ab to BBMV

The Cry1Ab protein showed specific binding to BBMV from both susceptible and resistant colonies (**Figure 31**). At the maximum concentration of BBMV tested, a total of 10.7% of the labelled protein bound to BBMV from the ACB-BtS colony, whereas only a total of 5.5% of the labelled protein bound with BBMV from the ACB-AbR colony.

In both samples, the non-specific binding at the maximum concentration of BBMV tested was ca. 3%. The resistant insects apparently lost approximately half the specific binding to Cry1Ab compared to the susceptible insects. The dissociation constants (K_d) obtained from the homologous competition assays were 7.75 (±1.97) nM and 2.40 (±0.98) nM (mean ± SEM), and the concentration of binding sites (R_t) were 1.95 (±0.28) and 0.60 (±0.16) pmol/mg (mean ± SEM) for the susceptible and resistant insects, respectively. The concentration of receptors (R_t) for Cry1Ab is decreased by almost 3-fold in the resistant colony, compared to the susceptible colony.

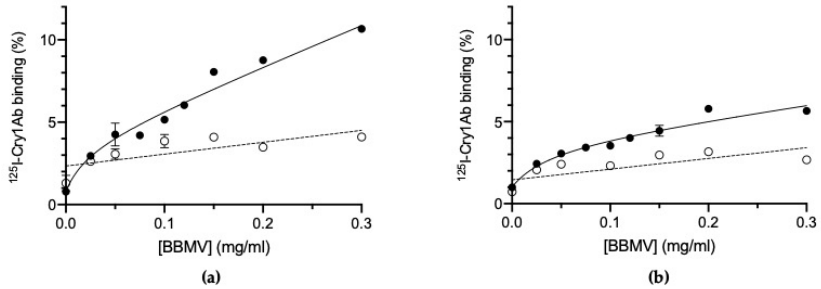

Figure 31. Binding of ^{125}I-Cry1Ab at increasing concentrations of BBMV proteins. **(a)** *Ostrinia furnacalis* susceptible colony (ACB-BtS). **(b)** *O. furnacalis* Cry1Ab-resistant colony (ACB-AbR). Full dots represent total binding and empty dots represent non-specific binding. Each binding experiment was replicated at least twice and the error bars represent the standard error of the mean.

The results from competition binding assays between labelled Cry1Ab and the other proteins are shown in **Figure 32**. For the susceptible colony (**Figure 32a**), the competition curve of Cry1Aa is very similar to that of the homologous competitor (Cry1Ab), indicating that Cry1Aa can bind to the same sites and with a similar affinity as Cry1Ab. For Cry1Ac, the competition curve indicates that, although it can displace all Cry1Ab binding sites, a higher concentration is required indicating a lower affinity for the Cry1Ab sites. For Cry1F, the curve indicates a much lower affinity for Cry1Ab binding sites and it is not clear if it would compete for all sites at higher concentrations. In the case of the resistant colony, both Cry1Aa and Cry1Ac are unable to

compete for all Cry1Ab binding sites, strongly suggesting that one of the binding sites, shared by the three Cry1A proteins, has been altered, preventing Cry1Aa and Cry1Ac binding, though still allowing Cry1Ab to bind (**Figure 32b**). However, since all the heterologous proteins are still able to compete partially with Cry1Ab, other shared binding sites must still remain. Since competition of Cry1F occurs at such high concentration, it is not possible to draw any conclusion whether the alteration of the shared biding site affects this protein.

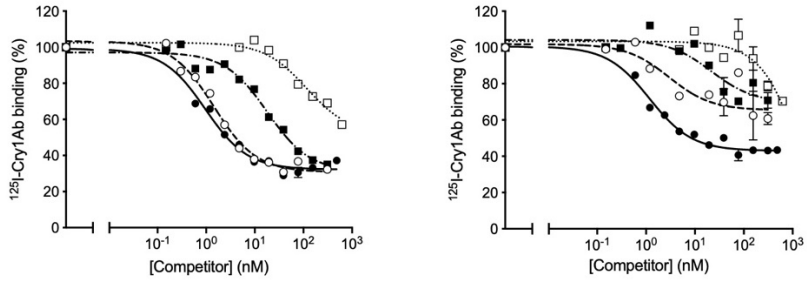

Figure 32. Competition binding assays with ^{125}I-Cry1Ab. **(a)** With BBMV from *Ostrinia furnacalis* susceptible colony (ACB-BtS) and **(b)** with BBMV from *O. furnacalis* Cry1Ab-resistant colony (ACB-AbR). Curves represent total binding of ^{125}I-Cry1Ab at increasing concentrations of unlabelled competitor: Cry1Aa (empty circles), Cry1Ab (full circles), Cry1Ac (full squares) or Cry1F (empty squares). Each competition experiment was replicated three times and the error bars represent the standard error of the mean.

Binding of ^{125}I-labelled Cry1Aa to BBMV

Since the competition curves using labelled Cry1Ab with the susceptible BBMV showed a similar behaviour of Cry1Ab and Cry1Aa unlabelled competitors, to validate the binding site model we further labelled Cry1Aa. To directly determine whether its binding was also affected in the resistant insects, we tested labelled Cry1Aa with BBMV of both colonies. **Figure 33** shows that both colonies showed specific binding of Cry1Aa to BBMV, with a clear reduction in the resistant colony compared to the susceptible one. The K_d values obtained were 0.48 (± 0.03) and 0.79 (± 0.24), and the R_t values were 0.22 (± 0.02) and 0.13 (± 0.02), for the susceptible and resistant colonies, respectively.

The concentration of receptors (R_t) for Cry1Aa is decreased by almost half in the resistant colony, compared to the susceptible colony.

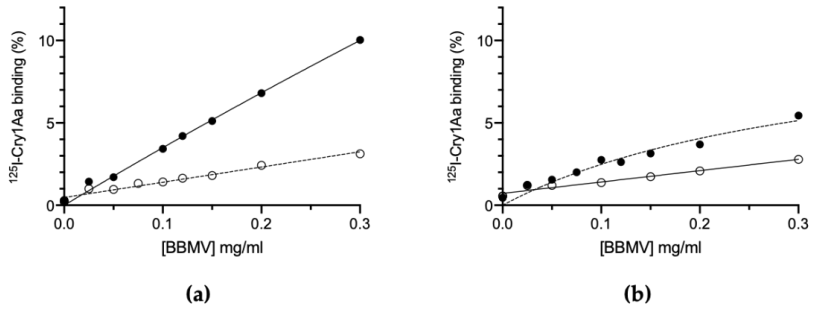

Figure 33. Binding of ^{125}I-Cry1Aa at increasing concentrations of BBMV proteins. **(a)** *Ostrinia furnacalis* susceptible colony (ACB-BtS). **(b)** *O. furnacalis* Cry1Ab-resistant colony (ACB-AbR). Full dots represent total binding and empty dots represent non-specific binding. Each binding experiment was replicated at least twice and the error bars represent the standard error of the mean.

Competition binding assays between labelled Cry1Aa and the other proteins is shown in **Figure 34**. Again, for the susceptible colony, both Cry1Aa and Cry1Ab compete with similar affinity for all sites occupied by labelled Cry1Aa (**Figure 34a**). In contrast, Cry1Ac could only compete for part of the Cry1Aa binding sites. The Cry1F protein shows a low ability to displace the labelled Cry1Aa binding, indicating a low affinity for certain Cry1Aa sites. For the resistant colony, although all heterologous proteins compete for Cry1Aa binding, both Cry1Ab and Cry1Ac cannot compete for all Cry1Aa binding, reinforcing the idea that at least one common binding site to Cry1A proteins is lost in the ACB-AbR colony (**Figure 34b**).

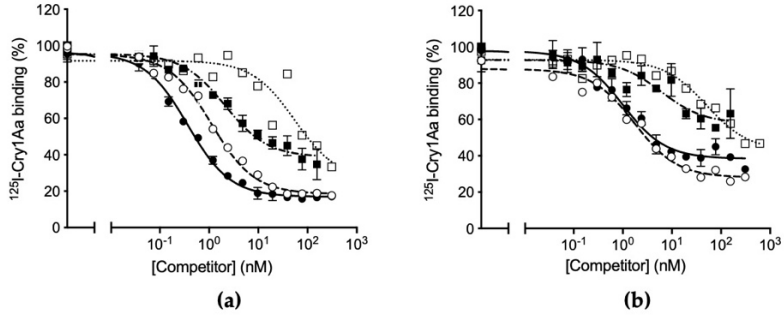

Figure 34. Competition binding assays with ^{125}I-Cry1Aa. **(a)** With BBMV from *Ostrinia furnacalis* susceptible colony (ACB-BtS) and **(b)** with BBMV from *O. furnacalis* Cry1Ab-resistant colony (ACB-AbR). Curves represent total binding of ^{125}I-Cry1Aa at increasing concentrations of unlabelled competitor: Cry1Aa (empty circles), Cry1Ab (full circles), Cry1Ac (full squares) or Cry1F (empty squares). Each competition experiment was replicated three times and the error bars represent the standard error of the mean.

Discussion

The bioassays of the Cry1Ab-resistant colony (ACB-AbR) showed a resistance ratio for Cry1Ab of >714-fold, and cross-resistant levels of >192-fold for Cry1Ac and Cry1F. In addition, we also assessed the cross-resistance to Cry1Aa, which was 178-fold. In earlier characterizations of the resistant colony, where the Cry1Ab resistance ratio reached 40-fold, an initial cross-resistance of 37-fold to Cry1Ac was observed (Xu et al., 2010), which increased later to 113-fold (Zhang et al., 2014), indicating that the selection for resistance to Cry1Ab produced a remarkable cross-resistance to Cry1Ac in the ACB-AbR colony. Interestingly, for the Cry1F protein an initial low cross-resistance of 6-fold was detected in the ACB-AbR colony (Xu et al., 2010), which increased to a notable 48-fold resistance in the latter study (Zhang et al., 2014) achieving very high levels in the present study.

The use of competition binding experiments can provide binding site models that help us predict or understand the biochemical basis of patterns of cross-resistance in a resistant colony (Ballester et al., 1999; Hernández-Rodríguez et al., 2013). In *O. furnacalis*, our results from competition binding assays suggest that there are at least two major binding sites for Cry1Aa and Cry1Ab which are shared by these two proteins (**Figure 35**). According to the competition curves, Cry1Ac competes for all Cry1Ab binding sites (**Figure 32a**), whereas it cannot compete for all Cry1Aa binding sites (**Figure 34a**), suggesting the occurrence of additional binding sites for this protein. The heterologous competition of Cry1F only at very high concentration may indicate that, though it can recognize with low affinity some of the Cry1A receptors, it must have singular receptors to exert its toxic action. This binding site model shares certain similarities with the model reported for the phylogenetically close species *Ostrinia nubilalis*, in which Cry1Ab and Cry1Ac competed for all binding sites and Cry1F only competed at higher concentrations (Hernández-Rodríguez et al., 2013; Jakka et al., 2015). The competition of Cry1F for the binding sites of Cry1A proteins has been shown to be of low affinity in many insect pests, such as *Plutella xylostella*, *Heliothis virescens*, *Helicoverpa*

armigera and *Helicoverpa zea* (Granero et al., 1996; Jurat-Fuentes et al., 2001; Hernández and Ferré, 2005).

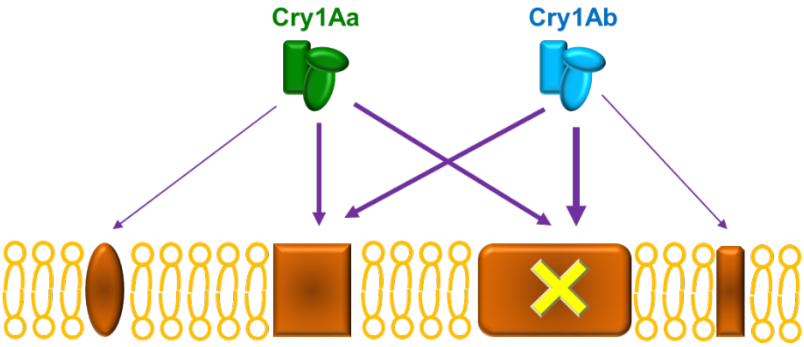

Figure 35. Binding site model for Cry1Aa and Cry1Ab proteins in *Ostrinia furnacalis*. The yellow cross represents the altered binding site in the ACB-AbR colony. The width of the arrows represents relative relevance of the binding sites. Cry1Aa and Cry1Ab may have, in addition to the shared sites, other sites with minor contribution to the toxicity and which can be shared with other Cry proteins.

For the resistant colony, the binding results indicate that Cry1Ab and Cry1Aa have lost part of its binding capacity, presumably by alteration of one of its shared binding sites (**Figures 31** and **33**). The fact that the heterologous competitors can only displace part of the ^{125}I-Cry1Ab and ^{125}I-Cry1Aa binding in the resistant insects (**Figures 32b** and **34b**) is indicative of the occurrence of, at least, two types of binding sites, one that will be shared with the heterologous competitors (the one that they are able to compete) and another one that is not shared (the part of the labelled protein that cannot be displaced). Taking into account the results with the susceptible insects, in which Cry1Aa and Cry1Ac could completely displace binding of ^{125}I-Cry1Ab (**Figure 32a**), the most plausible explanation is that one of the two Cry1Ab binding sites has suffered an alteration in the resistant insects that, though still allowing binding of Cry1Ab, prevents binding of the other Cry proteins (**Figure 33**).

It is well known that Cry1 proteins use several membrane proteins as receptors in the midgut of lepidopteran larvae, such as aminopeptidase N, cadherin and ABC transporters (Knight et al., 1994; Luo et al., 1997;

Vadlamudi et al., 1995; Tanaka et al., 2013). In *O. furnacalis*, a cadherin was first found altered in a Cry1Ac-resistant colony (ACB-AcR) (Jin et al., 2014). A later study, characterized the alteration of aminopeptidase N and ABC type G transcripts in both the ACB-AbR colony (studied here) and the ACB-AcR colony (Zhang et al., 2017). Recently, the involvement of the *O. furnacalis* cadherin in the toxicity of Cry1Aa and Cry1Ac was proven through CRISPR knock-outs (Jin et al., 2021) as well as the ABCC2 in the toxicity of Cry1F and Cry1Ab/c (Wang et al., 2020). The binding sites here proposed could be located in some of these receptors or even in different epitopes of the same one, as it has been shown for Cry1A proteins and the ABCC2 transporter in *Spodoptera exigua* (Chapters 1 and 2). Previous studies have linked the resistance to Cry1Ac to the modulation of the expression of many midgut genes by *trans*-regulatory signaling mechanisms in *Plutella xylostella* (Guo et al., 2015 and 2020). In our case, this possibility is plausible since the inheritance of the resistance in the ACB-AbR colony is polygenic (Zhang et al., 2014).

According to our binding site model, *O. furnacalis* has, at least, two major shared binding sites for Cry1A proteins. As a consequence, the alteration of one of the binding sites may contribute to the cross-resistance observed among Cry1A proteins. The cross-resistance to Cry1F seems not to be due to the alteration of Cry1A binding sites, thus other selected mechanisms may be responsible. Binding site models, along with cross-resistance data from laboratory-selected resistant colonies, are important tools that can help decision-making for effective pyramiding of Bt-crops to combat resistant evolution in insect pest populations.

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CHAPTER 6

Reduced membrane-bound alkaline phosphatase does not affect binding of Vip3Aa in a *Heliothis virescens* resistant colony

Adapted from:
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Introduction

The polyphagous pest *Heliothis virescens* (L.) (Lepidoptera: Noctuidae) is well known for producing substantial economic losses, particularly in cotton production, due to its ability to evolve resistance to different synthetic control products such as methyl parathion or pyrethroids (Wolfenbarger et al., 1981; Terán-Vargas et al., 1996). As an alternative approach, genetically modified crops expressing Cry and Vip3A insecticidal protein genes from *Bacillus thuringiensis* (Bt crops) were introduced in 1996 for the control of this and other pests. However, extensive use threatens their effectiveness and cases of field-evolved practical resistance have already been reported for some lepidopteran and coleopteran pests (Tabashnik et al., 2019). Gene pyramiding has been proposed as an effective strategy for insect resistance management in Bt crops (Roush, 1997). This approach consists of combined production of distinct insecticidal Bt proteins in the same plant, and its success heavily relies on the expressed insecticidal proteins having distinct mode of action, commonly defined as not sharing binding sites in target tissues (Moar and Anilkumar, 2007; Carrière et al., 2015). Although the mechanism of action and receptors for Cry proteins have been widely studied (Adang et al., 2014), little is known about the biochemical mechanisms that underlie the action of Vip3A proteins. Several studies have shown that Vip3A proteins do not share binding sites with Cry1 or Cry2 proteins, yet their damage to the midgut epithelium resembles Cry action (Lee et al., 2006; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014). Supported by the lack of shared binding sites, transgenic corn and cotton varieties pyramided with Cry1, Cry2, and Vip3A genes are currently commercialized in several countries. Knowledge of the biochemical and genetic factors involved in resistance is crucial to design management practices that delay the appearance of resistance and allow its rapid detection and ways to overcome it. The genetic potential to evolve resistance to Vip3A has already been shown in some laboratory-selected insect species such as *H. virescens* (Pickett, 2009),

Spodoptera litura (Barkhade and Thakare, 2010), *Helicoverpa armigera* and *Helicoverpa punctigera* (Mahon et al., 2012), *Spodoptera frugiperda* (Bernardi et al., 2016; Yang et al., 2018), and *Helicoverpa zea* (Yang et al., 2020). However, the biochemical basis of resistance to Vip3A has only been studied in a laboratory-selected colony of *H. armigera*, for which alteration of binding sites was not the cause of resistance (Chakroun et al., 2016).

In the present study, we aimed to determine the biochemical basis of >2000-fold resistance to Vip3A in a *H. virescens* colony (Vip-Sel). In a previous study with this colony, resistance was shown to be polygenic, conferring little cross-resistance to Cry1Ab and no cross-resistance to Cry1Ac (Pickett et al., 2017). A transcriptomic analysis detected significant differences in gene expression compared to a susceptible strain, with 420 over-expressed and 1569 under-expressed genes in Vip-Sel (Ayra-Pardo et al., 2019). Results herein support that Vip3Aa binding is not significantly altered in Vip-Sel compared to susceptible *H. virescens* and that membrane bound alkaline phosphatase (mALP) is not involved in Vip3Aa binding.

Materials and Methods

Insects

Two colonies of *H. virescens* originating from the same field population collected in Arkansas (USA) were used in this study: Vip-Sel (Vip3Aa-resistant) and Vip-Unsel (Vip3Aa susceptible). The process of selection of the Vip-Sel colony with Vip3Aa has been previously described (Pickett, 2009; Pickett et al., 2017). After 13 generations of selection, the LC_{50} of the Vip-Sel colony was 2300 μg/mL, representing a 2040-fold resistance ratio relative to the control Vip-Unsel colony. Both colonies were reared at the Imperial College London, Silwood Park campus (UK), and frozen larvae were sent for analysis to the Universitat de València (Spain).

BBMV Preparation and Enzyme Activity Assays

Brush border membrane vesicles (BBMV) from 3rd instar *H. virescens* larval midguts from Vip-Sel and Vip-Unsel colonies were prepared according to the differential magnesium precipitation method (Wolfersberger et al., 1987). Isolated BBMV were flash frozen in liquid nitrogen and kept at −80 °C until used. The protein concentration of the BBMV preparations was determined by the method of Bradford using bovine serum albumin (BSA) as a standard (Bradford, 1976). Alkaline phosphatase (ALP) and leucine aminopeptidase (APN) activities were used as brush border membrane enzymatic markers to determine the quality of the BBMV preparations (Hernández et al., 2004). Specific ALP activity was determined by chromogenic detection of *p*-nitrophenyl phosphate (*p*NPP) substrate hydrolysis into *p*-nitrophenol, and specific APN activity was detected by hydrolysis of L-leu-*p*-nitroanilide substrate into *p*-nitroanilide. In both cases, chromogenic variation was measured on 1 μg of either BBMV or midgut homogenate at 405 nm (Infinite m200, Tecan, Mannedorf, Switzerland). Two different batches of BBMV were used and all enzymatic activity assays were performed in triplicate. Means values for enzyme activities from Vip-Unsel and Vip-Sel were compared by

Student's *t*-test at a 5% level of significance. For measuring specific ALP enzymatic activity in cultured Sf21 cells, a 1.6-mL suspension of each cell type (non-transfected, transfected with empty plasmid, and transfected with plasmid with *HvmALP1*) was used. Culture cells were centrifuged, washed twice with 300 µL of phosphate buffered saline (PBS) and then resuspended in 50 µL of PBS. Protein concentration was determined by the method of Bradford and specific ALP activity measured as above.

Vip3Aa Protein Expression and Purification

The Vip3Aa16 (Vip3Aa) protein (NCBI Accession No. AAW65132) was overexpressed in recombinant *Escherichia coli* BL21 carrying the *vip3Aa16* gene. Protein expression and lysis was carried out following the conditions described elsewhere (Abdelkefi-Mesrati et al., 2009). Soluble Vip3Aa in the cell lysate was purified by two different methodologies. For binding and cell viability assays, Vip3Aa was partially purified by isoelectric point precipitation (IPP), activated with trypsin treatment and further purified by anion-exchange chromatography, as previously described (Chakroun and Ferré, 2014). For ligand assays, affinity chromatography purification was carried out using a HiTrap chelating HP column (GE Healthcare, Uppsala, Sweden) and then activated with trypsin, as described in Chakroun and Ferré (2014).

Vip3Aa Labelling and Binding Experiments

Purified Vip3Aa activated protein (25 µg) was labelled with 0.5 mCi of ^{125}I using the chloramine T method (Chakroun and Ferré, 2014). The labelled protein was separated from the excess of free ^{125}I in a PD10 desalting column (GE Healthcare, Uppsala, Sweden) and the purity of the ^{125}I-labelled Vip3Aa was checked by autoradiography. The specific activity of the labelled protein was 2.2 mCi/mg. Binding assays were performed as described by Chakroun and Ferré (2014). Prior to being used, BBMV were centrifuged and resuspended in binding buffer (20 mM Tris, 150 mM NaCl, 1 mM $MnCl_2$, pH 7.4, 0,04% Blocking reagent from Sigma Aldrich, St. Louis, MO, USA). Competition binding

experiments were conducted by incubating 1.4 µg of BBMV protein with 0.65 nM ^{125}I-Vip3Aa in a final volume of 0.1 mL of binding buffer for 90 min at 25 °C in the presence of increasing amounts of unlabelled Vip3Aa. After incubation, samples were centrifuged at 16,000× *g* for 10 min and the pellet was washed once with 500 µL of ice-cold binding buffer. Radioactivity retained in the pellet was measured in a model 2480 WIZARD^2 gamma counter. Data from the competition experiments were analyzed to determine equilibrium binding parameters, dissociation constant (K_d), and concentration of binding sites (R_t) using the LIGAND software (Munson and Rodbard, 1980).

Western and Ligand Blotting

For the detection of ALP proteins in BBMV by Western blotting, BBMV (20 µg) were suspended in ice-cold PBS and heat denatured before separation on a SDS–10% PAGE gel. The resolved BBMV proteins were transferred to a nitrocellulose filter (Protran 0.45 µm NC, GE Healthcare, Uppsala, Sweden) using a BioRad Mini Trans-Blot system (Bio-Rad, Hercules, CA, USA) at 4 °C in blotting buffer (39 mM Glycine, 48 mM Tris-HCl, 0.037% SDS, 10% methanol, pH 8.5) for 1 h at constant voltage (100 V). After transfer, the nitrocellulose filter was blocked in blocking buffer (PBS, 0.1% Tween 20, 5% skimmed milk powder) overnight at 4 °C. After blocking and washing with PBST (PBS, 0.1% Tween 20) three times (5 min each), incubation with primary antibody against the membrane-bound form of ALP from *Anopheles gambiae* (generously provided by M. Adang, University of Georgia, USA) was performed for 90 min at a 1:5000 dilution at room temperature (RT). The membrane was then washed with PBST three times for 5 min each and then incubated with secondary antibody (goat anti-rabbit conjugated to horseradish peroxidase (HRP) at a 1:10,000 dilution) for 1 h at RT. After being washed with PBST three times for 5 min each, the membrane was developed using enhanced chemiluminescence (ECL Prime Western Blotting detection reagent, GE Healthcare, Uppsala, Sweden) in an ImageQuant LAS 4000 (GE Healthcare, Uppsala, Sweden), according to the manufacturer's instructions.

Ligand blotting for the detection of BBMV proteins binding Vip3Aa protein was performed with BBMV proteins resolved and immobilized as described previously for Western blotting. The nitrocellulose membrane was blocked for 1 h at 4 °C in blocking buffer (5% skimmed milk), and after three washes for 5 min each with PBST buffer, it was incubated overnight at 4 °C with blocking buffer (1% skimmed milk) supplemented with affinity chromatography-purified Vip3Aa at a final concentration of 4 μg/mL. After washing with PBST three times for 5 min each, the membrane was incubated with primary antibody against Vip3Aa at a 1:5000 dilution for 1 h at RT. After three washing steps with PBST (5 min each), membranes were incubated with secondary antibody (goat anti-rabbit conjugated to HRP) for 1 h at RT. To visualize the marker, Precision Protein™ Streptactin-HRP conjugate (Bio-Rad, St. Louis, MO, USA) was used following the manufacturer's instructions. Upon washing three times (5 min each) with PBST, the membrane was developed as described for Western blotting.

Proteomic Analysis

After resolving BBMV proteins from Vip-Sel and Vip-Unsel colonies by SDS–10% PAGE, the gel was stained with Coomassie blue (Thermo Scientific™, Waltham, MA, USA). The band corresponding to the expected molecular weight of ALP (~66 kDa) was cut out and subjected to analysis by nano-electron spray ionization (nano-ESI) followed by tandem mass spectrometry (qQTOF) in a 5600 TripleTOF (AB Sciex, Madrid, Spain) system. Results were analyzed with ProteinPilot v5.0 software and the relative amount of the proteins detected was estimated using the exponentially modified protein abundance index (emPAI) as described elsewhere (Ishihama et al., 2005).

RT-qPCR

Relative expression levels for *HvmALP* and *HvmALP2* isoforms (accession numbers FJ416470 and FJ416471, respectively) were

determined by reverse transcription quantitative polymerase-chain reaction (RT-qPCR). For this purpose, total RNA of dissected midguts from both colonies (Vip-Unsel and Vip-Sel) was isolated using RNAzol (MRC Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Each RNA (1 μg) was reverse-transcribed to cDNA using random hexamers and oligo (dT) by following the instructions provided in the Prime-Script RT Reagent Kit (Perfect Real Time from TaKaRa Bio Inc., Otsu Shiga, Japan). RT-qPCR was carried out in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Reactions were performed using 5× HOT FIREpol EVAGreen qPCR Mix Plus (ROX) from Solis BioDyne (Tartu, Estonia) in a total reaction volume of 25 μL. Specific primers for *HvmALP1*, *HvmALP2* and *Rps18*(endogenous control) genes were as described elsewhere (Jurat-Fuentes et al., 2011). The REST MCS software was used for gene expression analysis (Pfaffl et al., 2002).

Expression Vector Construction

The full-length *HvmALP1* transcript was amplified from cDNA of *H. virescens* larvae and cloned into pET30a as described in Perera et al. (2009). Purified plasmid DNA was digested with *Eco*RI and *Not*I to excise the full-length sequence and ligate it in frame into *Eco*RI-*Not*I sites of the pIZT/V5His vector (Thermo Scientific™, Waltham, MA, USA), to generate the pIZT/V5His/*HvmALP1* construct. Ligation products were transformed into *E. coli* strain DH5α and transformants checked for correct insertion by sequencing (University of Tennessee Sequencing Facility, Knoxville, TN, USA). Purified plasmid was used to transform *E. coli* strain DH10β and liquid cultures of LB medium supplemented with Zeocin (25 μg/mL) were used to amplify the vector. To purify the plasmids for transfection, the NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany) was used. Double digestion with *Eco*RI and *Not*I (which cleaved the full-length *HvmALP1* insert) and 1% agarose gel electrophoresis were performed to check plasmid and/or insert integrity. The concentration of plasmid DNA was measured with a Thermo Scientific™ Nanodrop™ 2000 Spectrophotometer.

Transient Expression of HvmALP1 in Sf21 Cells

Cultured Sf21 insect cells, originally derived from *S. frugiperda*, were maintained in 25 cm^2 tissue culture flasks (Nunc T25 flasks, Thermo Scientific™, Waltham, MA, USA) at 25 °C with 4 mL of Gibco® Grace's Medium (1×) (Life Technologies™, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS). For transient expression, cells were seeded on 12-well plates with the same medium without FBS at ca. 70% confluency and transfected with 0.5 µg of the pIZT/V5His/*HvmALP1* or pIZT/V5His plasmid using Cellfectin® II Reagent (Thermo Scientific™, Waltham, MA, USA), following manufacturer's instructions. Five hours post-transfection, the medium was replaced with fresh medium containing 10% FBS. After 24 h, cells were examined using a confocal microscope (Olympus, FV1000MPE, Tokyo, Japan) equipped with the appropriate filter for green fluorescent protein (GFP) detection as transfection marker. The enzymatic activity of alkaline phosphatase was then measured as explained above.

Cell Viability Assays

Viability of transfected Sf21 cells exposed for 24 h to Vip3Aa was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Preliminary assays were performed to determine a final Vip3A concentration of 300 µg/mL as resulting in ~50% loss of viability in the control cell line (data not shown). Briefly, cells (100 µL per well) were transferred to 96-well ELISA plates (flat bottom) and incubated at 25 °C for at least 45 min. Then, 10 µL of trypsin-activated Vip3Aa toxin was added to each well (300 µg/mL final concentration). As negative and positive controls, 10 µL of Tris buffer (Tris 20 mM, NaCl 150 mM, pH 9) and 10 µL of 2% Triton X-100 were used, respectively. After 24 h of incubation at 25 °C, cell viability was assessed by applying 20 µL of CellTiter 96® AQueous One Solution Reagent (Promega, Madison WI, USA) to each well and incubating for 2 h at 25 °C. Absorbance was measured at 490 nm (Infinite m200, Tecan, Mannedorf, Switzerland). The percentage of viable cells was obtained as described in Chapter 1. Mean values in the

transfected cells against the non-transfected cells were compared by Student's *t*-test at 5% level of significance.

Results

Vip3Aa Binding to Midgut Brush Border Membrane Vesicles (BBMV)

In testing whether binding of Vip3Aa was altered in larvae from the Vip3A-resistant (Vip-Sel) compared to the reference susceptible (Vip-Unsel) colony, we measured binding of radiolabelled Vip3Aa to BBMV from the two colonies. Binding analyses showed specific Vip3Aa binding for BBMV from both colonies, with similar homologous competition curves (**Figure 36a**). A high percentage (35–40% of the input labelled toxin) of non-specific binding, i.e., not blocked by high concentrations of unlabelled Vip3Aa competitor, was detected, as previously reported (Chakroun and Ferré, 2014; Chakroun et al., 2016). The K_d and R_t values estimated from the competition curves (Table 3) indicated that Vip3Aa binds with low affinity to a high number of binding sites in BBMV from *H. virescens*. No major differences were found for these equilibrium binding parameters between the two *H. virescens* colonies, suggesting that binding alteration is not mechanistically related to Vip3Aa resistance in Vip-Sel.

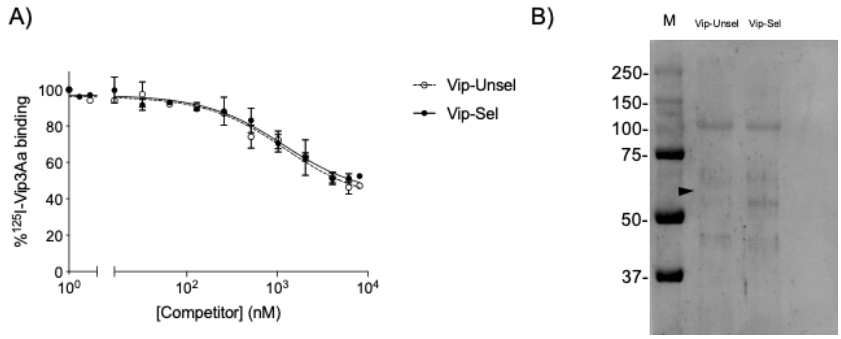

Figure 36. Analysis of ^{125}I-Vip3Aa binding to BBMV from susceptible (Vip-Unsel) and resistant (Vip-Sel) colonies of *H. virescens*. (**a**) Homologous competition binding assays of BBMV from the two colonies with ^{125}I-Vip3Aa, using increasing concentrations of unlabelled Vip3Aa as a competitor. Each data point represents the mean of two replicates performed in technical duplicates (±SEM). (**b**) Ligand blot of BBMV proteins from Vip-Unsel and Vip-Sel colonies probed with Vip3Aa. Lane M, protein molecular weight marker (in kDa) (Precision Plus Protein ™ Dual Color Standards, Bio-Rad, St. Louis, MO, USA). The black arrow indicates expected molecular weight of mALP (*ca*. 66 kDa).

Table 3. Equilibrium K_d (dissociation constant) and R_t (concentration of binding sites) binding parameters estimated from Vip3Aa homologous competition assays with BBMV from resistant (Vip-Sel) and susceptible (Vip-Unsel) *H. virescens* insects.

	Mean ± SEM [1]	
Strain	*Kd* (nM)	*Rt* (pmol/mg) [2]
Susceptible	138 ± 18	443 ± 66
Resistant	161 ± 34	443 ± 109

[1] Values are the mean of two replicates. [2] Values are expressed in picomoles per milligram of BBMV protein.

Reduced ALP Levels in the Vip3Aa-Resistant H. virescens Colony

During the evaluation of BBMV quality, we determined and compared the specific activities of alkaline phosphatase (ALP) and aminopeptidase-N (APN) as brush border membrane marker enzymes in midgut homogenates and BBMV preparations from Vip-Unsel and Vip-Sel colonies (**Figure 37**). The specific APN activity in midgut homogenates from both colonies was around 12 mU/mg, while in the BBMV preparations it was around 70 mU/mg, indicating an enrichment of APN activity of around 5.8 folds. Importantly, no significant differences (Student's *t*-test, $p > 0.05$) in APN activity were observed between the midgut homogenates or BBMV from Vip-Unsel and Vip-Sel colonies. In agreement with the 5.8-fold enrichment value from APN activity comparisons, specific ALP activity was 7.44 mU/mg in midgut homogenates and 42.5 mU/mg in the BBMV from the Vip-Unsel colony. In contrast, dramatically reduced ALP activity was detected in both midgut homogenate (1.15 mU/mg) and BBMV (1.88 mU/mg) samples from the Vip-Sel colony. While unexpected, this observation is in line with reports of reduced ALP levels in Cry1-resistant lepidopteran species, including *H. virescens* (Caccia et al., 2012; Guo et al., 2015; Jakka et al., 2016; Jurat-Fuentes et al., 2011). Consequently, we further explored the extremely reduced ALP activity in Vip-Sel to determine whether it was due to a loss of enzymatic function or reduced gene expression.

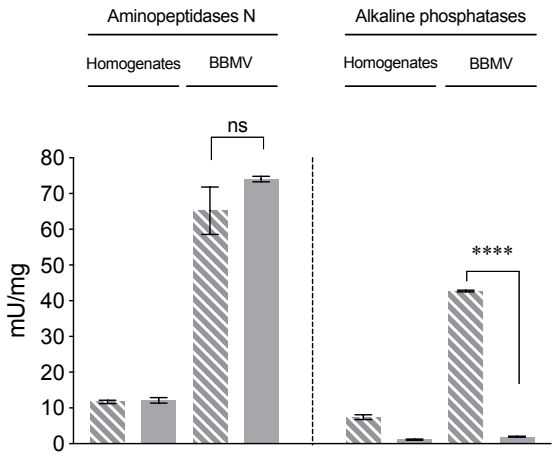

Figure 37. Enzymatic activities in homogenates and BBMV from the two colonies of *H. virescens* (dashed-grey bars: Vip-Unsel; grey bars: Vip-Sel). Each bar represents the mean of three replicates (±SEM). Asterisks represent significant difference (Student's *t*-test, **** $p < 0.0001$).

Electrophoretic comparison of BBMV proteins from the two *H. virescens* colonies showed a protein band of ~66 kDa for the Vip-Unsel colony that was almost imperceptible in the BBMV from the Vip-Sel colony (**Figure 38a**). Western blotting indicated the presence of ALP in the ~66-kDa protein band, and confirmed the highly reduced levels of this protein in the Vip-Sel colony (**Figure 38b**). The composition of the ~66-kDa protein band and its relative abundance in the two *H. virescens* colonies were determined by liquid chromatography coupled to mass spectrometry (LC-MS) analysis. The spectra for the most abundant protein detected and identified in the ~66-kDa band matched to membrane-bound alkaline phosphatase (mALP) from *H. virescens* (Genbank Accession No. ABR88230). According to the exponentially modified protein abundance index (emPAI) expressing the proportional protein content in a protein mixture, the abundance ratio of mALP between Vip-Unsel and Vip-Sel was 22.7 folds.

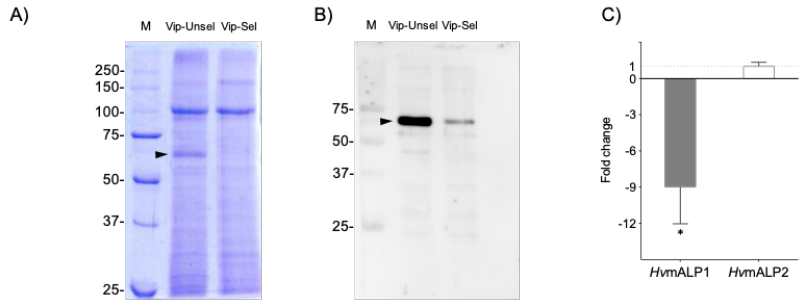

Figure 38. Analysis of membrane ALP levels in the susceptible (Vip-Unsel) and resistant (Vip-Sel) colonies of *H. virescens*. (**a**) Protein gel electrophoresis (SDS–PAGE) of BBMV from the two colonies. (**b**) Western blot performed with anti-ALP antibody against BBMV from the two colonies. The black arrow indicates mALP (ca. 66 kDa). Lane M, protein marker (molecular weight in kDa). (**c**) Membrane ALP expression levels in Vip-Sel colony using transcript levels in Vip-Unsel colony as a reference. Fold changes calculated by REST-MCS Software. Bars represent the mean of three independent experiments (±SD, * $p < 0.05$).

To test if the reduced mALP protein levels in Vip-Sel were controlled at the transcriptional level, we performed real-time quantitative PCR (RT-qPCR) with mRNA extracted from total RNA from the two colonies. Transcript levels for two *H. virescens* mALP genes, *HvmALP1* (Accession No. FJ416470.1) and *HvmALP2* (Accession No. FJ416471.1), were analyzed. Compared to insects from the Vip-Unsel colony, larvae from the Vip-Sel colony had significant (p-value < 0.05) nine-fold downregulation of the *HvmALP1* gene, while transcript levels for *HvmALP2* were not different between colonies (**Figure 38c**). These results support that reduced ALP enzyme activity in BBMV from Vip-Sel compared to Vip-Unsel is due to reduced expression of *HvmALP1* in the Vip-Sel colony.

Functional Role of HvmALP1 in Vip3Aa Binding

Since *H. virescens* ALP was proposed to play a role in binding of Cry1 proteins to the midgut membrane (Jurat-Fuentes and Adang, 2004), we used ligand blotting to test whether mALP was involved in Vip3Aa binding. Binding of Vip3Aa to blots of resolved BBMV

proteins was detected with anti-Vip3Aa antisera. No differences in the Vip3Aa-binding band pattern were detected between both colonies, in agreement with the binding results with radiolabelled Vip3Aa. However, no Vip3Aa binding was observed at the mALP position (~66 kDa) (**Figure 36b**). To further discard mALP as a functional Vip3Aa receptor, we cloned and transiently expressed the *HvmALP1* gene in cultured (Sf21) insect cells and performed cell viability tests after challenge with Vip3Aa. Transfection was successful, as transfected cells showed ~5-fold increased specific ALP activity compared with non-transfected cells or cells transfected with the empty plasmid (**Figure 39a**). However, after a challenge with Vip3Aa, the viability of transfected cells was not significantly different (Student's *t*-test; $p > 0.05$) from that of the control cells (**Figure 39b**), confirming that mALP does not serve as a functional receptor for Vip3Aa during the toxicity process.

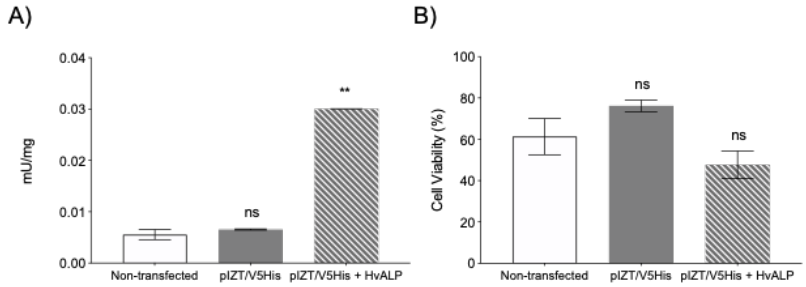

Figure 39. Specific ALP enzymatic activity and viability assays of Sf21 cells producing the HvmALP1 isoform. (**a**) Alkaline phosphatase enzymatic activity on non-transfected cells (empty bars), cells transfected with empty plasmid (grey bars, and plasmid with *HvmALP1* (dashed-grey bars). (**b**) Cell viability after 24 h of Vip3Aa intoxication (300 µg/mL final concentration) on the same three cell types. Each value represents the mean (±SEM). Means were compared by Student's *t*-test (** $p < 0.01$).

Discussion

The use of resistant insect strains isolated from the field or selected in the laboratory has been a powerful tool to understand the biochemical and genetic bases of resistance to Bt insecticidal proteins. Many studies have found that the alteration of membrane receptors is a common mechanism conferring high levels of resistance to Cry proteins (Ferré and Van Rie, 2002; Ferré et al., 2008; Peterson et al., 2017). In the case of Cry1 proteins, an important body of literature identifies aminopeptidase N, ABC transporters, cadherins and membrane alkaline phosphatases as main receptors, and identifies their alterations in association with resistance (Pigott and Ellar, 2007; Sato et al., 2019). In contrast, three candidate receptors have been proposed for Vip3A proteins, including the *Spodoptera spp*. ribosomal protein S2 (Singh et al., 2010), the fibroblast growth factor receptor-like protein (Jiang et al., 2018a) and the scavenger receptor class C-like protein (Jiang et al., 2018b), yet their role in resistance has not been established. In the present work, we aimed to determine whether alteration of membrane receptors was the basis for the observed 2040-fold resistance to Vip3Aa in the Vip-Sel colony of *H. virescens*. Results from binding assays with BBMV and radiolabelled Vip3Aa did not detect significant differences between the susceptible and resistant colonies, suggesting no involvement of binding site alteration in resistance. This conclusion was further supported by results from ligand blotting, where no differences between the binding patterns of Vip3Aa to BBMV proteins from the two colonies were observed. Similar results were reported for a laboratory-selected Vip3A-resistant colony of *H. armigera* (Chakroun et al., 2016), suggesting that high levels and narrow spectrum of Vip3A resistance may develop by mechanisms other than alteration of Vip3Aa binding sites.

Even though differences in binding were not found, a dramatic reduction in the ALP enzymatic activity was detected in midgut samples from the resistant compared to susceptible colony. Western blotting and RT-qPCR analyses showed that the decreased activity was

due to a reduction in the amount of mALP protein, which was controlled at the transcriptional level, in agreement with a previous study (Ayra-Pardo et al., 2019). Downregulation or reduced levels of mALP in the midgut membrane have been observed as a common phenomenon in resistance to Cry1Ac in *H. virescens* (Jurat-Fuentes and Adang, 2011), *Helicoverpa zea* (Caccia et al., 2012), *Plutella xylostella* (Guo et al., 2015), and *Helicoverpa armigera* (Jurat-Fuentes et al., 2011); to Cry1F in *S. frugiperda* (Jakka et al., 2016); to Cry1C in *Spodoptera litura* (Gong et al., 2015); and even in *Aedes aegypti* resistant to Bt subsp. *israeliensis* (Bti) (Tetreau et al., 2012). The fact that Cry1Ac and Cry1C do not share binding sites (Jakka et al., 2015) suggests that the role of ALP downregulation in resistance may not be related to reduced Cry binding, but may represent a physiological response to resistance. In agreement with this hypothesis, susceptibility of Sf21 cells expressing HvmALP1 was not significantly different to Vip3Aa, supporting that ALP is not a functional receptor for Vip3Aa in *H. virescens*. In addition, in a Cry1Ac-resistant strain of *P. xylostella*, altered expression of different genes (including the *PxmALP*) was reported to be *trans*-regulated by upregulation of a mitogen-activated protein kinase, which was linked to resistance (Guo et al., 2015). Similar *trans*-regulation of genes involved in resistance to Bt has also been observed for APN in *Trichoplusia ni* resistant to Cry1Ac (Tiewsiri and Wang, 2011) and *Ostrinia nubilalis* resistant to Cry1Ab (Coates et al., 2013), and for both APN and an ABCC transporter in *Bombyx mori* resistant to Cry1Ab (Chen et al., 2014). Further research should test the involvement of this control mechanism in downregulation of *mALP* in Vip-Sel and other Bt-resistant colonies.

The two studies so far focused on the underlying mechanism of resistance to Vip3Aa proteins share a similar feature in that in vitro binding is not reduced (Chakroun et al., 2016 and the present work). According to these results, mechanisms other than binding site alteration seem to be responsible for conferring specific and high-level resistance to Vip3A. This contrasts with the fact that the alteration of membrane receptors is a common mechanism conferring high levels of resistance to Cry proteins. Better knowledge of the mode of action of

Vip3A proteins will help shed light on the biochemical basis of resistance to these proteins.

To sum up, the results herein show lack of significant Vip3Aa binding alterations in a resistant colony of *H. virescens*. These observations are in contrast to most cases of high levels of resistance to Cry proteins for which decreased binding is commonly detected. In addition, this study provides evidence of downregulation of membrane bound alkaline phosphatase (mALP) in the Vip3Aa-resistant colony, although results do not support involvement of mALP as a receptor for the Vip3A protein.

CHAPTER 7

Response mechanisms of invertebrates to *Bacillus thuringiensis* and its pesticidal proteins

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Introduction

Several pathogenic bacteria produce virulence factors against their host to help them replicate and disseminate by eluding host defenses. The pore-forming toxins (PFT) are one of the most common virulence factors among bacterial pathogens (Alouf, 2003; Gonzalez et al., 2008). PFT production not only occurs in human bacterial pathogens (including *Streptococcus pneumoniae*, group A and B streptococci, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium tuberculosis*) but also in insect and nematode bacterial pathogens such as *Bacillus thuringiensis*, *Lysinibacillus sphaericus*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Photorhabdus luminescens* (Los et al., 2013; Ffrench-Constant et al., 2007; Chattopadhyay et al., 2004). PFT's primary action is to disrupt host cell membranes by forming lytic pores (Gonzalez et al., 2008; Bischofberger et al., 2009; Iacovache et al., 2010). However, its action may involve other effects in addition to pore formation (Los et al., 2013).

Bacillus thuringiensis (Bt) produces proteinaceous virulence factors that are toxic to several insect, mite, and nematode species (Schnepf et al., 1998; Van Frankenhuyzen, 2009; Palma et al., 2014). Currently, two different *B. thuringiensis* PFT groups (the crystal proteins [Cry] and the vegetative insecticidal proteins [Vip3]) are widely used to control insect pests both in formulated sprays and in transgenic crops (the so-called Bt crops) (Crickmore, 2006; Sanchis, 2011; Romeis et al., 2019). Cry proteins are produced during the sporulation phase as crystalline inclusions, whereas Vip3 proteins are produced and secreted during the bacterial vegetative growth phase. Due to the commercial interest of these proteins, understanding their mode of action has gained considerable attention (Vachon et al., 2012; Adang et al., 2014; Jurat-Fuentes and Crickmore, 2017). To date, most studies on the Cry proteins' mode of action have been carried out with the Cry1A and Cry5B insecticidal and nematocidal proteins, respectively (Wei et al., 2003; Pardo-López et al., 2013), and with the Vip3Aa protein for the Vip3 family (Chakroun et al., 2016).

B. thuringiensis proteins are toxic by ingestion, and their target is the host midgut epithelium (Palma et al., 2014; Jurat-Fuentes and Crickmore, 2017; Chakroun et al., 2016; Ruan et al., 2015). If the protein is ingested as a crystalline inclusion (the bacterial parasporal crystal containing Cry and Cyt proteins), the crystals are solubilized to release the proteins in the protoxin form. In the host midgut, protoxins are activated by midgut proteases. The resulting activated protein must then cross the peritrophic matrix and bind to specific receptors on the midgut epithelial membrane. *B. thuringiensis* proteins oligomerize and form pores, with each pore thought to consist of four toxin monomers assembled into a pore-forming oligomer for both Cry (Gómez et al., 2002) and Vip3 proteins (Quan and Ferré, 2019; Núñez-Ramírez et al., 2020). Once the oligomer is inserted into the epithelial membrane, the membrane is disrupted, allowing gut bacteria to invade the hemolymph leading to septicemia and insect death (Adang et al., 2014; Raymond et al., 2010; Nielsen-LeRoux et al., 2012; Caccia et al., 2016).

Substantial efforts have been devoted to identify the key molecules in the interactions between *B. thuringiensis* toxins and the midgut (Pigott and Ellar, 2007; Sato et al., 2019; Huang et al., 2020), whereas less attention has been directed to studying pore formation (Vachon et al., 2012; Kirouac et al., 2002; Girard et al., 2009; González-Cabrera et al., 2006; Heckel, 2006) or how insect cells respond upon B. thuringiensis toxin intoxication. The main reasons for this are as follows: (i) interaction between *B. thuringiensis* proteins and their midgut specific receptors is considered the main specificity determinant (Jurat-Fuentes and Crickmore, 2017; Van Rie et al., 1989), and (ii) binding site alteration is the most common mechanism associated with high levels of insect resistance to Cry proteins (Ferré and Van Rie, 2002; Tabashnik and Carrière, 2017; de Bortoli and Jurat-Fuentes, 2019; Heckel, 2020; Jurat-Fuentes et al., 2021). However, insect susceptibility to *B. thuringiensis* proteins depends on binding interactions with the surrogate membrane receptors and on a series of concatenated events related to the complex mode of action (Adang et al., 2014; Jurat-Fuentes and Crickmore, 2017; Heckel, 2020).

Indeed, after the ingestion of *B. thuringiensis* and its proteins, different host response mechanisms are activated to counteract the toxic effects. A summary of the response mechanisms which have been found altered in resistant invertebrates to *B. thuringiensis* and/or its proteins is shown in Table 4. As a means of improving the efficacy of *B. thuringiensis* pesticidal proteins in controlling insect pests, several studies have been conducted to understand and characterize the repair mechanisms that allow midgut epithelium to recover after exposure to pore-forming toxins (as discussed below). However, it is worth mentioning that, in addition to Cry and Vip3 proteins, other virulence factors contribute to the *B. thuringiensis* pathogenesis. Therefore, host responses observed when only using *B. thuringiensis* toxins might be different from the ones observed when bacteria, or a mixture of spores and crystals, are used (Tetreau, 2018). Overall, host responses will vary depending on the effector, the concentration, the exposure time, and even the host used. Here, we gather and discuss the available information on invertebrate responses (with emphasis on insects and *Caenorhabditis elegans*) to *B. thuringiensis* and its toxins that may counteract the detrimental effects of *B. thuringiensis* intoxication. Some of the studies reviewed here made use of gene silencing or mutants to shed light on the role of specific genes in invertebrate defense responses (Table 5). We have defined eight categories in which the host can respond to palliate the effect of B. thuringiensis exposure, either to its pesticidal proteins or to the bacterium. The molecules involved in the binding interactions (surrogate receptors) will not be included here since that topic has been extensively reviewed (Pigott and Ellar, 2007; Sato et al., 2019). A general view of the mechanisms elicited after *B. thuringiensis* intoxication is summarized in **Figure 40**, and they are described in detail in the following sections.

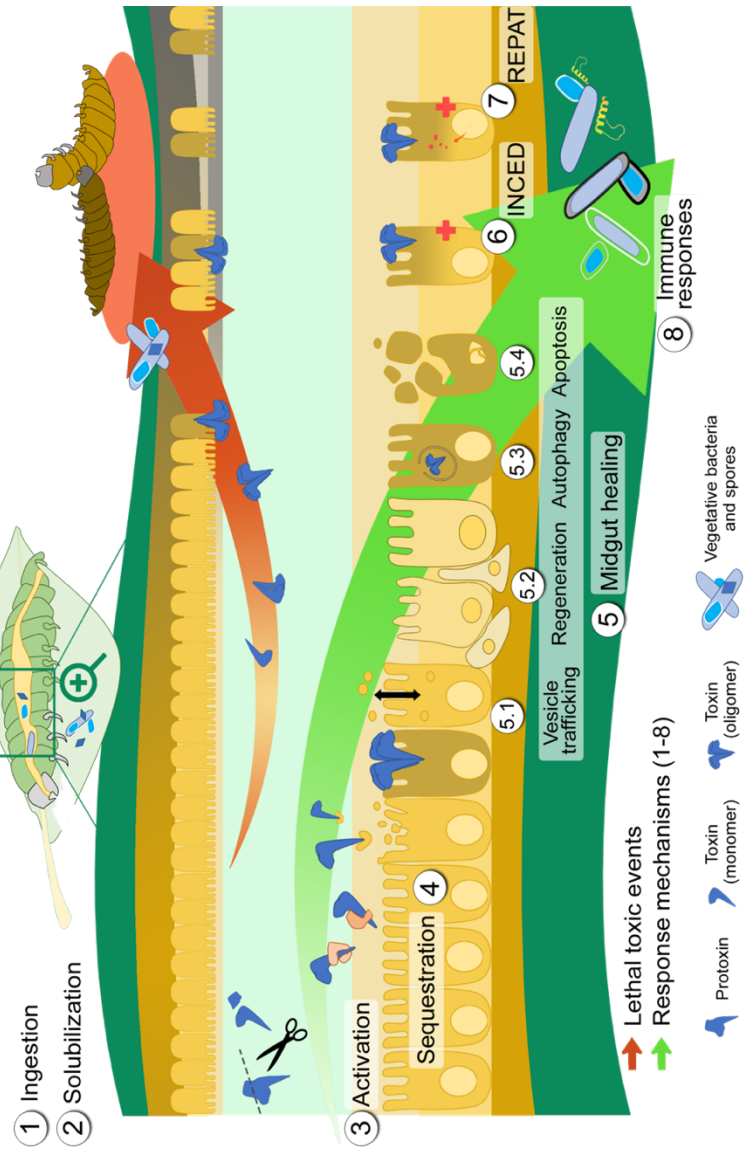

Figure 40. Schematic description of the general host response mechanisms occurring after *B. thuringiensis* intoxication. The red arrow represents the sequential steps leading to a lethal outcome. The green arrow represents the response mechanisms triggered after intoxication, which can help overcome the toxic effects after sublethal doses. The mechanisms have been classified in different groups and described in detail in the main body of this review. INCED, epithelium intrinsic cellular defense; REPAT, REsponse to PAThogens.

Table 4. Response mechanisms found altered in resistant invertebrates to *Bacillus thuringiensis* and/or its proteins

Altered mechanism	Species	Strain	Exposure condition(s)	Response	Outcome (level of resistance)	Other mechanisms studied	Reference(s)
Protoxin activation	*Plodia interpunctella* (Indianmeal moth)	133-r	Bt *aizawai*	Lack of proteinase activity	Decreased susceptibility	ND	Oppert et al., 1997
		198-r	Bt *entomocidus*		Decreased susceptibility, 11-fold Cry1Ab (protoxin over toxin)	Physiological adaptation, higher immune status	Oppert et al., 1997; Herrero et al., 2001
		Dplr	Dipel (Bt *kurstaki* HD-1)	Unknown	4-fold Cry1Ab (protoxin over toxin)	ND	Herrero et al., 2001
	Ostrinia nubilalis (European corn borer)	KS-SC-R	Dipel (Bt *kurstaki* HD-1)	Reduced proteinase activity	70-fold to Dipel, 200-fold to Cry1Ab protoxin, 500-fold to Cry1Ac protoxin	Ingestion/higher avoidance ability	Huang et al., 2001, Li et al., 2004
	Heliothis virescens (Tobacco Budworm)	YHD2-B	MVP (compound containing Cry1Ac protoxin), and Cry1Ac	Reduced proteinase activity	> 2,000-fold Cry1Ac, 9.5-fold Cry2Aa	Loss of binding	Karumbaiah et al., 2007
		CXC	Cry1Ac, Cry2Aa		290-fold Cry1Ac, > 250-fold Cry2Aa	Enhanced regeneration/healing; reduced ALP	
	Mythimna unipuncta (True Armyworm)	MR	Bt maize (MON810)	Reduced proteinase activity	Resistance to Bt maize and Cry1Ab protoxin	ND	González-Cabrera et al., 2013
	Helicoverpa armigera (Corn Earworm)	Akola-R	Cry1Ac protoxin	Downregulation of a protease, improper protoxin processing	72-fold resistance to Cry1Ac	ND	Rajagopal et al., 2009
		LF5		Downregulation of trypsin	110-fold to Cry1Ab protoxin, 39-fold to Cry1Ab toxin	ND	Liu et al., 2014
		SP85	Vip3A	Slower activation	Vip3Aa protoxin resistance	ND	Chakroun et al., 2016
		XJ10	Cry1Ac protoxin	Reduced trypsin levels	147-fold to Cry1Ac protoxin, 45-fold to Cry1Ac toxin	ND	Jin et al., 2019
	Helicoverpa zea (Cotton Bollworm)	GA	Cry1Ab corn	Reduced proteinase activity	7.8-fold to MVPII (Cry1Ac protoxin-based compound)	ND	Zhang et al., 2019
		GA-R	Cry1Ac		110-fold to MVPII (Cry1Ac protoxin-based compound)	ND	
	Plutella xylostella (Diamondback Moth)	SZ-R			662-fold to Cry1Ac protoxin, 442-fold to Cry1Ac toxin	ND	Gong et al., 2020
		R	Bt *kurstaki*		ND	ND	Talaei-Hassanloui et al., 2013
Toxin sequestration (in the midgut lumen)	*Helicoverpa armigera* (Corn Earworm)	"Silver selected"	Cry1Ac	Sequestration of Cry1Ac protoxin and toxin by esterases	275-fold to Cry1Ac toxin and resistance to transgenic cotton (Ingard)	ND	Gunning et al., 2005
		R (ISOC4)	*B. thuringiensis* HD73, Cry1Ac	Insoluble aggregate formation of Cry1Ac through hexamerin production	100-fold Cry1Ac and resistance to transgenic cotton	Humoral response (higher melanization rates); transgenerational immune priming (TGIP)	Ma et al., 2005
	Spodoptera exigua (Beet armyworm)	Xen-R	Bt formulation, Xentari	Shedding of cell surface proteins (APN)	> 1,000-fold resistance to Xentari	*repat* (response to pathogens) and *arylphorin* genes altered	Hernández-Martínez et al., 2010

	Helicoverpa zea (Cotton Bollworm)	AR1	Cry1Ac	High alkaline phosphatase levels in midgut lumen	> 100-fold Cry1Ac toxin	ND	Caccia et al., 2012
Midgut regeneration	*Heliothis virescens* (Tobacco Budworm)	CP73-3	Cry1Ac	Better replacement of damaged cells at sublethal concentrations of Cry1Ac	20-fold Cry1Ac toxin	Altered proteinase activity	Martinez-Ramirez et al., 1999
INCED (MAPK)	*Plutella xylostella* (Diamondback Moth)	DBM1Ac-R	Bt *kurstaki*, Cry1Ac	Transregulation of expression of midgut receptors by MAPK; activation of MAPK by insect hormones (20E, JH)	> 3,500-fold Cry1Ac	High phenol oxidase activity	Guo et al., 2015, 2020 Liu et al., 2019
		SZ-R	Cry1Ac		458-fold Cry1Ac	ND	
		SH-R	Bt *kurstaki*		1,890-fold Bt *kurstaki*	ND	
		NIL-R	Bt *kurstaki*, Cry1Ac		> 3,900-fold Cry1Ac, > 2,800-fold Bt *kurstaki*	ND	
Innate response	*Galleria mellonella* (Greater Wax Moth)	R line	Bt spore-crystal mixture	Antimicrobial factor secretion	8.8-fold Bt	ND	Dubovskiy et al., 2016
	Ostrinia furnacalis (Asian Corn Borer)	ACB-AhR	Cry1Ah	High phenol oxidase expression; reduced binding sites	200-fold Cry1Ah	Reduced binding sites; increased immune response	Shabbir et al., 2020
Immune priming	*Ephestia kuehniella* (Flour Moth)	"Bt-tolerant"	Delfin WG (Cry1Aa, 1Ab, 1Ac, 2Aa, spores)	Elevated immune status	7.9-fold resistant to Delfin WG	Coagulation by complex formation of lipophorin with toxin	Rahman et al., 2004, 2007
	Galleria mellonella (Greater Wax Moth)	-	Previous injection of heat-killed Bt	Elevated immune status	Higher survival to *Photorhabdus luminescens* TT01	ND	Wu et al., 2014
		-	Previous exposure to live Bt		Overcoming lethal injection to Bt, specific	ND	Taszlow et al., 2017
	Tribolium castaneum (Red Flour Beetle)	-	Previous exposure to supernatant from toxic Bt culture	Extensive transcriptomic changes of immune genes after Bt spore exposure	ND	ND	Greenwood et al., 2017
		-	Previous injection of Bt	Immune priming toward Bt	Higher survival rates	ND	Medina Gomez et al., 2018
		-		Strong specific priming effect toward Bt		ND	Ferro et al., 2019
Transgenerational immune priming (TGIP)	*Tenebrio molitor* (Mealworm Beetle)	-	Injection of inactivated Bt to females	Maternal transfer of antimicrobial activity (toward Gram+ bacteria) in eggs	Eggs with higher antimicrobial activity and AMP presence	ND	Dubuffet et al., 2015; Tetreau et al., 2020
	Tribolium castaneum (Red Flour Beetle)	-	Injection of Bt to males and/or females	TGIP through both maternal and paternal effect to next generations	Offspring with increased survival	ND ND ND	Roth et al., 2010 Eggert et al., 2014 Schulz et al., 2019
				TGIP transmitted to F_2 generation			

Table 5. Genes involved in different host response mechanisms whose alteration (mutations and/or silencing) modify the susceptibility to *Bacillus thuringiensis* and/or its proteins.

Altered mechanism	Species	Target	Specifications/ function of the target	Approach	Outcome (level of resistance)[b]	Reference(s)
Vesicle trafficking pathways	*Caenorhabditis elegans*	*Rab-5* and *Rab-11*	Small GTPases, intracellular vesicle transport events regulation	Knockdown by RNAi	Hypersensitivity to Cry5B	Los et al., 2011
Autophagy	*Caenorhabditis elegans*	Autophagy-related genes (*atg*)	ATG proteins, involved in the autophagy process	Knockdown by RNAi	Hypersensitivity to Cry5B	Chen et al., 2017
	Spodoptera litura Sl-HP cell line	Autophagy-related genes (*atg*): *atg13*	Involved in the autophagy process	Knockdown by RNAi	Swollen cells decreased after Cry1Ca and Cry1Ac treatment	Yang et al., 2020
	Trichoplusia ni TN5B1-4 (Hi5) cell line	Vacuolar H+-ATPase; autophagosome fusion with lysosomes	Involved in the last steps of the autophagy process	Autophagy inhibitors: bafilomycin A1 and chloroquine, respectively	Swollen cells decreased after Cry1Ca treatment	
MAPK	*Caenorhabditis elegans*	*sek-1*	MAPKK in the p38 MAPK pathway	*sek-1*(*km4*) mutant strain	10-fold more sensitive to Cry5B and hypersensitive to Cry21A	Huffmann et al., 2004
		kgb-1	JNK-like MAPK	*kgb-1* (*um3*) mutant strain	Hypersensitivity to Cry5B and Cry21A	
	Manduca sexta	*p38*	p38 MAPK	Knockdown by RNAi	8-fold higher susceptibility to Cry1Ab (mixture spores and crystals)	Cancino-Rodezno et al., 2010
	Aedes aegypti	*p38*	p38 MAPK	Knockdown by RNAi	10-fold higher susceptibility to Cry11Aa (mixture spores and crystals)	Cancino-Rodezno et al., 2010
	Chilo suppressalis	*p38*	p38 MAPK	Knockdown by RNAi	Higher susceptibility to Cry1Ca	Qiu et al., 2017
	Plutella xylostella (resistant strain)	*MAP4K4*	MAP4K, upstream of p38, JNK, and ERK MAPKs	Knockdown by RNAi	Restored susceptibility to Cry1Ac	Guo et al., 2015, 2020
ER UPR	*Caenorhabditis elegans*	*xbp-1*	Xbp-1 (X-box binding protein-1); IRE-1 RNASE; after IRE-1 RNASE action on Xbp-1 mRNA, mature Xbp-1 protein activates UPR genes	*xbp-1* (*zc12*) mutant strain	Hypersensitivity to Cry5B (6-fold)	Bischof et al., 2008
	Aedes aegypti	*ire-1*		Knockdown by RNAi	2.6-fold higher sensibility to Cry11Aa (mixture spores and crystals)	Bedoya-Pérez et al., 2013
		xbp-1			3.1-fold higher sensibility to Cry11Aa (mixture spores and crystals)	
Hypoxia response pathway	*Caenorhabditis elegans*	*egl-9*	EGL-9 protein (prolyl hydroxylase); HIF-1 (master hypoxia response activation regulator); in normal oxygen conditions, HIF-1 is degraded through EGL-9	*egl-9* mutants	3- to 5-fold resistance to Cry21A (mixture spores and crystals) and > 3-fold to Cry5B	Bellier et al., 2009
		hif-1		*hif-1*(*ia04*) mutant strain	Hypersensibility to Cry5B	
Insulin/insulin like growth factor 1 signaling pathway (IIS)	*Caenorhabditis elegans*	*daf-2*	DAF-2 (insulin-like receptor) DAF-16 (transcription factor); DAF-2 activation inhibits transcription of DAF-16 dependent genes	*daf-2 (e1370)* mutant strain	Higher survival rate to *B. thuringiensis* strain NRRL B-18247 (spore and crystal mixture) than *daf-16*(*m26*) or wild type	Hasshoff et al., 2007
					7-fold resistant to Cry21 (spore and crystal mixture), > 10-fold resistant to Cry5B	Chen et al., 2010
					Greater longevity to Cry6Aa than *daf-16*(*m26*) *daf-2*(*e1370*);*daf-16*(*m26*) or wild-type	Wang et al., 2012
		daf-16		*daf-16*(*m26*) mutant strain	ND Shorter longevity to Cry6Aa than *daf-2* (*e1370*);*daf-16*(*m26*) or wild type	Hasshoff et al., 2007; Wang et al., 2012
				daf-16(*mu86*) mutant strain	ND	Chen et al., 2010

		Double mutant: *daf-2/daf-16*		*daf-2(e1370);daf-16(m26)*	Higher survival rate to *B. thuringiensis* strain NRRL B-18247 (spore and crystal mixture) than *daf-16(m26)* or wild type, less than *daf-2(e1370)*	Hasshoff et al., 2007;
					Greater longevity to Cry6Aa than *daf-16 (m26)*, lower than *daf-2(m26)*, same as wild type	Wang et al., 2012
				daf-2(e1370);daf-16(mu86)	2.3-fold more resistant to Cry21 (spore and crystal mixture) than *daf-16(mu86)* and 2.4-fold more resistant than wild type; 4.3-fold more resistant to Cry5B than *daf-16(mu86)* and 3.7-fold more resistant than wild type	Chen et al., 2017
	Maruca vitrata	InR	Insulin receptor	Knockdown by RNAi	Increased insecticidal activity in laboratory and field conditions of Bt *kurstaki*: 50% and Bt *kurstaki* + dsInR: 80%	Al Baki et al., 2020
Innate response	*Spodoptera littoralis*	*102SI*	Immune gene	Knockdown by RNAi	5-fold mortality Xentari/Cry1Ca	Caccia et al., 2016
	Spodoptera exigua	*gloverin*	Gene encoding an AMP	Knockdown by RNAi	Increased susceptibility to *B. thuringiensis*	Hwang and Kim, 2011

Ingestion

To exert its toxic effect, *B. thuringiensis* or their virulence factors penetrate via ingestion. Therefore, a first step to evade the toxic effect of *B. thuringiensis* is feeding behavior modification. Aversive taste responses have evolved in different organisms to prevent toxic compound ingestion (Dethier, 1980; Shivers et al., 2009; Yarmolinsky et al., 2009).

Early reports stated that larvae treated with either *B. thuringiensis* proteins or a mixture of spores and crystals modified their behavior by feeding cessation or becoming less active than nontreated larvae (Dulmage et al., 1978; Retnakaran et al., 1983). Indeed, these authors suggested that feeding cessation would allow larvae to clear the *B. thuringiensis* protein by degradation and repair the midgut epithelial cells by replacing damaged cells (Retnakaran et al., 1983). In *C. elegans*, feeding behavior inhibition was also observed in response to *B. thuringiensis* protein intoxication (including Cry5B, Cry6A, and Cry21A) (Los et al., 2013; Luo et al., 2013). Interestingly, both the insulin-like receptor (Hasshoff et al., 2007; Wang et al., 2012) and the neuronal Goa (Los et al., 2013) pathways are important in the *C. elegans* feeding cessation response.

Many studies have reported that larvae from different insect species consistently preferred noncontaminated diet rather than *B. thuringiensis* toxin-treated diet when diet choice was offered (Gould et al., 1991; Berdegué et al., 1996; Stapel et al., 1998; Zhang et al., 2004; Stoops and Adler, 2006; Bowling et al., 2007; Zhao et al., 2016). These studies concluded that larvae tend to avoid food with deleterious effects, highlighting larval ability to modify their feeding behavior. In contrast, other studies did not find differences in the behavioral responses (especially *B. thuringiensis* toxins avoidance) when the larvae were placed on an untreated or a *B. thuringiensis*-treated diet (Schwartz et al., 1991; Bilbo et al., 2019).

Feeding behavior involvement has been suggested as a resistance mechanism to *B. thuringiensis* toxins (Gould et al., 1991; Stapel et al., 1998; Huang et al., 2001). However, only a few behavioral resistance studies have been conducted with *B. thuringiensis*-resistant colonies (Gould et al., 1991; Huang et al., 2001; Luong et al., 2018). A recent review claims that most studies conducted could be explained by simple aversion behaviors rather than behavioral resistance (Zalucki and Furlong, 2017). Therefore, more experimental evidence would be necessary to support feeding behavior as a *B. thuringiensis* resistance mechanism.

Crystal solubilization

The parasporal crystals produced by *B. thuringiensis* must undergo solubilization to release the Cry proteins and initiate the intoxication process. Crystal solubilization is facilitated by the physicochemical conditions (mainly pH) in the host digestive fluids (Angus, 1954). Therefore, it was claimed that only hosts producing digestive fluids capable of solubilizing crystals would be susceptible to the particular *B. thuringiensis* crystal-producing strain (Du et al., 1994; Bradley et al., 1995). Although this may be critical in determining insect susceptibility to *B. thuringiensis* toxins, to date, no reports assert that susceptible hosts are able to alter their midgut pH to overcome the toxic effects of Cry proteins.

Activation

Bacillus thuringiensis Cry and Vip3A proteins are produced in the protoxin form, which are processed by midgut proteases to render an active toxin. Hence, significant research efforts have been made to understand the role of midgut proteases in *B. thuringiensis* toxin processing (Zalunin et al., 2015). Some studies have shown that *B. thuringiensis* protein toxicity is influenced by the composition/activity of midgut proteolytic enzymes (Lambert et al., 1996; Lightwood et al., 2000; Rouis et al., 2008; Caccia et al., 2014). Indeed, Cry1C is more toxic

to the *Spodoptera littoralis* first instar than to subsequent instars due to proteolytic activity enhancement (Keller et al., 1996). The different susceptibilities of *Helicoverpa zea* and *Heliothis virescens* to Cry1Ac were correlated with the insect species' proteolytic processing capability (Abdelgaffar et al., 2019). Thus, differential sensitivity to *B. thuringiensis* pesticidal proteins among different larval instars or populations might depend on midgut protease relative production.

Several studies have related protease activity alteration with the emergence of insect resistance to *B. thuringiensis* proteins (**Figure 41**). In a *Plodia interpunctella* Cry1Ab/c-resistant colony, protoxin activation was slower due to reduced expression levels and/ or reduced midgut protease activity (Oppert et al., 1997). This was later confirmed by the resistant insects' differential susceptibility to the protoxin and activated protein forms (Herrero et al., 2001). A study on an *Ostrinia nubilalis* Dipel-resistant strain (Dipel is trademark of a *B. thuringiensis*-based insecticide) showed a relationship between reduced trypsin-like protease activity and resistance (Li et al., 2004). Similar results were found in a *H. virescens* strain resistant to Cry1Ac and Cry2Ab, with slower protoxin processing and a reduced chymotrypsin-like enzyme amount (Karumbaiah et al., 2007). In a *Mythimna unipuncta* strain resistant to Bt maize, a reduction in proteolytic enzyme activity was correlated with lesser Cry1Ab protoxin activation (González-Cabrera et al., 2013). Similarly, a *Helicoverpa armigera* Vip3Aa-resistant strain exhibited significantly slower Vip3Aa protoxin activation into toxin compared to the susceptible strain (Chakroun et al., 2016). Similar results were found in two *H. zea* Cry1Ac-resistant strains, which showed less total protease, trypsin, and chymotrypsin activities and a reduced Cry1Ac activation rate compared to the susceptible control (Zhang et al., 2019). Recently a laboratory-selected *Plutella xylostella* Cry1Ac-resistant strain with reduced caseinolytic and trypsin protease activities that affect Cry1Ac protoxin activation was characterized. Downregulation of a trypsin-like serine protease gene was genetically linked to Cry1Ac resistance, which was further confirmed by RNAi experiments (Gong et al., 2020). Midgut total proteolytic and tryptic

activities were altered in another *P. xylostella* population resistant to a *B. thuringiensis* product compared to a susceptible population (Talaei-Hassanloui et al., 2013). Other studies have also shown differential expression of trypsin-like and chymotrypsin-like proteins in Cry1Ab resistant *Ostrinia furnacalis* and *O. nubilalis* strains compared to corresponding susceptible insect species (Khajuria et al., 2009; Xu et al., 2015). Furthermore, two studies from different Cry1Ac-resistant *H. armigera* strains found strong trypsin gene downregulation (Liu et al., 2014; Jin et al., 2019). Although this alteration was genetically linked to resistance in the first study, these authors indicated that additional mechanisms were likely involved, since the larvae were also resistant to the activated form.

Another aspect implicating proteases in resistance is the occurrence of improper protoxin processing, which translates into lower relative toxicity, as demonstrated in a *H. armigera* Cry1Ac-resistant strain (Rajagopal et al., 2009). Finally, it was proposed that the overproduction of certain proteases may cause activated toxin degradation, as observed with a *H. virescens* Cry1Ac-resistant strain (Forcada et al., 1999) (**Figure 41**). Altogether, these studies show that alterations of digestive proteases may be an active insect response to *B. thuringiensis* toxicity, although the exact adaptation mechanisms remain debatable.

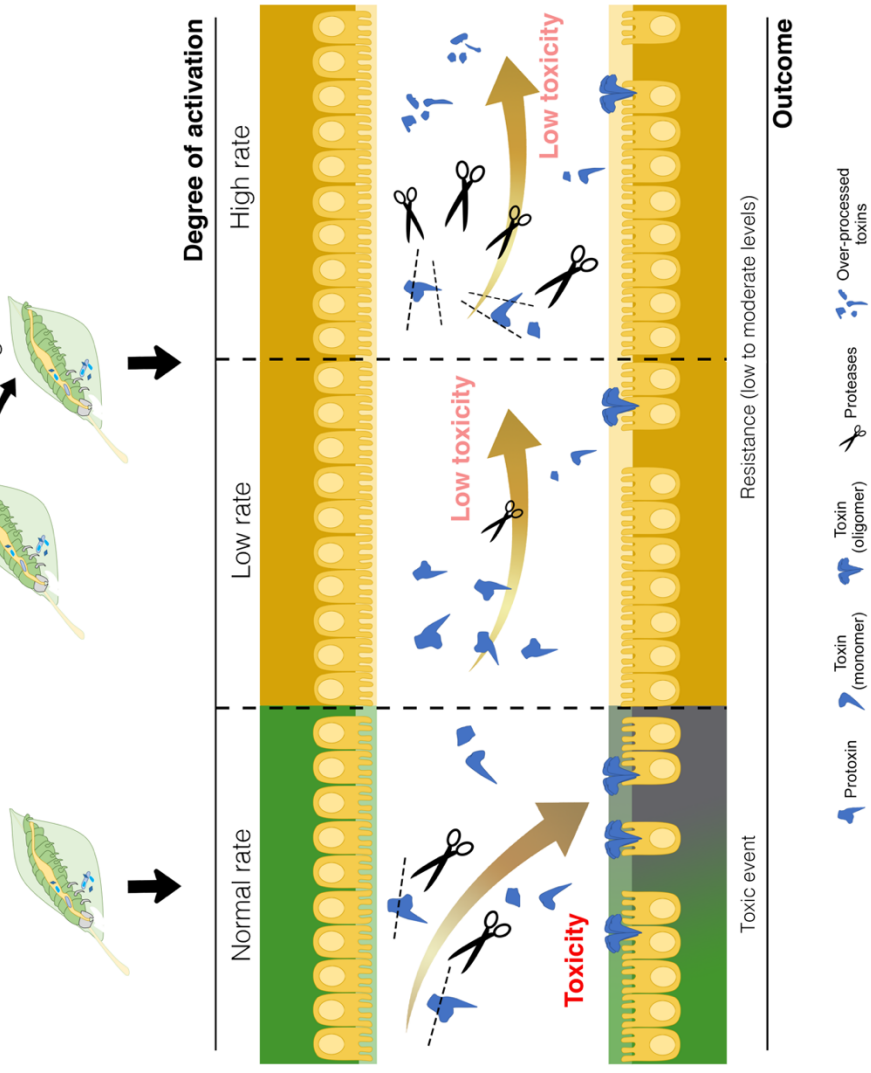

Figure 41. Alternative scenarios of processing of *Bacillus thuringiensis* toxins (Cry and Vip3) by midgut proteases after ingestion and solubilization. (Left panel) Normal activation rates cause an appropriate toxin processing that allows high concentration of activated molecules binding to membrane receptors. (Middle panel) Reduced processing activity would cause a decrease in the concentration of activated molecules, which, in turn, would result in a lower number of molecules reaching the membrane receptors. (Right panel) High protease levels can lead to toxin overprocessing, rendering inactive molecules unable to bind to membrane receptors. The outcome of the ɜ low rate› and ɜ high rate› scenarios is a decrease of toxicity.

The aforementioned ability of a susceptible species to alter the amounts of digestive proteases after *B. thuringiensis* toxin exposure was analyzed by several studies. The expression pattern of some genes encoding digestive enzyme was altered after *B. thuringiensis* challenge in several insect pests such as *Choristoneura fumiferana*, *Manduca sexta*, *Tenebrio molitor*, *O. nubilalis*, *Spodoptera exigua*, *Aedes aegypti*, *Spodoptera litura*, *Bombyx mori*, *Achaea janata*, and *P. xylostella* (Meunier et al., 2006; Van Munster et al., 2007; Oppert et al., 2012; Yao et al., 2012; Bel et al., 2013; Canton et al., 2015; Song et al., 2016; Wu and Yi, 2018; Dhania et al., 2019; Lin et al., 2020). Mainly, downregulation of serine protease encoding genes, such as trypsin and chymotrypsin was observed, as well as upregulation of different serpin encoding genes (serine protease inhibitors), when larvae were exposed to *B. thuringiensis* or to its toxins at sublethal concentrations. However, it is important to note that the results from the different transcriptional studies provided different response profiles, which could be dependent on the different experimental conditions (insect species, larval stage, intoxication method, etc.). The importance of the infection method on this experiment type was put forward in a *Tribolium castaneum* study. Larvae injected with *B. thuringiensis* subsp. *tenebrionis* showed upregulation of serine-type peptidases, endopeptidases, and endopeptidase inhibitors compared to orally infected larvae (Behrens et al., 2014). Notably, the alteration in the expression profile of genes encoding digestive proteases or their inhibitors after exposure to *B. thuringiensis* and/or to its toxins is typically related to the process of protoxin activation or degradation. However, to our knowledge, it has not been experimentally validated whether these alterations directly affect protoxin processing. Furthermore, it is also worth noting that both molecules, serine proteases and serpins, have other significant roles in a wide range of physiological processes, including signal transduction and invertebrate defense responses (Ross et al., 2003). Indeed, some innate responses are dependent on serine proteases cascades that in turn are tightly regulated by serpins (Meekins et al., 2017).

Toxin sequestration

Another opportunity for invertebrates to counteract *B. thuringiensis* proteins toxicity, prior to pore formation in the midgut epithelial cells, is toxin sequestration. This process can occur through certain molecules found in the midgut, either in the midgut lumen or in the peritrophic matrix. It is believed that this phenomenon would help to detoxify the organism when it is exposed to *B. thuringiensis* proteins, as toxin-receptor interactions would be reduced.

Sequestration in the Midgut Lumen

Currently, different toxin-binding molecules have been characterized in the insect midgut lumen. One example are esterases, which have been reported to sequester the Cry1Ac protein in a *H. armigera* strain resistant to Bt cotton (Gunning et al., 2005). Other molecules that participate in this process are the hexamerins (a type of inducible immune proteins). These proteins were found in the *H. armigera* midgut lumen and hemolymph, and they bind to Cry1Ac and GalNAc-specific lectins, forming insoluble aggregates (Ma et al., 2005). Another case of Cry protein sequestration in the insect gut lumen was due to glycolipids. A lipid carrier, lipophorin, was able to interact with Cry proteins in the gut lumens of immune-induced insects, suggesting that this interaction might be the consequence of a coagulation reaction (Rahman et al., 2007). Later, aggregation and sequestration of Cry1Aa and Cry2Ab by lipid particles were also observed by Ma et al. (Ma et al., 2012). These authors suggested that the toxin-lipid aggregation observed resembles immune-mediated lipid particle aggregation around lectins (Schmidt et al., 2010), which would explain gut lumen Cry protein inactivation and subsequent prevention of its interaction with specific membrane receptors.

The ability of some Cry proteins to trigger massive shedding of cell surface proteins, such as glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N (APN) and alkaline phosphatase (ALP),

into the midgut lumen was reported in different insect species (Valaitis, 2008; Hernández-Martínez et al., 2010; Caccia et al., 2012; Hernández-Martínez et al., 2017). Although the role of this shedding has not been clarified yet, it might act as a defense mechanism against the toxic action of Cry proteins, since membrane-bound APN and ALP were described as surrogate Cry protein receptors (Pigott and Ellar, 2007; Lu and Adang, 1996). This hypothesis is based on the assumption that the soluble APN or ALP molecules present could act as competitive inhibitors, preventing the interaction of these proteins with the cell surface receptors. Indeed, high levels of soluble APN and ALP in the lumen of some *B. thuringiensis*-resistant *S. exigua* and *H. zea* insects have been observed (Hernández-Martínez et al., 2010; Caccia et al., 2012). Moreover, high midgut lumen ALP levels were correlated with *B. thuringiensis* resistance (Caccia et al., 2012). Another way in which shedding of GPI-anchored receptors might contribute to attenuate the *B. thuringiensis* proteins toxic action is by simply depleting some cell membrane receptors. Cleavage of APN by phospholipase C from brush border membrane vesicles attenuated Cry1Ab protein insertion (Bravo et al., 2004). In contrast, Valaitis (2008) proposed that shedding might be due to *B. thuringiensis* toxin cytocidal activity. In agreement with this hypothesis, Valaitis (2008) reported that the shedding inhibition of GPI-anchored receptors by cyclic AMP did not affect Cry1A toxicity against *Lymantria dispar* larvae, suggesting that shedding of GPI-anchored receptors may not be involved in Cry protein defense.

Sequestration by the Peritrophic Matrix

The midgut epithelium is protected by a peritrophic matrix, which in fact is not a membrane but a dense net composed mainly of chitin and highly glycosylated proteins, in which peritrophins are the most abundant. This semipermeable structure, besides improving digestion, protects the epithelium from mechanical abrasion and efficiently protects against pathogens (Terra, 2001).

Bacillus thuringiensis toxins cross this barrier to reach their specific midgut epithelial receptors to exert their toxic effects. Besides constituting a physical defense, the peritrophic matrix binds *B. thuringiensis* proteins in a nonspecific way, thus reducing the toxin amounts that can reach their membrane targets (Bravo et al., 1992; Aranda et al., 1996; Hayakawa et al., 2004; Rodrigo-Simón et al., 2006) (**Figure 42**). Peritrophic matrix disruption by chitinase or protease action increased the susceptibility to Cry proteins and different pathogens (Sampson and Gooday, 1998; Liu et al., 2002; Jakubowska et al., 2010; Han et al., 2015). Downregulation of chitin deacetylases, enzymes which increase matrix permeability, has generally been found after a *B. thuringiensis* challenge. This has been reported in *S. exigua* after Vip3Aa intoxication (Bel et al., 2013), in *H. armigera* after *B. thuringiensis* spore and crystal feeding (Liu et al., 2002), and in *O. nubilalis* after Cry1Ab intoxication (Yao et al., 2017). In contrast, chitin deacetylase genes were upregulated in *T. castaneum* with oral or systemic infections with *B. thuringiensis* spores (Behrens et al., 2014). Also, the chitin deacetylase level was higher in a *H. armigera* Cry1Ac-resistant strain compared to the susceptible strain, and the authors suggested that in this manner chitin rigidity may impede Cry1Ac from crossing the peritrophic membrane (Jin et al., 2019). Interestingly, the role of chitin deacetylases can go beyond peritrophic matrix structural maintenance, since they are involved in converting chitin into chitosan, a strong antimicrobial agent (Kong et al., 2010); thus, its implication in *B. thuringiensis* intoxication should be further studied.

Gut epithelium healing

The insect gut epithelium has at least two main roles: (i) in nutrient uptake and (ii) as a physical barrier that protects the host against different stress factors (Huang et al., 2015). During *B. thuringiensis* toxin intoxication, the gut epithelial barrier is disrupted, allowing gut bacterial entry and eventually leading to death by septicemia (Raymond et al., 2010; Caccia et al., 2016; Broderick et al., 2009). After exposure to sublethal Cry protein concentrations,

invertebrates can recover by repairing the injured membranes (restore membrane integrity) or by regenerating their midgut epithelium (Spies and Spence, 1985; Cancino-Rodezno et al., 2010; Tanaka et al., 2012; Los et al., 2013; Castagnola and Jurat-Fuentes, 2016). Therefore, the host ability to regenerate damaged epithelial cells will contribute to the insect susceptibility to diferent pathogens and, in particular, to *B. thuringiensis* toxins. The most well-studied healing mechanisms, including vesicle trafficking pathways, midgut regeneration, autophagy, and apoptotic death, are reviewed here.

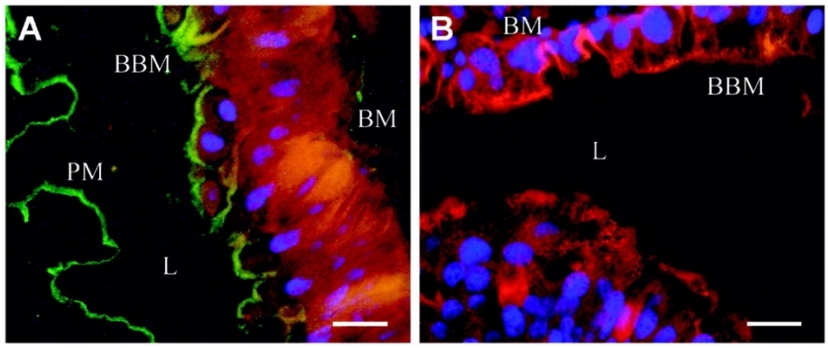

Figure 42. Cry1Ac immunodetection in *Helicoverpa armigera* larval midguts. (A) Larvae exposed to a high dose of Cry1Ac (7mg) for 20min. The green fluorescence shows Cry1Ac trapped in the peritrophic matrix (PM) and bound to the brush border membrane (BBM). (B) Control larvae not exposed to the toxin. An Alexa Fluor 488-anti-rabbit antibody was used as secondary antibody. BM, basal membrane; L, lumen. Scale bars, 50mm. (Reproduced from Rodrigo-Simón et al., 2006)

Midgut Regeneration

Insect gut epithelium integrity is maintained through intestinal stem cell regulation, since these cells have unlimited self-renewal capacity (Loeb and Hakim, 1996; Lu and Yi, 2015; Caccia et al., 2019). In mammals and *Drosophila melanogaster*, the mechanisms that regulate gut epithelial integrity maintenance have been extensively characterized (Chung and Kasper, 2010; Buchon et al., 2013). In contrast, the molecular regulation of midgut homeostasis is lacking in Lepidoptera and other insect pests.

Insect midgut cell regeneration after exposure to sublethal *B. thuringiensis* protein concentrations has been studied *in vivo*. One of the earliest *in vivo* studies reporting a regenerative mechanism upon exposure to sublethal *B. thuringiensis* crystal protein concentrations was described in *M. sexta* (Spies and Spence, 1985). Martínez-Ramírez et al. (1999) found that exposure to sublethal Cry1Ac concentrations rendered the same amount of midgut cellular damage to Cry1Ac-resistant and Cry1Ac-susceptible *H. virescens* larvae, suggesting the resistant strain more readily replaced its damaged cells. In a *B. mori* study, apoptosis and subsequent healing were clearly observed when larvae were exposed to sublethal Cry1Aa concentrations (Tanaka et al., 2012). Increased stem cell proliferation rate was also reported when *A. janata* larvae were exposed to sublethal concentrations of a *B. thuringiensis* formulation (Chauhan et al., 2017). These authors suggested that this higher proliferation rate could allow larvae to repair their gut epithelium facilitating survival.

Midgut regeneration has also been studied using primary midgut cell cultures from insect larvae as an *ex vivo* model. Loeb et al. (2001) reported that when *H. virescens* primary midgut cell cultures were exposed to sublethal *B. thuringiensis* protein concentrations, the mature cultured midgut cells were destroyed, whereas the number of stem and differentiating cells increased. The dynamics of depletion and replacement were dependent on the protein type and its concentration. Castagnola et al. (2017) reported that the culture fluid (secretome) of *H. virescens* midgut cells exposed to the Cry1Ac protein induced a greater stem cell proliferation and an overall reduction in total cell mortality in primary cultures. In general, results from *ex vivo* studies support the idea that enhanced gut stem cell production might allow insects to overcome the loss of mature cells destroyed by sublethal doses of *B. thuringiensis* proteins.

The mitogenic effect of monomeric α-arylphorin was described ex vivo using *H. virescens* midgut stem cells and also *in vivo* using different insect pests (Blackburn et al., 2004; Hakim et al., 2007).

Feeding this purified protein to *H. virescens* larvae resulted in midgut hyperplasia and significant susceptibility reduction to the Cry1Ac protein (Castagnola et al., 2017), suggesting that it may act as a healing regulatory factor. Furthermore, the results from transcriptional *B. thuringiensis* intoxication studies showed changes in *α-arylphorin* gene expression levels, suggesting an active *α-arylphorin* role after *B. thuringiensis* challenge. However, the relationship between *α-arylphorin* expression levels and midgut healing is still not clear, since contradictory results have been reported. *α-arylphorin* gene downregulation was observed in *L. dispar* after *B. thuringiensis*-based insecticide ingestion (Sparks et al., 2013), in *S. exigua* after Vip3Aa intoxication (Bel et al., 2013), and in *O. nubilalis* exposed to Cry1Fa (Vellichirammal et al., 2015). Upregulation was detected in *H. armigera* (Ma et al., 2005) and *H. virescens* (Castagnola et al., 2017) after Cry1Ac intoxication, as well as in *S. exigua* and *A. janata* after exposure to different *B. thuringiensis*-based insecticides (Hernández-Martínez et al., 2010; Chauhan et al., 2017). Discrepancies found in *α-arylphorin* gene expression throughout the literature could be attributed to either protein function diversification (versatility) in a concentration-dependent manner or different experimental conditions or insect species used (Dhania et al., 2019). Other studies have shown upregulation of the *α-arylphorin* gene in resistant strains of *Diatraea saccharalis* (Guo et al., 2012) and *S. exigua* (Hernández-Martínez et al., 2010) compared to corresponding susceptible insect species.

The expression of some genes involved in signal pathways regulating epithelial layer proliferation after exposure to *B. thuringiensis* proteins was further examined by quantitative real-time PCR. Tanaka et al. (2012) reported an increase in the *Bm-Socs* expression level after 6 h of exposure to a sublethal Cry1Aa concentration in *B. mori*. *Bm-Socs* is an ortholog of the *D. melanogaster Socs36E* gene, a negative regulator of the JAK-STAT pathway (Callus and Mathey-Prevot, 2002; Karsten et al., 2002). Similarly, a gene encoding a component of the JAK-STAT pathway was downregulated after 24 h of exposure of *S. exigua* to sublethal Vip3Ca concentrations

(Hernández-Martínez et al., 2017). The JAK-STAT pathway is associated with regulation of midgut cell renewal via insect stem cell proliferation and differentiation after exposure to different stress factors (Callus and Mathey-Prevot, 2002; Jiang et al., 2009; Jiang and Edgar, 2011). Based on all these observations, Hernández-Martínez et al. (2017) speculated that negative JAK-STAT pathway regulation might affect midgut cell renewal upon exposure to *B. thuringiensis* proteins. Moreover, it is worth noting that the JAK-STAT pathway orchestrates other many functions, such as immune responses (Buchon et al., 2014; Cytrynska et al., 2016). Therefore, it is difficult to predict the outcomes that might result from its negative regulation.

Vesicle Trafficking Pathways

One of the mechanisms observed to be involved in repairing and restoring membrane integrity after PFT exposure is the vesicle trafficking pathway (Brito et al., 2019). Membrane resealing is achieved through endocytic and/or exocytic events. During endocytosis, the membrane is invaginated and excised leading to internalization of the cargo (molecules in the plasma membrane, e.g., PFT, lipids, and proteins) from the cell surface. Once inside the vesicle, the cargo can be either degraded or recycled. Recycling of the cargo back to the membrane is eventually undertaken by exocytic events (Fletcher and Rappoport, 2009). Los et al. (2011) showed that vesicle trafficking pathways are utilized in *C. elegans* protection against Cry proteins. These authors showed that, after Cry5B intoxication, endocytosis rates in intestinal cells were greater than in non-intoxicated worms. These results are in agreement with results obtained in mammalian cells when treated with PFT (Idone et al., 2008; Husmann et al., 2009; Thiery et al., 2010). In order to shed light on how this mechanism can counteract mechanical membrane wounds caused by Cry proteins, the *rab-5* and *rab-11* genes were silenced. Rabs are small GTPases that regulate intracellular vesicle transport events, in particular, several endocytic, transcytic, and exocytic pathways (Bhuin and Roy, 2014). Gene silencing resulted in significant *C. elegans* hypersensitivity to Cry5B and

decreased the endocytosis rate. Subsequent analysis showed that both Rab-5- and Rab-11-mediated vesicle trafficking pathways helped to remove Cry5B protein pores, suggesting that these pathways were required to restore cell surface integrity after Cry5B exposure (Los et al., 2011). Furthermore, p38 mitogen-activated protein kinase (MAPK) was not involved in modulating Rab-5 activity when worms were exposed to Cry5B. This result is in opposition to what was previously shown by Cavalli et al. (2001) and Macé et al. (2005), who showed that p38 MAPK might regulate both the Rab-5 cytosolic cycle and the phosphorylation of its effectors.

The involvement of vesicle trafficking pathways were also described in an *A. aegypti* culture cell line (Mos20) after exposure to sublethal doses of Cry11Aa (toxic to Diptera) and Cry1Ab (toxic to Lepidoptera) (Vega-Cabrera et al., 2014). These authors reported that both proteins, regardless of their specificity, were internalized by endocytosis, although they were not degraded in lysosomes. Based on the results, the authors suggested that vesicle pathways regulated by Rab-5 and Rab-11 might be a mechanism to eliminate bound Cry proteins by internalization.

Autophagy

Autophagy is a conserved eukaryotic catabolic process occurring in the intracellular space by which double-membrane vesicles engulf material to be eliminated within lysosomes. The term "autophagy" was coined in 1963 by de Duve (1963) and was described to play a major role in a wide range of biological events (de Duve, 1966). For instance, autophagy is involved in the maintenance of cellular homeostasis, differentiation and developmental processes (Levine and Klionsky, 2004), response to extra- and intracellular stress conditions (such as nutrient starvation, hypoxia, and oxidative stress) (Mizushima, 2005; Filomeni et al., 2015), and endoplasmic reticulum (ER) stress (Yorimitsu et al., 2006), as well as in larval tissue and organ disassembly during metamorphosis in holometabolous insects (Tettamanti et al.,

2008, 2011; Malagoli et al., 2010). It is also considered part of the insect innate immune response against bacteria, viruses, and fungi, since pathogens can be eliminated via autophagy (Kuo et al., 2018). Furthermore, autophagy is also involved in determining mammalian cell susceptibility to different PFT (Gutierrez et al., 2007; Opota et al., 2011; Maurer et al., 2015).

An early *ex vivo S. litura* cell (Sl-HP) study suggested autophagy induction occurred after *B. thuringiensis* HD-73 strain infection (Gai et al., 2013). Recently, another study showed that different insect cell lines, which are susceptible to Cry1 toxins, induced autophagy in an AMPK (AMP-activated protein kinase) and JNK (c-Jun N-terminal kinase) pathway-dependent manner after exposure to Cry1 proteins (Yang et al., 2020). These authors suggested that autophagy might have a prodeath role as the number of swollen cells after exposure to Cry1 proteins decreased when autophagy inhibitors were used in the cell assay or RNAi was conducted to silence autophagy-related genes (Yang et al., 2020).

To gain insight into the role of autophagy in C. elegans host defense against *B. thuringiensis* proteins, Chen et al. (2017) performed *in vivo* experiments. Autophagy induction was specific to Cry5B and Cry21A, but not to other non-pore-forming cellular stressors (heavy metals, osmotic pressure, heat shock, or non-PFT pathogens). Indeed, autophagy was abolished when pore forming mutants were used, suggesting that the pore-forming activity was required to induce autophagy. Furthermore, when Cry5B toxicity was analyzed in different *C. elegans* autophagy defective mutants, these mutants were hypersensitive to Cry5B. Based on these findings, the authors concluded that autophagy serves as a cellular defense system. In addition, they showed that autophagy contributed to repairing the membrane after Cry5B membrane injury (Chen et al., 2017).

By *in silico* analysis, the authors identified a Cry5B activated transcriptional factor (HLH-30) that regulates autophagy-related

genes. In addition to this regulatory function, HLH-30 also regulates gene expression involved in vesicle-mediated transport, membrane invagination, and endocytosis (e.g., *rab-5*), suggesting a central modulating membrane-pore repair system against Cry5B intoxication. Likewise, other genes like *daf-16* (involved in the insulin/insulin-like growth factor 1 pathway [see below]) were identified as HLH-30 dependent (Chen et al., 2017).

Apoptotic Death

Membrane permeabilization by *B. thuringiensis* proteins action may trigger several pathways leading to either cell survival or cell death (Aroian and Van der Goot, 2007; Gonzalez et al., 2011). Apoptosis has been described as a cellular response mechanism after exposure to different PFT (Cancino-Rodezno et al., 2010; Chen and Zychlinsky, 1994; Galindo et al., 2004; Genestier et al., 2005; Timmer et al., 2009). Furthermore, the pore formation activity of PFT is necessary to induce the apoptotic response. This was established by using pore-forming defective mutants and by using inactivated proteins (Cancino-Rodezno et al., 2010; Genestier et al., 2005; McClane and Chakrabarti, 2004; Katayama et al., 2007; Portugal et al., 2017).

In vivo assays showed that apoptosis was observed in insect midgut epithelial cells when sublethal Cry protein concentrations were administered to both *Culex pipiens* and *B. mori* larvae (Tanaka et al., 2012; Smouse and Nishiura, 1997). However, when *B. mori* larvae were exposed to lethal Cry1Aa concentrations, apoptosis was not observed. Instead, midgut columnar cells swelled and burst, resulting in midgut disruption. More recently, TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labelling) staining showed that apoptosis also occurs in *S. exigua* larvae treated with sublethal Vip3Aa or Vip3Ca protein concentrations (Hernández-Martínez et al., 2017). In agreement with the B. mori study, no apoptotic cells were observed when *S. exigua* larvae were exposed to greater toxin concentrations. These results suggest that midgut epithelium

disruption by the *B. thuringiensis* protein pore formation activity occurs when insects are exposed to high doses of an active protein. On the other hand, when larvae are exposed to sublethal *B. thuringiensis* protein concentrations, apoptosis occurs as a host defense response, allowing the midgut epithelium to heal (Castagnola and Jurat-Fuentes, 2016; Aroian and Van der Goot, 2007). Therefore, whether apoptosis is involved in the host defense or it is just a consequence of *B. thuringiensis* protein toxicity needs further clarification.

Conversely, *ex vivo* experiments with *H. virescens* midgut primary culture cells and with other culture cell types (CF1 or Sf9) showed that *B. thuringiensis* proteins (including Cry1 and Vip3 proteins) can induce apoptosis (Portugal et al., 2017; Loeb et al., 2000; Jiang et al., 2016; Hou et al., 2020; Nimsanor et al., 2020). Portugal et al. (2017) reported that apoptosis inhibitors reduced Cry1A protein toxicity to CF1 cells, suggesting that apoptosis is involved in the death response. Similarly, Vip3A promotes apoptosis in Sf9 cells by mitochondrial dysfunction after being internalized (Jiang et al., 2016; Hou et al., 2020; Nimsanor et al., 2020). Therefore, at least under ex vivo conditions, apoptosis is not a general defense response, but it is related to the toxic events.

Epithelium intrinsic cellular defense pathways

It is well-known that the primary action of *B. thuringiensis* proteins is to disrupt host cell membranes by forming lytic pores (Adang et al., 2014; Heckel, 2020). Later, it was observed that the presence of pores at the cell surface triggers different intracellular signaling processes that allow cells to protect themselves against them, at least when hosts are exposed to sublethal *B. thuringiensis* protein concentrations (Los et al., 2013). In *C. elegans*, to obtain a deeper and wider understanding of the effectors and molecules involved in the signaling cascades, a proteomic analysis was performed comparing nematodes challenged with pathogenic and nonpathogenic *B. thuringiensis* strains. A relative protein increase related to innate

immune defense, proteolysis, apoptosis, MAPK pathways, unfolded protein response (UPR), and autophagy was observed, suggesting a complex host-Bt response (Treitz et al., 2015).

In this section, we summarize different pathways (**Figure 43**) involved in counteracting the toxic effects of *B. thuringiensis* proteins, such as the MAPK pathways, the UPR, the hypoxia pathway, and the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway (Hasshoff et al., 2007; Wang et al., 2012; Chen et al., 2017; Huffman et al., 2004; Bischof et al., 2008; Bellier et al., 2009; Chen et al., 2010). It is worth noting that other authors have also included autophagy and vesicle trafficking pathways as epithelium intrinsic cellular defense (INCED) mechanisms (Aroian and Van der Goot, 2007) (**Figure 43**).

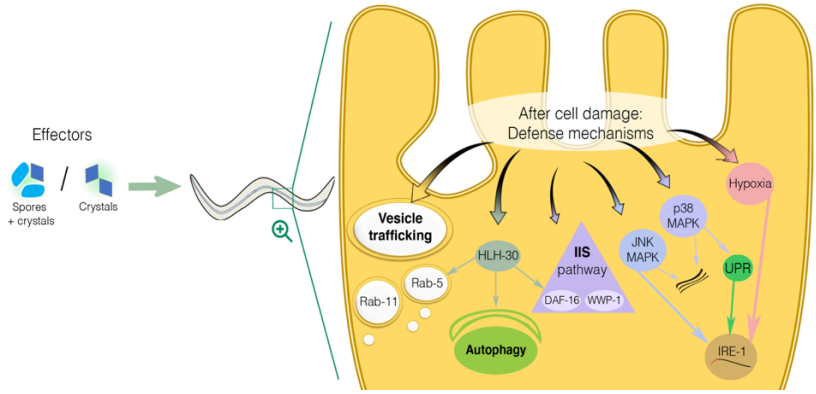

Figure 43. Schematic representation of the epithelium intrinsic cellular defense (INCED) in *Caenorhabditis elegans* as a response to the toxic effect of B. thuringiensis and/or its toxins. The vesicle trafficking pathways mediated by Rab-5 and Rab-11, autophagy and the Insulin/insulin-like growth factor 1 (IIS) pathway are interlinked by an upstream effector, the transcription factor HLH-30. A second set of cellular responses involving the hypoxia pathway, and two MAPKs pathways (p38 and JNK) converge on a downstream target: the IRE-1-XBP-1 branch of the unfolded protein response (UPR).

Mitogen-Activated Protein Kinase Pathways

In general, MAPKs have a multifaceted role acting as the control center of several cellular processes such as development, proliferation, differentiation, endocytosis, apoptosis, and migration (Cavalli et al., 2001; Macé et al., 2005; Kim et al., 2002, 2004; Andrusiak and Jin, 2016). In turn, MAPKs are controlled by a signaling cascade of MAPK kinase (e.g., MAP4Ks, MAP3Ks, and MAP2Ks) (Dong et al., 2002; Horton et al., 2011; Krishna and Narang, 2008). In multicellular organisms, MAPKs are classified into three subfamilies: ERK (extracellular signal- regulated kinase), JNK, and p38 (Dong et al., 2002). In particular, in *C. elegans*, p38 and JNK MAPKs were reported as the main component of innate immune response (Kim et al., 2002, 2004) and are also known as "stress-activated protein kinases" for their activation under stress conditions such as osmotic stress or heat shock (Andrusiak and Jin, 2016).

The involvement of p38 and JNK MAPK pathways in defense against Cry5B in *C. elegans* was reported by Huffman et al. (2004). In agreement with previous eukaryotic studies, these pathways have also been linked to a defense response to different PFT (Cancino-Rodezno et al., 2010). Transcriptomic analysis showed the upregulation of two genes: *sek-1* (MAPKK in the p38 MAPK pathway) and *kgb-1* (JNK-like MAPK) after a Cry5B challenge. Moreover, *sek-1* and *kgb-1* deletion allele mutants were hypersensitive to Cry5B and Cry21A, suggesting that both processes were important in the host defense against Cry5B intoxication.

To address the question of how extensive and coordinate is the host response to *B. thuringiensis* proteins, a genome-wide RNA interference (RNAi) screen for genes whose privation leads to *C. elegans* Cry5B hypersensitivity was carried out (Kao et al., 2011). Surprisingly, many genes had a protective function against sublethal concentrations of Cry5B. To unravel the network underlying the Cry5B response, an interactome database was used to clarify the connections between those

defense genes. In this regard, the p38 and JNK MAPK pathways seemed to play a central role in the network. Further analysis revealed that the JNK pathway controls most of the transcripts, including some induced by p38 MAPK, although JNK does not activate p38. It is also remarkable that KGB-1 JNK-like MAPK regulates *xbp-1* (*X-box binding protein-1*) splicing, which is involved in the UPR pathway (this pathway is addressed below). Kao et al. (2011) suggested that the JNK pathway might be the first master regulator of the Cry5B-induced transcriptional response. Similarly, the synthesis of p38 was activated in *M. sexta* and *A. aegypti* after sublethal Cry1Ab and Cry11Aa exposure (as spore and crystal mixtures), respectively (Cancino-Rodezno et al., 2010). Interestingly, p38 MAPK was activated at the posttranslational level (p38 was phosphorylated) in both insect species. These authors also silenced p38 and found that both insects were more susceptible to *B. thuringiensis* spore-crystal suspensions, in agreement with the *C. elegans* results (Huffman et al., 2004; Kao et al., 2011). Likewise, the p38 MAPK pathway has also been implied in *Chilo suppressalis* resistance to Cry1Ca; in this case, it seemed that the JNK and ERK pathways were not essential (Qiu et al., 2017a). In contrast to what was described after Cry1Ca oral intoxication, further analysis with injected *B. thuringiensis* bacteria revealed an increase in the phosphorylation level of p38, JNK, and ERK (Qiu et al., 2017b). A greater activation in the phosphorylated state of p38 and JNK (but not of ERK) after *B. thuringiensis* infection of *Galleria mellonella* was also reported (Wojda et al., 2014). Therefore, it seems that the in vivo protective function of MAPK pathways against *B. thuringiensis* and/or its proteins may be a general mechanism in several insect orders, as well as in species from other phyla.

Transcript profiling has shed light on the mechanism underlying host response to sublethal concentrations of *B. thuringiensis* proteins. In this context, a transcriptomic approach revealed that in a coleopteran, *T. molitor*, Cry3Aa protoxin intoxication provoked the induction of different kinase signaling pathways, p38 MAPK among them, during the initial 24 h of treatment (Oppert et al., 2012).

Similarly, the *A. aegypti* response after Cry11Aa ingestion was determined. Signaling processes such as the MAPK pathway, vesicular trafficking, and lipid metabolism were upregulated within 12 h of exposure (Canton et al., 2015). Genes in the JNK pathway were upregulated after *B. thuringiensis* oral infection in *P. xylostella*. In the same way, the *C-Jun1* gene was also upregulated at the translational level (Lin et al., 2020). *S. litura* treatment with Vip3Aa also activated signal transduction-related genes involved in MAPK pathways after 24 h of treatment (Song et al., 2016). In contrast, no signs of transcriptional regulation were observed in *S. exigua* larvae exposed to sublethal Vip3Aa concentrations (99% growth inhibition) (Bel et al., 2013). According to these authors, the lack of transcriptional regulation might be due to short-term Vip3Aa exposure or to the fact that most immune signaling pathway activation relies mainly on posttranslational modifications (e.g., phosphorylation, ubiquitination, and proteolytic cleavage) (Cancino-Rodezno et al., 2010; Welchman et al., 2009).

Recently, it was reported that the MAPK signaling pathway can modulate the expression of different midgut genes related with *B. thuringiensis* Cry protein resistance in *P. xylostella* (Guo et al., 2015). Interestingly, some of these genes encode putative Cry receptors such as the APN, ALP, and ATP-binding cassette transporter subfamily C (ABCC). Changes to their expression resulted in Cry1Ac resistance in *P. xylostella*, while suppression of MAP4K4 gene expression (upstream of p38) restored the susceptibility to Cry1Ac (Guo et al., 2015, 2020). In this context, a recent study has revealed that the MAPK signaling pathway is activated and modulated by the cross talk of two insect hormones: 20-hydroxyecdysone (20E) and juvenile hormone (JH). However, the upstream mechanism involved in controlling the ratio of these two hormones is still unknown (Guo et al., 2020). Interestingly, 20E also activates apoptosis and autophagy in insects during larval development and metamorphosis (Mané-Padrós et al., 2010; Romanelli et al., 2016; Tian et al., 2012, 2013). Hence, there may be a connection among hormone levels, the MAPK signaling pathway, midgut receptor expression, and *B. thuringiensis* resistance (Guo et al., 2020).

Endoplasmic Reticulum Unfolded Protein Response

The ER UPR is a conservative stress response to balance ER capability to synthesize and fold proteins correctly to maintain cellular homeostasis. When unfolded or misfolded proteins accumulate in the organelle, the UPR is activated. This response is orchestrated by three main transducers: PERK (double-stranded RNA-activated protein kinase-like ER kinase), ATF6 (activating transcription factor 6), and IRE-1 (inositol required enzyme 1), which mediate the different response signaling branches. In other words, those sensors activate different genes necessary to counteract the impact of the imbalance. The IRE-1 branch senses the ER stress, and then the IRE-1 RNase is acti- vated to perform *xbp-1* mRNA splicing, resulting in a mature transcription factor (XBP-1) capable of UPR gene activation (Walter and Ron, 2011).

The implication of UPR in *C. elegans* Cry protein defense was documented by Bischof et al. (2008). UPR target genes were mainly activated via IRE-1 after Cry5B intoxication, whereas the ATF6 pathway was only partially activated. Conversely, the PERK branch was not activated after Cry5B intoxication. In terms of sensitivity to Cry5B, mutants which were defective in the IRE-1 branch (known as *xbp-1* mutants) were hypersensitive compared to wild-type worms. Interestingly, the UPR branch activation patterns upon Cry5B exposure differed from the canonical UPR response to unfolded proteins. To explain this result, these authors hypothesized that after the *B. thuringiensis* challenge, the main role of the IRE-1 branch could be related to its phospholipid biogenesis activity, possibly involved in a defense role, rather than mitigating unfolded protein stress. Remarkably, the authors also suggested that UPR might be a p38 MAPK downstream factor, revealing a complex and coordinated response (Bischof et al., 2008).

In agreement with the *C. elegans* results, *A. aegypti* exposure to a mixture of spores and Cry11Aa crystals (sublethal concentration)

resulted in IRE-1 branch activation. In addition, RNAi was conducted to silence the *xbp-1* and *ire-1* genes, which confirmed UPR participation in Cry defense, since susceptibility to spore-crystal mixtures of Cry11Aa was higher in RNAi-treated larvae (Bedoya-Pérez et al., 2013). However, whether UPR is induced in *A. aegypti* in a p38 MAPK-dependent manner remains unknown.

Hypoxia Response Pathway

The hypoxia response pathway was found to play a role in *C. elegans* resistance to *B. thuringiensis* proteins (Bellier et al., 2009). In this pathway, HIF-1 (hypoxia-inducible factor 1) is a transcription factor and a master regulator of the hypoxia response activation. Under normal oxygen conditions, HIF-1 is degraded in the proteasome after being hydroxylated by EGL-9. *egl-9* mutants prevented the degradation of HIF-1 and conferred 3- to 5-fold resistance to Cry21A and Cry5B. Also, authors highlighted that HIF-1 stimulation resulted in resistance to the *Vibrio cholerae* cytolysin (another PFT). However, HIF-1 was not involved in other stress responses, like heat stress, *Pseudomonas aeruginosa* infection, heat shock, or oxidative stress. Interestingly, *hif-1* mutants showed hypersensibility to Cry5B but not to Cry21A (in a spore-crystal mixture). This study also emphasized the IRE-1 UPR branch as a downstream hypoxia pathway effector, to counteract the Cry21A attack.

Insulin/Insulin-Like Growth Factor 1 Signaling Pathway

Another INCED mechanism that is related to *C. elegans* and *B. thuringiensis* protein resistance is the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway (Chen et al., 2010). We simplified the insulin/IGF-1 signaling (IIS) pathway in two main components: the DAF-2 insulin-like receptor and the DAF-16 transcription factor. The DAF-16 component is antagonized by DAF-2 receptor activity: when the DAF-2 receptor is activated, DAF-16 is phosphorylated and subsequently sequestered in the cytoplasm, unable to initiate gene

transcription in the nucleus; in the opposite situation, under DAF-2 downregulation or inactivation, DAF-16 translocates into the nucleus, promoting gene transcription involved in stress response, bacterial pathogen defense, metabolism, longevity, growth, or behavior (Murphy and Hu, 2013).

Hasshoff et al. (2007) not only associated the IIS pathway with *B. thuringiensis* evasion and uptake (see "Ingestion" section) in *C. elegans* but also with *B. thuringiensis* defense. In this study, the effect of different mutations in the IIS cascade on the worm's survival rate after *B. thuringiensis* spore-crystal mixture exposure was assayed. As expected, the *daf-2* mutant survived longer than either control or *daf-16* mutant, while survival of the latter was less than that of the control worms. Surprisingly, in comparison with the *daf-16* mutant, the *daf-2*; *daf-16* double mutant exhibited an increased survival rate. This compelling evidence caused the authors to speculate on the existence of an extra downstream target, parallel to the already described DAF- 16. In agreement with these results, Chen et al. (2010) showed that reduction of DAF-2 insulin-like receptor function conferred *C. elegans* Cry5B and Cry21A resistance, and the *daf-2*; *daf-16* double mutant was more resistant than the daf-16 mutant or the wild-type in response to either Cry protein. Interestingly, the authors pointed out that the cellular defense against *B. thuringiensis* proteins might in part depend on a novel IIS output, namely, the WW domain Protein 1 (WWP-1), since the lack of this protein led to Cry5B hypersensitivity. Hence, the IIS pathway involved in *B. thuringiensis* defense will be composed of DAF-2, which exerts antagonist action on two downstream targets: DAF-16, and the newly described WWP-1. Later, another study confirmed the activation of DAF-16 in response to B. thuringiensis spore-crystal mixture in *C. elegans*; in fact, increased levels of DAF-16 were correlated with higher survival rate and reduced feeding activity (Wang et al., 2012). Furthermore, a proteomic analysis after challenging *C. elegans* with Cry6Aa showed that proteins participating in innate immune defense and IIS pathway were altered. In agreement with previous results from exposure of worms to Cry5B, the *daf-2*

mutant exhibited greater longevity than the wild-type, the *daf-16* mutant, or the *daf-2*; *daf-16* double mutant. This finding suggests that the IIS pathway is also part of the defense response to Cry6Aa in *C. elegans* (Wang et al., 2012).

The role of the IIS pathway in response to *B. thuringiensis* intoxication was also studied in the lepidopteran *Maruca vitrata* (Al Baki et al., 2020). These authors observed that a reduction in the gene expression of different components of the IIS pathway by dsRNA enhanced the insecticidal activity of *B. thuringiensis kurstaki* (spores) in laboratory and field conditions. These authors also suggested that the strategy (dsRNA 1 *B. thuringiensis*) might be useful for delaying resistance against *B. thuringiensis* in *M. vitrata* (Al Baki et al, 2020).

REPAT proteins

REPATs (from REsponse to PAThogens) are glycoproteins with around a 15-kDa molecular size, typically differentially expressed in midgut cells after a *B. thuringiensis* protein challenge. They were first observed upregulated in S. exigua after Cry1C intoxication (Herrero et al., 2007). Later, several REPAT family members were constitutively upregulated in a *B. thuringiensis*-resistant *S. exigua* population (Hernández-Martínez et al., 2010), suggesting a correlation between REPAT gene expression and resistance. Interestingly, a transcript with homology to the *S. exigua* repat family was among the most overexpressed UniTags in a *H. virescens* Vip3Aa-resistant strain compared to the susceptible control (Ayra-Pardo et al., 2019).

Since the discovery of the first REPAT member, several new members have been identified in *Spodoptera* spp., mainly in *S. exigua* (Navarro-Cerrillo et al., 2013) and *Spodoptera frugiperda* (Machado et al., 2016). The REPAT members were divided into two main groups according to their conserved motifs: αREPAT proteins and βREPAT proteins (Navarro-Cerrillo et al., 2013). According to this classification, the corresponding *αrepat* genes are mainly found in *Spodoptera* spp.

and other closely related Noctuidae family members, such as *Mamestra* spp. In contrast, the corresponding *βrepat* genes have a large representation among Lepidoptera (e.g., *H. armigera* or *B. mori*), and homologs have been found in phylogenetically distant insects, such as those in Diptera order (e.g., *D. melanogaster*) (Navarro-Cerrillo et al., 2013).

Also, *repat* genes may play a role in insect response, as their transcriptional patterns were altered after *B. thuringiensis* protein exposure (Bel et al., 2013; Dhania et al., 2019; Hernández-Martínez et al., 2010, 2017; Navarro-Cerrillo et al., 2013); however, their functions still remain unknown. Some REPAT members can form heterodimers, as well as translocate into the nucleus (Navarro-Cerrillo et al., 2012). Because of this and their homology to lepidopteran transcriptional coactivators, it has been speculated that different REPAT protein combinations could be linked to a variety of processes that involve REPAT-mediated transcriptional coactivation (Navarro-Cerrillo et al., 2012; Zhou et al., 2016). Different REPAT proteins differ not only in their expression pattern but also their subcellular localization (Navarro-Cerrillo et al., 2012). Although the presence of a signal peptide was predicted for all REPAT members, *repat* gene expression in insect culture cells (Sf21) indicated that the targeting occurs in certain cellular organelles (Herrero et al., 2007). Further research will help to shed light on the precise role that these proteins play within the insects' defense mechanisms.

Immune responses

Immune responses are divided into two categories: innate and adaptive. Innate responses are considered mainly nonspecific, lacking immunological memory. In contrast, adaptive responses have been associated exclusively with vertebrates due to the need of an antigen-specific memory developed after a first encounter with the pathogen, which allows the vertebrate to produce a faster and stronger immune response following the second encounter. Therefore, it was assumed

that insects rely exclusively on innate immune mechanisms, which were thought to be identical after a repeated challenge with the same stimulus. However, to date there is enough supporting evidence that demonstrates that most invertebrates, including insects, have the ability to produce a stronger immune response as a consequence of a previous exposure to a determined stress condition, known as immune priming (reviewed in references Contreras-Garduño et al., 2016; Milutinović et al., 2016). Notably, this new research area remains somewhat controversial, since some studies have found such response to be non-specific under certain effectors (such as mild stressors, e.g., thermal shock) (Le Bourg et al., 2009; Browne et al., 2014). Thus, the specificity of the response against *B. thuringiensis* and/or its proteins in different insects species needs to be further studied and clarified. In the subsequent sections of this review, we will focus on the studies where an encounter with *B. thuringiensis* and/or its proteins has caused either an innate or an immune priming response (**Figure 44**). Furthermore, it is also important to stress that some of the reported studies analyze the host immune responses via cuticular injection, and this is not how the intoxication/infection naturally occurs. Therefore, the immune response observed might not be relevant in natural infections.

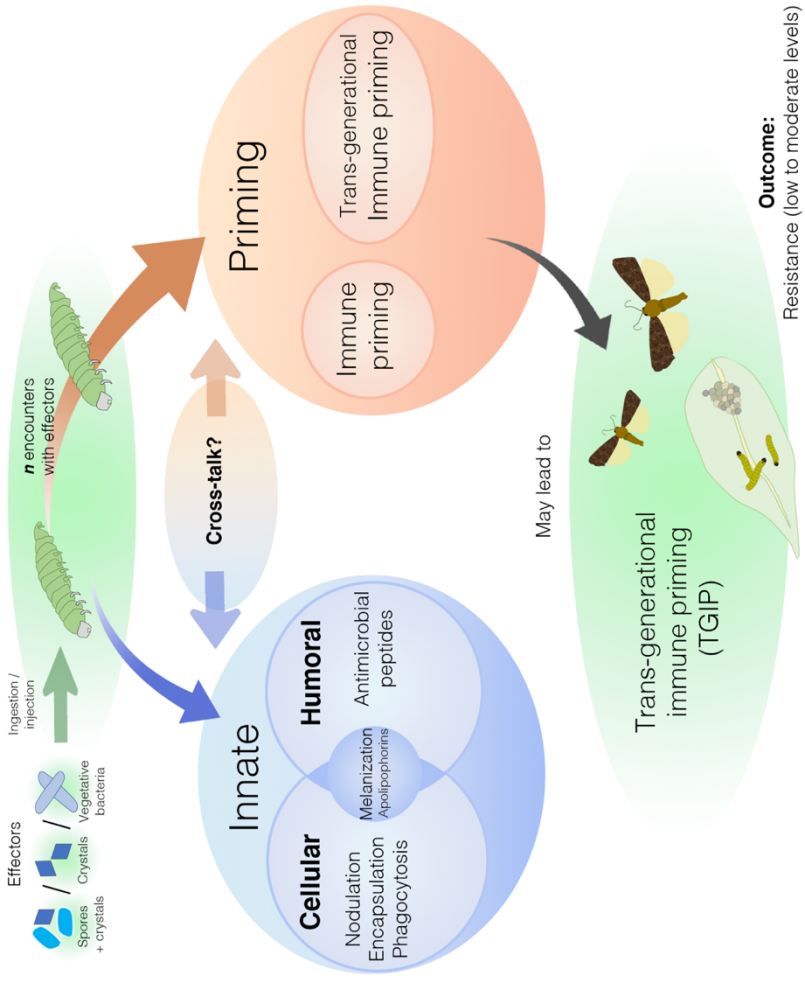

Figure 44. Immune response types that can occur after (i) a single encounter causing an innate immune response and (ii) more than one encounter, leading to an immune primed status. Immune priming to B. thuringiensis can be transmitted to the offspring generally by both progenitors (known as transgenerational immune priming).

Innate Responses

Once the midgut epithelium and the basal lamina are disrupted by the toxic action of *B. thuringiensis* proteins, bacteria residing in the host midgut can reach the hemolymph, causing septicemia and eventually host death (Caccia et al., 2016). However, insects have evolved different defense responses to cope with pathogen attack coming from their midgut. In general, these defense responses have been classified as humoral or cellular responses, though it is important to highlight that both responses are interlinked in many aspects.

Cellular response. Traditionally, the insect cellular response includes phagocytosis, nodulation, and encapsulation (Kavanagh and Reeves, 2004; Lundgren and Jurat-Fuentes, 2012). The nodulation or encapsulation processes usually start within the first minutes after pathogen entry in the hemolymph. The nodulation process is elicited by small structures (such as bacteria or fungi), which are entrapped by hemocyte clusters (Dunn, 1986; Jiang et al., 2010; Satyavathi et al., 2014; Dubovskiy et al., 2016). In contrast, the encapsulation process con- sists of capsule formation by overlapping hemocyte layers around large structures such as nematodes, eggs, and larvae, which cannot be phagocytized (Kavanagh and Reeves, 2004; Dubovskiy et al., 2016).

It is noteworthy that much research concerning the innate immune response against microbial pathogens, including *B. thuringiensis*, has been performed in the model organism *G. mellonella* (Kavanagh and Reeves, 2004, Dubovskiy et al., 2016). Dubovskiy et al. (2008) showed that injecting a spore and crystal mixture from *B. thuringiensis* (at LC_{15}, the lethal concentration causing a 15% mortality) into the *G. mellonella* hemolymph increased phagocytic activity and the encapsulation rates. Similar results were observed when sublethal concentrations (LC_{25}) of a spore and crystal mixture from *B. thuringiensis* were administered by oral intoxication (Grizanova et al., 2014). In contrast, the same study showed that greater *B. thuringiensis* concentrations (LC_{50}) decreased the encapsulation and phagocytosis

response. Therefore, the *G. mellonella* cellular response against *B. thuringiensis* was dose dependent (Grizanova et al., 2014).

Silencing of the immune gene *102 Sl* in *S. littoralis* triggered a reduction in the nodulation response after exposure to sublethal concentrations of either a *B. thuringiensis* biopesticide (Xentari) or Cry1C, decreasing larval survival (Caccia et al., 2016). However, neither hemocyte phagocytic activity nor the humoral antimicrobial responses were altered. Also, the insecticidal activity of Xentari on *S. littoralis* larvae was enhanced when combined with bacteria expressing dsRNA to knock out the immune gene *102 Sl* (Caccia et al., 2020).

Humoral response. The humoral response involves pathways such as the activation of both prophenoloxidase (ProPO) and reactive oxygen species, as well as soluble pep- tide production, which can kill, or at least stop, pathogen growth, besides acting as immunomodulators (Easton et al., 2009; Eleftherianos et al., 2013). Several studies have been carried out in different hosts to determine whether humoral responses are important in defense against the toxic effects of *B. thuringiensis*. Here, we will review the works studying the role of ProPO pathway and the production of antimicrobial peptides (AMPs) in response to *B. thuringiensis* intoxication.

The prophenoloxidase-activated system (ProPO-AS) is a complex enzyme-cascade reaction system associated with the insect immune defense induced by exogenous stimuli (Nappi and Christensen, 2005). The ProPO is activated to phenoloxidase (PO) mainly by serine proteases and is responsible for initiating the biosynthesis of quinones to melanin (Ashida and Dohke, 1980; Bidla et al., 2009). For this reason, the expression levels of some components of this cascade and enzyme activity levels of PO have been usually employed as markers for immune activation after exposure to *B. thuringiensis* and/or its proteins. Some studies have shown an increase in the transcripts of any of the effectors in this system or in PO enzyme activity (Bel et al., 2013; Wu and Yi, 2018; Lin et al., 2020; Contreras et

al., 2013; Valadez-Lira et al., 2012; Ferro et al., 2019; Sulek et al., 2019), whereas other studies have found the contrary or no effect (Lin et al., 2020; Valadez-Lira et al., 2012; Li et al., 2018). The high variability observed depending on the species, larval stage, or doses employed, as well as the time of exposure, make it difficult to draw conclusions, although an early activation of this route as a response after exposure has been observed. In the same way, high expression or activity levels of PO could be correlated with resistance to some extent (Ma et al., 2005; Rahman et al., 2004; Ma et al., 2005; Liu et al., 2019; Shabbir et al., 2020) but not in other cases (Rahman et al., 2007; Candas et al., 2003; Wang et al., 2007; Gassmann et al., 2009). Therefore, the implication of the ProPO-AS as a response mechanism to defend the insects against *B. thuringiensis* and its proteins, and the relation with the appearance of resistance should be studied in a deeper way.

AMPs are mainly produced in the fat body (humoral response) and, to a lesser extent, in other somatic cells (local defenses) (Hoffmann, 1995; Lavine and Strand, 2001; Govind, 2008). Since the discovery of the first insect AMP (Steiner et al., 1981), many AMPs have been identified and described as highly pathogen specific. Studies have been done to extend the knowledge of AMP diversity expressed in the larval midgut, as well as to unravel their specific activity in the midgut immune response during a *B. thuringiensis* challenge (Lin et al., 2020; Crava et al., 2015). A correlation between the humoral response and *B. thuringiensis* resistance in *G. mellonella* was reported for the first time by Dubovskiy et al. (2016). The resistance to *B. thuringiensis* spore-crystal mixture was mainly due to antimicrobial factor secretion when comparing the defense mechanism between resistant and susceptible insects. In another study, silencing of one AMP gene, *gloverin*, increased *S. exigua* susceptibility to the bacterium *B. thuringiensis*, supporting the importance of AMPs in defense against bacteria (Hwang and Kim, 2011).

Results from transcriptional assays showed that several AMPs were overexpressed in different tissues after *B. thuringiensis* challenge.

In the *S. exigua* midgut, different transcripts coding for diapausins, spodoptericin, lebocin 1, moricin, cobatoxin A, or gloverin were upregulated after exposure to sublethal concentrations of Cry1Ca, Vip3Aa, or Vip3Ca (Bel et al., 2013; Hernández-Martínez et al., 2017; Crava et al., 2015). Further research is needed to understand how exposure to *B. thuringiensis* proteins can activate an AMP response. To date, two possible mechanisms have been proposed to explain this phenomenon: (i) *B. thuringiensis* proteins release damage-associated endogenous pattern molecules (DAMPs), when disrupting the gut cells, that sense the presence of specific pathogens and trigger an immune response, or (ii) *B. thuringiensis* proteins themselves are interpreted as "danger molecules" by the innate immune system of the host (Tetreau, 2018). In agreement with the first argument, Crava et al. (2015) reported that the local immune response observed in *S. exigua* was only triggered after ingestion of Vip3Aa or Cry1Ca but not after ingestion of WK6 *Escherichia coli* lysate. The authors concluded that bacterial peptidoglycans and/or lipopolysaccharides which can be possibly present in the lysates containing the toxins were not affecting the immune status of the larvae. In *B. mori* hemocytes, significant upregulation of transcripts coding for gloverin, moricin, lebocin, and attacin were found when heat-killed *B. thuringiensis* suspension cells were injected into the larval hemocoel (Wu and Yi, 2018). Similarly, three different transcripts coding for defensins were upregulated in *T. castaneum* larvae injected with heat-killed *B. thuringiensis* bacteria (Tonk et al., 2015). Remarkably, downregulation of *P. xylostella* genes encoding cecropins, moricins, and gloverins was observed after B. *thuringiensis* treatment (feeding bacteria at LC50) (Li et al., 2018). Reduced antimicrobial peptides expression in response to pathogens has scarcely been reported (Nielsen-LeRoux et al., 2012; Khattar et al., 2009; Xu et al., 2017), and it is hypothesized that this response could be triggered by secondary metabolites to benefit bacterial survival and infection.

Cross talk between cellular and humoral responses. Although cellular and humoral responses have different action mechanisms, they

are tightly interconnected. These two defense systems work together to produce melanin, a compound toxic against extracellular pathogens in the hemolymph (Soderhäll and Ajaxon, 1982; Leger et al., 1988). In fact, in the earlier steps of capsule/nodule formation (cellular response), hemocyte degranulation occurs, destroying cells and releasing ProPO (humoral response), which eventually leads to capsule/nodule melanization due to melanin formation. In addition, cytotoxic molecules like reactive oxygen or nitrogen species (ROS or RNS) (humoral response) are also produced during melanogenesis (cellular response). In turn, the reaction products generated lead to the stimulation of other antimicrobial peptides (Bidla et al., 2009; Dubovskiy et al., 2013).

Rahman et al. (2004) showed that a low level of resistance (referred to as "tolerance" by the authors) to a *B. thuringiensis* product in a *Ephestia kuehniella* laboratory strain was associated with an inducible increase in the hemolymph melanization rate. Further studies in *E. kuehniella* suggested that hemolymph melanization was correlated with an elevated immune status, but it was not responsible for the *B. thuringiensis* resistance (Rahman et al., 2007).

The apolipophorin III (apoLp-III) protein family has also been implicated in different defense mechanisms, such as hemolymph clotting, as well as humoral or cellular immune stimulation (Jiang et al., 2010; Kim et al., 2004; Whitten et al., 2004; Zdybicka-Barabas and Cytryńska, 2013). Furthermore, this protein activates the ProPO cascade in *Locusta migratoria* and *G. mellonella* (Mullen and Goldsworthy, 2003; Park et al., 2005). *apoLp-III* gene transcripts were upregulated in *T. castaneum* upon oral intoxication using spore-crystal mixtures from *B. thuringiensis* (Behrens et al., 2014; Contreras et al., 2013). In the same line, an apolipophorin precursor was significantly upregulated in a *D. saccharalis* Cry1Ab-resistant strain compared to a susceptible strain (Guo et al., 2012), and apoLp-III transcripts were detected in Cry3Aa-treated *T. molitor* larvae but not in nontreated larvae (Oppert et al., 2012).

Immune Priming

The appearance of immune priming after insect exposure to either *B. thuringiensis* and/or its proteins has been described in many studies. In an *E. kuehniella* laboratory strain, an increase in resistance to a *B. thuringiensis* formulation correlated with an elevated immune status caused by a first low-concentration exposure (Rahman et al., 2004). This priming effect has also been observed in *G. mellonella* with a systemic infection of heat-killed *B. thuringiensis* by injection, which led to a protective effect by extending the survival of subsequently infected insects (Wu et al., 2014). Another study showed that the priming with live *B. thuringiensis* allowed the insects to overcome a lethal reinjection of the same pathogen but did not cause any change in the response to other pathogens (Taszlow et al., 2017). Furthermore, exposure of *T. castaneum* larvae to *B. thuringiensis* spores caused extensive transcriptomic changes of immune-related genes in larvae that had been primed with a culture supernatant of a *B. thuringiensis* toxic strain, compared to non-primed larvae. Interestingly, larvae that were orally primed with the supernatant of a nontoxic *B. thuringiensis* strain showed only minor changes (Greenwood et al., 2017). It is worth noting that, as reviewed by Milutinovic and Kurtz (2016), the induction of a primed response may vary largely depending on the route of infection used in the study (oral or injection) and is one of the main reasons why not all studies can be extrapolated to different insect species.

In addition, the results from two independent studies showed that *T. molitor* immune priming development was dependent on the bacterial type used for the challenge. Greater immune responses were observed when *T. molitor* larvae were challenged with *B. thuringiensis* than in larvae challenged with Gram-negative bacteria. The authors suggested that this result can be related to the fact that Gram-positive bacteria is the main pathogenic group against this coleopteran and may have led to a specific coevolution of immune processes (Dhinaut et al., 2018; Medina Gomez et al., 2018). This hypothesis was later proved by

studies on *T. castaneum* primed with *B. thuringiensis*. In a first study, insects were primed with different bacteria, and those challenged with *B. thuringiensis* showed a stronger priming induction (Ferro et al., 2019). The authors argued that the priming development and its specificity might be related to the probability of pathogen encounter. A latter study demonstrated that populations that were more susceptible to *B. thuringiensis* infection produced a stronger priming response, indicating a greater survival benefit (Khan et al., 2019). Although it is true that immune priming offers certain protection to subsequent infections in a species-specific manner (Cooper and Eleftherianos, 2017), it is metabolically costly to initiate and maintain. This fact reinforces the idea that an immune priming response is more relevant when a specific pathogen has a high probability of infecting the same host (Sheehan et al., 2020).

Transgenerational Immune Priming

Several studies have shown that B. thuringiensis immune priming can be transmitted to offspring, generally by both progenitors. This phenomenon is called transgenerational immune priming (TGIP). It was first proposed in *E. kuehniella*, in which the TGIP was due to immune elicitor incorporation into the oocyte by an immune-induced female. The elicitor could interact with embryonic tissues to induce the neonate immune system (Rahman et al., 2004). In the case of *T. molitor*, females primed with inactivated Gram-positive bacteria (*Arthrobacter globiformis* or *B. thuringiensis*) resulted in increased antimicrobial activity in eggs caused by tenecin-1, an AMP specific against Gram-positive bacteria (Dubuffet et al., 2015). Although it was first proposed that these antimicrobial substances were produced in the eggs and were not directly provided by the mother (Dhinaut et al., 2018), a recent study has demonstrated that, at least for this coleopteran, egg protection essentially relies on the active transfer of peptides and protein effectors from primed mothers to the offspring (Tetreau et al., 2020). In addition, experimental evidence supported that these offspring immune mechanisms triggered by a maternal challenge could

be pathogen dependent, suggesting *T. molitor*-pathogen coevolution (Dhinaut et al., 2018). Although less is known about the role of male progenitors in the TGIP, recent reports demonstrate that seminal fluid and spermatozoid transferred compounds that can contribute to offspring protection in *T. castaneum* primed with *B. thuringiensis* (Roth et al., 2010; Eggert et al., 2014). Similarly, Schulz et al. (2019) showed that TGIP was transmitted by a paternal effect and that it persists for multiple generations, with similar survival advantage in the second filial generation compared to the first filial generation. Overall, the development of insect transgenerational immune priming was associated with certain pathways related to antimicrobial peptide regulation, ProPO or vitellogenin, but the link between these and transgenerational immune priming remains to be elucidated (Gourbal et al., 2018).

Final considerations

The benefits of *B. thuringiensis* based products, including Bt crops, are continuously threatened by pest resistance evolution in the field (Ferré and Van Rie, 2002; Jurat-Fuentes et al., 2021; Tabashnik and Carrière, 2020). Indeed, resistance monitoring reports documented a sustained loss of field control or increased resistance levels over time in some Bt crop fields (Whitburn and Downes, 2009; Yang et al., 2019; Tabashnik and Carrière, 2019; Reay-Jones et al., 2020). Therefore, understanding the mechanisms developed by susceptible hosts to counteract the detrimental effects of *B. thuringiensis* and its toxins can help researchers to design better strategies to maintain the long-term efficiency of *B. thuringiensis*-based products.

Many studies have reported that alterations in genes encoding midgut proteases and toxin-binding molecules (surrogate receptors) are important in *B. thuringiensis* resistance development (Ferré and Van Rie, 2002; de Bortoli and Jurat-Fuentes, 2019; Jurat-Fuentes et al., 2021). Mutations in either ABC transporters or cadherins confer the highest levels of resistance to *B. thuringiensis* toxins (Gahan et al., 2001; 2010). However, the genetic basis of resistance in insects is far from simple. Indeed, in some cases, resistance cannot be explained by mutations or alterations in toxin-binding molecules, and it seems that it is conferred by multiple mechanisms that can be under independent genetic control (Table 4) (Lawrie et al., 2020 and Chapter 6 in this thesis). As discussed in this review, other mechanisms, such as defense responses, insect immunization, or midgut healing, are also involved in contributing to resistance to *B. thuringiensis* and its toxins (Ma et al., 2005a, 2005b; Hernández-Martínez et al., 2010; Martínez-Ramírez et al., 1999; Guo et al., 2015, 2020; Rahman et al., 2004) (Table 4). Unfortunately, the role of these additional mechanisms has been often overlooked as the development of genetic tools in insect pests has lagged far behind model organisms such as *Drosophila* and also because they all too often are associated with fitness costs. In recent years, research on these mechanisms progressed substantially, and some

studies have shown that continued exposure to *B. thuringiensis* and its toxins can lead to constitutive expression of defense responses without incurring significant trade-offs (Guo et al., 2015; 2020). An in-depth exploration of other host response mechanisms included in this review will be necessary to test whether they may be involved in the evolution of resistance in the long term. It is worth noting that the current picture illustrating all the events contributing to the host defense against *B. thuringiensis* and, even more important, their interplay, has still to be completely elucidated. Therefore, a thorough understanding of the intersections and cross talk between pathways triggered by *B. thuringiensis* and its proteins are foremost objectives to address in the coming years.

GENERAL DISCUSSION

Since the first Bt-based commercial product was available in 1938, the use of Bt and its pesticidal proteins as a treatment against various pests has increased, either as Bt formulates or Bt crops. As an example, the hectares planted globally of Bt corn, cotton, and soybean grew from 1.1 million in 1996 to 101 million in 2017 (ISAAA 2017). There is no doubt that *B. thuringiensis* is by far the most widely used and well-known microbial control agent among biotechnology-based solutions at present. Although researchers have been working for many years in deciphering how the proteins of this outstanding organism behave in the midgut environment of insects, it is clear that it is far from simple. Understanding the mode of action of pesticidal proteins from Bt in detail is a key point to ensure their long-term use for many reasons. It lets us know to some extent which, where, and why are the different mechanisms altered in the development of resistance, and it can allow us to combine Bt proteins with different modes of action to maximize their effect. In the present thesis, we have deepen into these interactions through different approaches.

In the **first** and **second** chapter we have focused on the interactions that occur between Cry1 proteins themselves, and with the ABC transporter C2 from the beet armyworm, *S. exigua.* Since this pest is of great agricultural relevance, and is widely distributed around the world, it is of high interest to understand how Cry1 proteins interact with the receptors from the midgut, since Bt-based products and Bt-crops containing Cry1 proteins are often used to control this pest. In these two chapters, we chose an *ex vivo* approach, since the use of *ex vivo* systems such as insect cell cultures or Brush Border Membrane Vesicles (BBMV) preparations, can allow the researcher to understand and deepen into specific interactions that sometimes can be overlooked in other kinds of *in vivo* assays. To this end, an ovary-derived insect cell line stably expressing the full length *SeABCC2* (Sf21-FRA) was used, and different binding assays with iodine-labelled Cry1A proteins as well as cell viability assays were performed. We found that Cry1A proteins bind specifically and with high affinity to cells expressing the transporter, and that its presence in the cells is sufficient to cause the

toxicity of the different Cry1A proteins tested. These results supported the role of the ABCC2 of *S. exigua* as a functional receptor for Cry1A proteins, as observed in other pest species (Gahan et al., 2010; Ren et al., 2016; Tanaka et al., 2013; Banerjee et al., 2017). Through both chapters, the lack of competition of the Cry1C protein, as well as the lack of toxicity to HEK cells expressing the transporter, discarded the ABCC2 as a receptor for this protein. These results indicate that Cry1C might have a different binding site in the midgut of *S. exigua*, since it is highly effective to control this pest.

Furthermore, an unusual phenomenon was observed in ^{125}I-Cry1Aa binding assays where low doses of the non-labelled Cry1Aa used as competitor produced a stimulatory response, increasing the total binding achieved by the ^{125}I-Cry1Aa to the cells expressing the transporter. Since higher doses of the competitor competed as usual, the nature of the biphasic behavior was further characterized. The stimulation at low doses was also observed in a more drastic way when Cry1Ab, Cry1Ac or the hybrid H04 protein (which has the same domains I and II as Cry1Ab/c, but domain III as Cry1C) were used as competitors against ^{125}I-Cry1Aa, but not with Cry1C or the hybrid H205 (with domains I and II from Cry1C and domain III from Cry1Aa). These results indicate the importance of a common sequence of domain I to produce the stimulatory binding phase. Moreover, the stimulation was present but to a lesser extent when using ^{125}I-Cry1Ac and Cry1Aa as competitor. To reveal the nature of the agent causing the stimulation phase, SDS-PAGE and autoradiographies of the pellets of the competition assays were carried out. The molecular size along with the intensity of the bands were used to determine that the oligomeric form of the protein is responsible of the stimulatory response, and pointed the ability of these proteins to form either homo- or hetero-oligomers. To confirm it, we used two mutant versions of Cry1Aa and Cry1Ab, with an amino acid substitution of Arg to Glu in position 99 of domain I that impedes oligomerization. The absence of any stimulatory phase or oligomer-sized band in the autoradiographies when using both mutants as competitors confirmed the ability of the

wild-type proteins to homo- and hetero-oligomerize through domain I, as well as to bind with higher affinities to the ABCC2 transporter.

Moreover, the toxicities observed with Cry1Ab and Cry1Ac proteins to cells expressing the transporter as well as the competition assays performed with ^{125}I-Cry1Ac in the first chapter, led us to conclude that these two proteins share a binding site that has very low affinity for the Cry1Aa protein. The presence of this shared binding site was further confirmed with the use of the hybrid H04, indicating that domain II of Cry1Ab/c is the domain with the highest affinity for this site. Since Cry1Aa appeared to be less relevant in the binding to this site but is toxic to cells expressing the transporter, we performed competition experiments with both ^{125}I-Cry1Aa and ^{125}I-Cry1Ac using Cry1Aa and the hybrid H205 as competitors. We could conclude that Cry1Aa must have another binding site in the transporter that has a high affinity for domain III of Cry1Aa. To this binding site the Cry1Ab protein can also bind with high affinity, since it shares the same domain III as Cry1Aa. Lastly, we observed through different combinations of Cry1A proteins in toxicity assays that the advantage obtained in forming hetero-oligomers over homo-oligomers was not only transferable to increased binding values, but also to higher toxicities when using the appropriate ratios of proteins. Altogether, the first chapter highlights the importance of the SeABCC2 as a multivalent receptor for Cry1A proteins, and the second chapter demonstrates the ability to form hetero-oligomers by Cry1A proteins, taking advantage of this ability towards the multivalence of the transporter.

So far, it is evident that the ABCC2 transporter from *S. exigua* and from other lepidopterans plays a relevant role in the binding and toxicity of at least Cry1A proteins, but few other members of this transporter family have been studied. It is plausible that we are currently missing many ABC transporters that could interact with pesticidal proteins from *B. thuringiensis*, since up to now more than 400 ABC transporters have been described in arthropods (Dermauw and Van Leeuwen, 2014). Thus, describing novel interactions between ABC

transporters and pesticidal proteins is currently a major challenge in Bt research. Besides ABCC2, few other ABC transporters have been related with Bt proteins, some examples are the ABCB1 from the leaf beetle, *Chrysomela tremulae*, which was identified as a functional receptor for Cry3Aa (Pauchet et al., 2016), the ABCA2 from the lepidopterans *H. armigera* and *B. mori* which acts as receptor for Cry2A proteins (Wang et al., 2017; Li et al., 2020), or the ABCC3 from *S. exigua, S. litura, P. xylostella*, *H. armigera,* and *S. frugiperda* which is related to the action of Cry1Ac (Endo et al., 2017; Chen et al., 2015; Guo et al., 2015; Wang et al., 2020; Jin et al., 2021). Interestingly, no other ABC member (besides the HvABCC2) has been characterized in *H. virescens*, in which ABC transporters were first related with *B. thuringiensis* pesticidal proteins more than a decade ago (Gahan et al., 2010).

In the **third** chapter of this thesis, we have performed a search of other non-characterized ABC transporters in *H. virescens* to address this gap of knowledge. We have identified and described two novel ABC transporters, the HvABCC3 and HvABCC4. The phylogenetic analysis together with the already described HvABCC2 for this species has shown a high similarity between the HvABCC2 and the HvABCC3, as recently observed for other species as *H. armigera* (Wang et al., 2020). However, the HvABCC4 is a more distant protein according to the amino acidic sequence. Based on the predicted models, both novel transporters are formed by two transmembrane domains and six regions facing the outer part of the membrane that correspond to the extracellular loops. The functionality of both transporters as possible receptors for Bt proteins was tested by performing CRISPR/Cas9 knock-outs of the *HvABCC3* or *HvABCC4* genes in *H. virescens* individuals and by conducting bioassays with Cry1Aa, Cry1Ac and Vip3Aa proteins. Preliminary results showed no reduction in toxicity for both knock-out strains compared to the *H. virescens* colony used as control for the three proteins tested here. However, lower mortalities in both KO strains were recorded at the lowest concentration of Cry1Ac tested, especially in the ABCC3-KO strain compared to the control

colony, but the degree of significance has yet to be studied due to low number of replicates in the bioassays. In addition, the correct knock-out of the transporters in both strains remains to be confirmed by mRNA sequencing of the genes. Since guide RNAs were designed in the first exon of the gene, and there could be alternative open reading frames, we must ensure that other functional versions of the transporters are not being expressed. Therefore, the possible implication of the novel ABC transporters with Bt proteins remains to be studied more deeply. For now, it is known that the ABCC3 of *S. exigua, H. armigera* and *S. frugiperda* shares functional redundancy with the ABCC2 in the mode of action of Cry1A proteins (Endo et al., 2017; Wang et al., 2020; Jin et al., 2021) and plays a minor, secondary role. To fully elucidate if this is also the case in *H. virescens*, further experiments are needed such as performing an ABCC2-KO strain, as well as double KO strains and assessing their binding abilities with labelled proteins. Lastly, other ABC transporters besides the HvABCC2, HvABCC3 and HvABCC4 remain to be characterized on *H. virescens*.

Besides studying the interaction between putative receptors and Bt proteins and searching for not yet known receptors, we must also seek to understand how and why some pest populations eventually develop resistance to certain Bt proteins, since resistance is the main threat to the long-term use of Bt crops or Bt-based biopesticides. It is widely accepted that binding alteration is the main cause of resistance to Bt proteins in crop pests, although other several mechanisms can occur. Binding alterations may result for different reasons, such as reduced gene expression of the membrane protein, deletions in the sequence, or amino acidic mutations that impede the interaction between the toxin and the receptor. For this, it is highly interesting to characterize the functionality of receptors that have been found altered in different resistant crop pests. There are many functional studies with wild-type versions of ABC transporters using various techniques, such as RNAi silencing, CRISPR knockout, or expression in an *ex vivo* system, but few studies have characterized the functionality of mutated

versions of ABC transporters from resistant pests. Furthermore, it was proposed that the ATP-switch mechanism of ABC transporters (an active conformational change of transmembrane domains by the binding and hydrolysis of ATP that allows the translocation of molecules) is necessary to correctly work as a receptor for Cry proteins (Heckel, 2012). In the **fourth** chapter, we characterized the functionality of a mutated version of the ABCC2 from an *S. exigua* colony resistant to the Bt-based product Xentari™. The transporter harbored a deletion in the nucleotide binding domain 2 (NBD2) that was genetically linked to resistance, but functionality assays to understand if this deletion was the cause of resistance were not carried out (Park et al., 2014). For this reason, we used an ovary-derived insect cell line expressing the truncated version of the transporter (Sf21-XenR), as well as the cell line with the full version of the ABCC2 (Sf21-FRA), previously used in chapters 1 and 2.

In addition to the NBD2 deletion, four amino acid substitutions were identified, three of them in the intracellular NBDs and one of them in an extracellular loop (ECL4). First characterizations through western blot and immunostaining showed the correct expression of the truncated transporter and its location on the cell membrane of the Sf21-XenR cells, which indicated that the mutations present on this transporter does not impede its presence in the membrane. Then, cell viability assays with Cry1Aa, Cry1Ab and Cry1Ac proteins were performed, both in the Sf21-XenR cell line and Sf21 cells as a control. The three proteins tested were toxic against cells expressing the truncated transporter, and to a highly similar level as to the toxicity observed previously for the line expressing the full form of the ABCC2 (Chapter 1). Furthermore, binding assays with ^{125}I-labelled Cry1Ac revealed specific binding to the truncated transporter, and similar dissociation constant (K_d) values to those found in the full-length SeABCC2 form, after performing homologous competition assays. Also, the Cry1Ab protein could partially compete for the labelled Cry1Ac binding sites, but Cry1Aa could not. These results confirm that the amino acid substitutions and the lack of part of the

NBD2 do not impede the binding and, consequently toxicity, produced by Cry1A proteins in the truncated form of the ABCC2 of *S. exigua*. Therefore, the results point out that the truncation of the transporter is not the direct cause of resistance to the Xentari™ product in the *S. exigua* colony studied here.

Another lepidopteran pest that currently causes a major impact on agriculture is the Asian corn Borer, *Ostrinia furnacalis*, which feeds largely on corn, but also sugarcane, pepper or cotton. The impact of larvae from this species is felt from China to Australia, where it can cause from 10 to 30% yield loss of corn grain each year (Wang et al., 2000). A promising tool for its effective control is the use of Bt-corn, which co-expresses Bt proteins to whom this species is susceptible to, such as Cry1Ab/c or Cry1F (ISAAA, 2014). Nevertheless, it is of high interest to understand the mode of action of these Bt proteins in *O. furnacalis* and possible mechanisms of resistance before they even occur in the field. Through the **fifth** chapter, we have first analyzed the binding parameters of a Bt-susceptible colony of *O. furnacalis* to constitute a preliminary binding site model. For this purpose, binding assays with ^{125}I-labelled Cry1Ab and Cry1Aa, as well as non-labelled Cry1Aa, Cry1Ab, Cry1Ac and Cry1F were performed. From this assays, we can conclude that there are at least two different binding sites in the membrane of midgut cells that can be shared mainly by Cry1Aa and Cry1Ab, but also to a lower extent with Cry1Ac. This model would share certain similarities to that previously proposed for the phylogenetically-close species *O. nubilalis*, the European corn borer, in that Cry1A proteins can bind to more than one site (Hernández-Rodríguez et al., 2013). Then, we analyzed a laboratory colony of *Ostrinia furnacalis* that was selected for resistance to the Cry1Ab protein. We assessed the appearance of cross-resistance by performing bioassays with the Cry1A and Cry1F proteins in both colonies. With a Cry1Ab-resistance of >700-fold, the analysis of cross-resistance showed moderate-to-high cross-resistance to Cry1Aa (178-fold), but also to Cry1Ac and Cry1F (>192-fold for both) when compared with the values obtained for the susceptible colony. Taking these data into account,

understanding the mechanism that produces resistance, but also cross-resistance to other proteins is a key step to allow the long-term use of Bt-corn. Since the main mechanism for resistance is the alteration of receptors from the membrane of midgut cells, we also performed binding assays between the proteins tested in bioassays and BBMV from the resistant colony. We observed a partial reduction in the specific binding of Cry1Ab and Cry1Aa towards BBMV, which indicates receptor alteration. The resistant colony would have one site altered, causing a deficiency in binding of, at least, the Cry1A proteins tested here, and consequently reducing the toxicity of the proteins. In regards to Cry1F, the minimal differences observed are inconclusive and we can just speculate that other resistance mechanisms may be involved, and further research is needed to understand the levels of cross-resistance observed for this protein. Still, it remains uncertain if the alteration of this binding site is solely responsible for the resistance levels observed here, and further studies identifying which is the altered receptor, as well as the nature of the alteration itself are needed.

A current strategy to avoid, or at least delay the appearance of resistance to Bt proteins in the field is the use of pyramided Bt crops (Carrière et al., 2015). It is based on the co-expression of Bt proteins that do not share their mode of action, generally Cry and Vip3 proteins, thus hindering the development of resistance (Moar and Anilkumar, 2007). Still, the pyramided strategy has been used for several years and, although the mode of action of Vip3 proteins is assumed to be different to that of Cry proteins, it is not yet well known. In laboratory conditions, resistance to Vip3A has been achieved in some lepidopterans. These resistant colonies are used to characterize the genetic or biochemical changes causing resistance and can also give us a glimpse on the mode of action of these Bt proteins. In the **sixth** chapter, we have explored the resistance to Vip3Aa in a >2,000-fold resistant *H. virescens* laboratory colony. By labelling Vip3Aa with ^{125}I, binding assays with BBMV from both, susceptible and resistant colonies were performed. No significant alterations were observed in binding parameters, and further ligand blotting confirmed the lack of

differences in binding between both colonies. These initial results indicate that the resistance towards Vip3Aa could be developed by mechanisms other than altered binding sites. Lack of alteration of binding sites in Vip3Aa-resistant colonies was also observed in *H. armigera* (Chakroun et al., 2016) and it has been documented recently in *Mythimna separata* (Quan et al., 2021). Despite the lack of differences in binding, a major reduction of the alkaline phosphatase (ALP) activity was detected in the midguts of the resistant colony. Moreover, western blotting and RT-qPCR confirmed the reduction of the amount of membrane ALP protein, as well as its reduction at a transcriptional level. This reduction had already been evidenced, although it went uncharacterized, in a transcriptomic study of the resistant colony (Ayra-Pardo et al., 2019). To discard that ALP is playing a role as receptor for Vip3Aa, the membrane ALP of *H. virescens* was expressed in cultured insect cells (Sf21 cells) and toxicity assays with Vip3Aa were performed. The susceptibility of the cell line expressing the membrane ALP was not significantly different from the control cell line, supporting that the ALP is not acting as a functional receptor for Vip3Aa in *H. virescens*. Although the alteration of ALP has been correlated with resistance to Cry1 proteins in different species, such as *H. virescens, H. zea, H. armigera, P. xylostella, S. frugiperda* or S. *litura* (Jurat-Fuentes and Adang, 2014; Caccia et al., 2012; Jurat-Fuentes et al., 2011; Guo et al., 2015; Jakka et al., 2016; Gong et al., 2015), it remains unclear if it is a functional receptor, or its alteration is due to a physiological response to resistance. From this study, we can rule out the membrane ALP as a receptor for Vip3Aa at least for *H. virescens*, and point out that the appearance of resistance to Vip3A may differ from the most common cases encountered with Cry1-resistant strains, that is, the alteration of binding sites. To confirm it, further studies with other Vip3A-resistant populations would be needed, as well as deepening in the knowledge of other, less common resistance mechanisms.

Currently, there is a large body of literature that has identified many response mechanisms of insects to overcome the infection of Bt

and its proteins, besides the alteration of receptors found in midgut cells. Nevertheless, there is a need to gather all this research to highlight it since many of it has been often overlooked. In the **seventh and final chapter** of the thesis, we have performed an intensive search of all known mechanisms that may contribute to the development of resistance to a certain extent. For that, we have written a comprehensive review that classifies these response mechanisms according to the step of the mode of action of *B. thuringiensis* and its proteins that is interfering with. We defined eight categories: (i) Ingestion, (ii) Crystal solubilization, (iii) Activation, (iv) Toxin sequestration, (v) Gut epithelium healing, (vi) Epithelium intrinsic cellular defense pathways, (vii) REPAT proteins, and (viii) Immune responses and we have discussed them and their interactions throughout the review. This piece of work aims to illustrate all the known events contributing to the defense of hosts against *B. thuringiensis* and its proteins, and seeks to highlight how characterizing the cross-talk of these response mechanisms will be one of the main objectives in the future of the Bt research world. To conclude, the results obtained in this thesis can give a better understanding of the interaction between insects, *B. thuringiensis,* and its pesticidal proteins, which can be useful to ensure the long-term use of this mighty organism.

CONCLUSIONS

1. The ATP-Binding Cassette transporter subfamily C member 2 from *Spodoptera exigua* (SeABCC2) acts as a functional receptor for Cry1A proteins from *Bacillus thuringiensis*, but not for the Cry1C protein.
2. The SeABCC2 has at least two binding sites for Cry1A proteins. The Cry1Ab and Cry1Ac proteins can bind to the main site through their domain II, and Cry1Aa and Cry1Ab can bind to the secondary, less relevant site through their domain III.
3. The Cry1A oligomer is the main state binding to the SeABCC2. This structure allows higher levels of binding than the monomer.
4. Cry1A proteins can hetero-oligomerize in the presence of the transporter, increasing their binding to the SeABCC2 and the toxicity in cells.
5. Two new ABC transporters have been characterized in *Heliothis virescens*, the HvABCC3 and the HvABCC4. Preliminary data shows no major implication as receptors for the main Cry1A and Vip3Aa proteins.
6. The amino acid changes and the lack of part of the Nucleotide Binding Domain II in the truncated SeABCC2 from a Bt resistant strain of *S. exigua* do not affect its functionality as a receptor for Cry1A proteins. These data support that the ATP-switch mechanism is not necessary for the receptor function.
7. Cry1Ab-resistance in *O. furnacalis* is due, at least in part, to the alteration of some binding sites of Cry1Ab, and may be responsible of the cross-resistance not only to other Cry1A proteins, but also to Cry1F.
8. The lack of binding alterations in a Vip3Aa-resistant strain of *H. virescens* and the reduction of the expression of its membrane-bound alkaline phosphatase suggests alternative mechanisms that can confer resistance to Vip3A proteins in this species.
9. The genetic basis of resistance to Bt in insects is highly complex. The need to explore mechanisms beyond the alteration of toxin-binding molecules has been highlighted.

10. Other Bt resistance mechanisms, some of them often overlooked, such as defense responses, insect immunization, or midgut healing, should be in the spotlight of Bt research in the coming years to ensure its long-term use in agriculture.

CONCLUSIONES

1. El transportador dependiente de ATP subfamilia C tipo 2 de *Spodoptera exigua* (SeABCC2) actúa como receptor funcional de las proteínas Cry1A de *Bacillus thuringiensis*, pero no para la proteína Cry1C.
2. El SeABCC2 tiene al menos dos sitios de unión para las proteínas Cry1A. Las proteínas Cry1Ab y Cry1Ac pueden unir al sitio principal mediante su dominio II y las proteínas Cry1Aa y Cry1Ab pueden unir al sitio secundario, menos relevante, mediante su dominio III.
3. El oligómero de proteínas Cry1A es la conformación principal en la unión al SeABCC2. Esta conformación permite alcanzar mayores niveles de unión que los monómeros de las mismas proteínas.
4. Las proteínas Cry1A son capaces de hetero-oligomerizar en la presencia del transportador, aumentando los niveles de unión al SeABCC2 y de toxicidad a las células.
5. Dos nuevos transportadores dependientes de ATP subfamilia C se han caracterizado en *Heliothis virescens*, el HvABCC3 y el HvABCC4. Los datos preliminares no muestran una implicación relevante como receptores de proteínas Cry1A o Vip3Aa.
6. Los cambios aminoacídicos y la falta de parte del dominio de unión a nucleótidos II en la versión truncada del SeABCC2 de la cepa de *S. exigua* resistente a Bt, no afectan en su funcionalidad como receptor para proteínas Cry1A. Estos datos apoyan que el mecanismo de transporte activo mediante hidrólisis de ATP no es necesario para su funcionalidad como receptor.
7. La resistencia a Cry1Ab en *O. furnacalis* se debe, al menos en parte, a la alteración de algunos sitios de unión de Cry1Ab, y puede ser responsable de la resistencia cruzada no solo a otras proteínas Cry1A, sino también a Cry1F.
8. La falta de alteraciones en la unión en una cepa de *H. virescens* resistente a Vip3Aa y la reducción de la expresión de su fosfatasa alcalina de membrana sugiere posibles mecanismos

alternativos en el desarrollo de la resistencia a proteínas Vip3A en esta especie.

9. La base genética de resistencia a Bt en insectos es altamente compleja. Se ha destacado la necesidad de explorar mecanismos más allá de la alteración de moléculas de unión a las toxinas.
10. Otros mecanismos de resistencia a Bt, algunos de ellos a menudo pasados por alto, como las respuestas de defensa, la inmunización de los insectos o la reparación del intestino medio, deben de estar en el punto de mira en la investigación de Bt en los próximos años para garantizar su uso a largo plazo en la agricultura.

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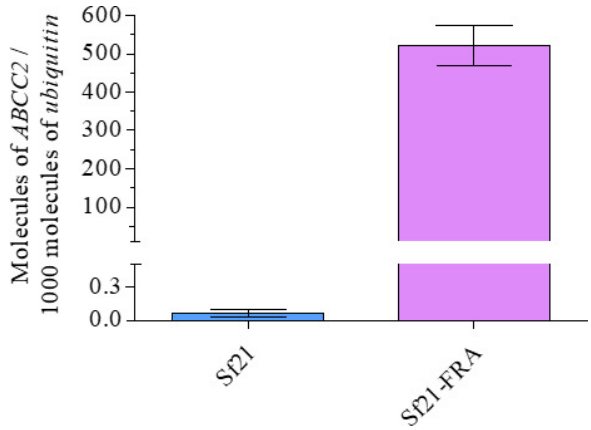
ANNEX

Supplementary Table S1. Sequence of primers used for cloning and RT-qPCR of ABCC2 from *Spodoptera exigua*.

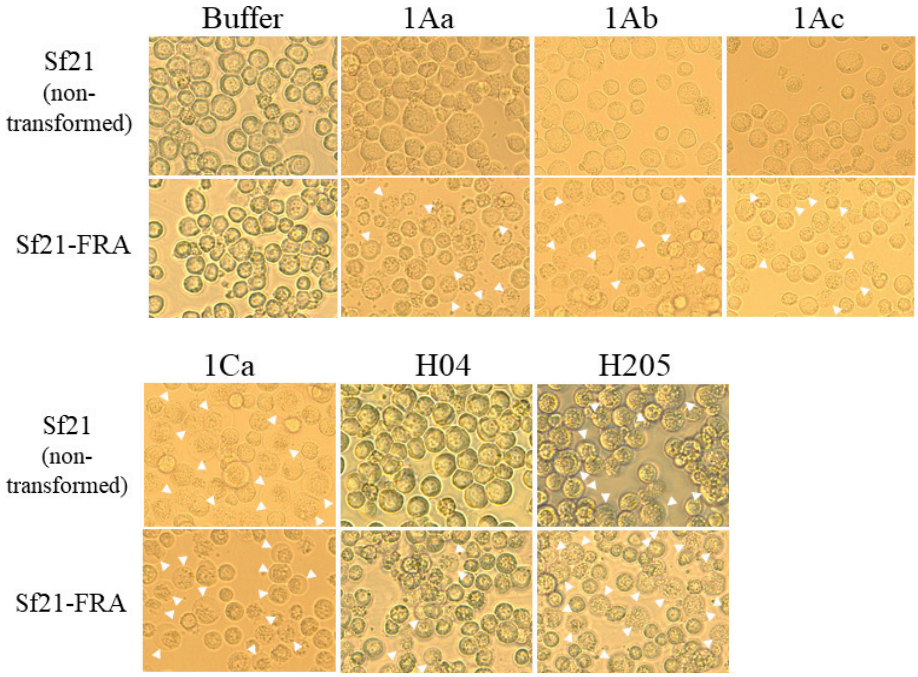
Primer name	Sequence (5'-3')
Cloning	
SeABCC2_SacI^{F}	CGAGCTCATGGACAAATCGAATAAA
SeABCC2_FLAG/XbaI^{R}	GTCTAGACTACTTGTCGTCATCGTCTTTGTAGTCAGCGGTTTTGGAATCACTTT
RT-qPCR	
qF_SeABCC2	AGCTACCGACCGAGGAAAAT
qR_SeABCC2	CTCTCCAGCACTAGGCCATC
qF-ubiquitin	GTTGCTGGTCTGGTGGGATT
qR-ubiquitin	AGGCCTCAGACACCATTGAAA

Supplementary Table S2. Primers used in PCR and sequencing, and guide RNAs used in CRISPR knock-outs for HvABCC3 and HvABCC4 from *Heliothis virescens*.

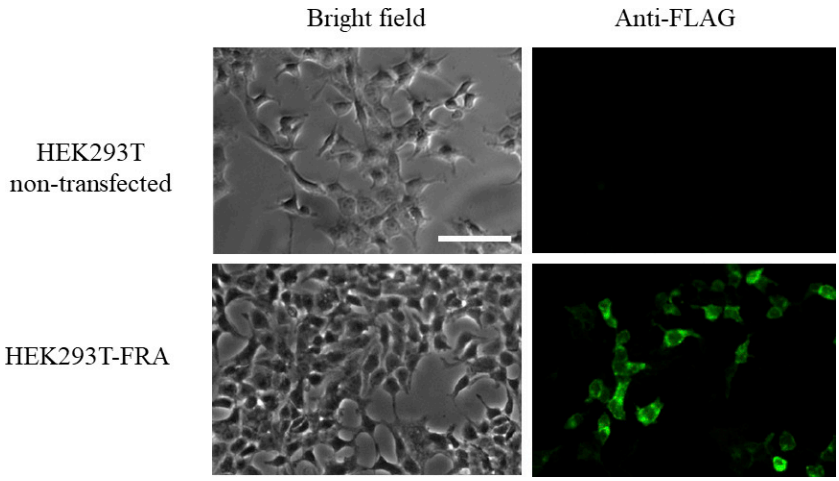
Target	Function	Name	Sequence (5'-3')
HvABCC3 **gene**	**Sequencing**	HvABCC3_5UTR3_F	GCGGAACTCTCCACATTTGC
		HvABCC3_526_R	CATCTTCATACCGAACTGCTGGC
		HvABCC3_356_F	GGCATCTCCATTGCTGTTTAGTC
		HvABCC3_1440_R	CAAAGCAGTGTCGTCCTGGTATG
		HvABCC3_1331_F	ATCGCAAATCGGATGGAGC
		HvABCC3_2272_R	GGTTGCCTGACTGCCTCTCC
		HvABCC3_2153_F	GAAGCATTGATGAGAAGGACAGGC
		HvABCC3_3468_R	TATCAGGGAAGATTTGCCAGC
		HvABCC3_3377_F	AGCCCGTATTGAAGAACCTGAAC
		HvABCC3_3UTR3_R	TGCCTACTCTACATTACTATTCACAAG
	CRISPR gRNA	gRNA_C3	AGAAAATTATGCTTATTGTTAGG
	Knock-out characterization	HvABCC3_F	CTCAGTCTCGCGATACCGTC
		HvABCC3_R	AGCAATGGAGATGCCGTTCT
HvABCC4 **gene**	**ABCC4**	HvABCC4_5UTR2_F	GGACTCTAAGCCCGAGGACAT
		HvABCC4_552_R	GAGTGAGCAGGCGGCGATG
		HvABCC4_464_F	TGTCGCTGGTCATCGCCTTC
		HvABCC4_1381_R	TGCACTCGAATCCAGGGTTG
		HvABCC4_1219_F	CGAGAGGATCTCCGCAGTGTG
		HvABCC4_2080_R	CCCTGTGCTCACTAATTCGTCG
		HvABCC4_1970_F	TCCTCGTCACGCATCAACTC
		HvABCC4_2794_R	ATTCATCGAGGGCTCCAATGTC
		HvABCC4_2673_F	CCGCAACCTCCACAACGAC
		HvABCC4_3611_R	CAACGTCGTCAATGAGCACTTC
		HvABCC4_3448_F	ACGCCCGAAGACTTGCCAG
		HvABCC4_3UTR5_R	ACGAAACTACATAGAGAAGGACACTG
	CRISPR gRNA	gRNA_C4	CAGGCGTTTCGCCTAGTTGCCGG
	Knock-out characterization	HvABCC4_F	GGCGCCGTGAATTCTTAACG
		HvABCC4_R	TGCATGTTTATAAACTTCATTGCCA



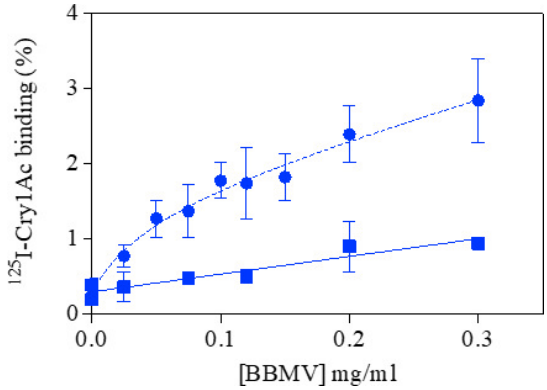
Supplementary Figure S1. Analysis of the expression levels of the *SeABCC2* gene by RT-qPCR in Sf21 and Sf21-FRA insect cell lines. The *ubiquitin* gene was used as housekeeping gene. The gene expression is given as copy number per 1000 molecules *ubiquitin* ± SEM.



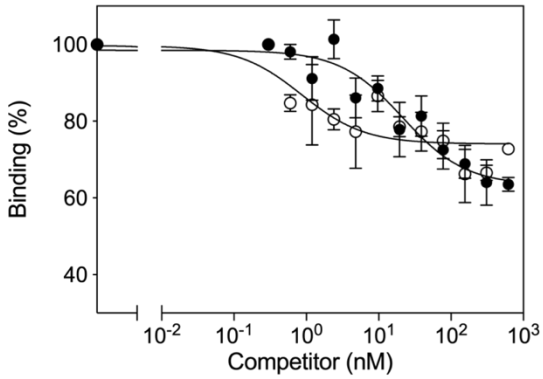
Supplementary Figure S2. Morphological changes observed after 3 h treatment with buffer (as a control), or 150 nM of each Cry1 protein. Arrowheads indicate swollen cells.



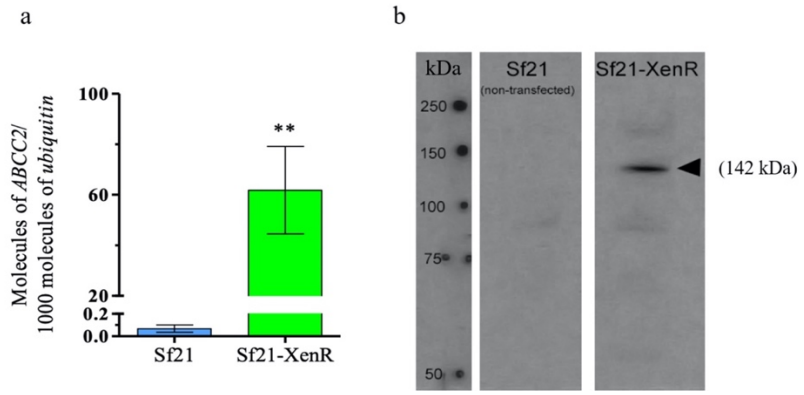
Supplementary Figure S3. Immunostaining of SeABCC2-FRA transporter expressed in HEK293T cells. Cells were stained with an anti-FLAG tag antibody followed by anti-mouse IgG conjugated to Alexa Fluor 488 (green signal).



Supplementary Figure S4. Binding assay with *S. exigua* BBMV. Total (circles) and nonspecific (squares) binding of ^{125}I-Cry1Ac to increasing concentrations of *S. exigua* BBMV.



Supplementary Figure S5. Binding of ^{125}I-Cry1AaR99E to Sf21-FRA cells in the presence of competitors. Curves represent total binding of ^{125}I-Cry1AaR99E to increasing concentrations of unlabelled Cry1AaR99E (full circles) and Cry1Aa (empty circles) as competitors. Each data point represents the mean of at least three independent experiments (± SEM).



Supplementary Figure S6. Detection of the truncated ABCC2. (a) Expression levels measured by RT-qPCR in Sf21 and Sf21-XenR insect cell lines. The *ubiquitin* gene was used as housekeeping gene. The gene expression is given as copy number per 1000 molecules ubiquitin ± SEM. Means were compared by T-test (p <0.01). Significant differences are indicated by asterisks. (b) Western blot analysis showing the presence of the truncated SeABCC2 transporter (black arrow, *ca*. 142 kDa) in the membrane vesicles of Sf21 and Sf21-XenR cells. First line, molecular weight marker (in kDa).

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Role of *Bacillus thuringiensis* Cry1A toxins domains in the binding to the ABCC2 receptor from *Spodoptera exigua*

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ABSTRACT

Cry proteins from *Bacillus thuringiensis* (Bt) have been used to control insect pests either as formulated sprays or as in Bt-crops. However, field-evolved resistance to Bt proteins is threatening the long-term use of Bt products. The *SeABCC2* locus has been genetically linked to resistance to a Bt bioinsecticide (Xentari™) in *Spodoptera exigua* (a mutation producing a truncated form of the transporter lacking an ATP binding domain was found in the resistant insects). Here, we investigated the role of SeABCC2 in the mode of action of Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, and two Cry1A-1Ca hybrids by expressing the receptor in Sf21 and HEK293T cell lines. Cell toxicity assays showed that Sf21 cells expressing SeABCC2 become susceptible to Cry1A proteins. HEK293T cells expressing the transporter were found susceptible to Cry1A proteins but not to Cry1Ca. The results with the Cry1A-1Ca hybrids suggest that domain II from Cry1Ab/c is crucial for the toxicity to Sf21 cells, whereas domain III from Cry1Aa/b is crucial for the toxicity to HEK293T cells. Binding assays showed that the Cry1Ac binding is of high affinity and specific to cells expressing the SeABCC2 transporter. Heterologous competition experiments support a model in which domain II of Cry1Ab/c has a common binding site in the SeABCC2 protein, whereas domain III of Cry1Aa/b binds to a different binding site in the SeABCC2 protein.

1. Introduction

Bacillus thuringiensis (Bt) is a bacterium which produces a wide range of insecticidal proteins which are useful in biological control of insect pests (Crickmore, 2006; Schnepf et al., 1998). One of the most studied proteins from Bt are the insecticidal crystal proteins (Cry proteins) which have been used to control insect pests both in formulated sprays or in insect-resistant genetically-modified crops (Bt-crops) (Roh et al., 2007). The extensive use of these proteins in agriculture has led to the emergence of resistance to some Cry proteins in target insects, threatening the long-term use of Bt products (Tabashnik and Carrière, 2017).

The mode of action of Cry proteins has been widely studied, though some aspects still remain unclear. In general, it is accepted that, after the ingestion of the protoxin by the insect, the protein is solubilized and activated by the action of digestive enzymes. The way in that the active forms exert their cytotoxicity is still controversial, though binding to specific receptors in the brush border of the epithelial midgut cells is accepted by either the sequential binding model (Bravo et al., 2007) or the Mg^{2+}-dependent signalling pathway (Zhang et al., 2006, 2005). In the sequential binding model, the activated Cry1A proteins (as monomers) go through a complex sequence of binding events with different midgut Cry-binding proteins, leading to the cleavage of helix α1 of the Domain I and to the oligomerization of monomers. Afterwards, the oligomer binds to other midgut membrane proteins, and finally is irreversibly inserted into the membrane, where it forms pores in the apical membrane of larvae midgut cells which eventually lead to septicemia and insect death (Adang et al., 2014; Bravo et al., 2007; Pardo-López et al., 2013). The second model proposes that the activation of an Mg^{2+}-dependent signalling pathway, after the binding of the Cry protein to a cadherin protein, leads to oncotic cell death (Zhang et al., 2006, 2005).

The specific toxin-receptor interaction has been reported as a crucial step for toxicity (Jurat-Fuentes and Crickmore, 2017). In addition, binding alteration in the insect midgut is the step of the mode of action that has more often been associated with insect resistance to Cry

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toxins

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Article

The *Spodoptera exigua* ABCC2 Acts as a Cry1A Receptor Independently of its Nucleotide Binding Domain II

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Abstract: ABC proteins are primary-active transporters that require the binding and hydrolysis of ATP to transport substrates across the membrane. Since the first report of an ABCC2 transporter as receptor of Cry1A toxins, the number of ABC transporters known to be involved in the mode of action of Cry toxins has increased. In *Spodoptera exigua*, a mutation in the *SeABCC2* gene is described as genetically linked to resistance to the Bt-product Xentari™. This mutation affects an intracellular domain involved in ATP binding, but not the extracellular loops. We analyzed whether this mutation affects the role of the SeABCC2 as a functional receptor to Cry1A toxins. The results show that Sf21 cells expressing the truncated form of the transporter were susceptible to Cry1A toxins. Moreover, specific Cry1Ac binding was observed in those cells expressing the truncated SeABCC2. Additionally, no differences in the irreversible Cry1Ac binding component (associated with the toxin insertion into the membrane) were observed when tested in Sf21 cells expressing either the full-length or the truncated form of the SeABCC2 transporter. Therefore, our results point out that the partial lack of the nucleotide binding domain II in the truncated transporter does not affect its functionality as a Cry1A receptor.

Keywords: mode of action; Sf21 cells; heterologous expression; truncated transporter; Bt resistance

Key Contribution: The truncated SeABCC2 (lacking part of the NBDII) of the previously associated Xentari™-resistant *S. exigua* colony acts as a functional receptor for Cry1A toxins. This study points out that a functional analysis is required to assess the effect of the mutations in receptors on Bt resistance.

1. Introduction

Bacillus thuringiensis (Bt) crystal proteins (Cry proteins) have been largely used in biological control as formulated sprays or in genetically-modified crops because of their high and specific toxicity against insect pests [1,2]. Due to the steady increase in the use of these proteins in agriculture, the appearance of resistance has been reported in some insect species, threatening the long-term use of Bt products [3].

Cry proteins are generally recognized as pore-forming toxins (PFTs), as their main action is to form pores in the membrane of the midgut epithelial cells of susceptible insects [4,5]. The mode of action of Cry proteins has been widely studied, especially for Cry1A proteins [6,7]. According with recent models, the mode of action of Cry proteins involves the sequential binding to different membrane

Modo de acción de las proteínas insecticidas de *Bacillus thuringiensis*

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RESUMEN

Actualmente, los insecticidas biológicos más vendidos son aquellos basados en proteínas de *Bacillus thuringiensis* (Bt), teniendo como principal diana las especies plaga de lepidópteros. Además de estar presentes en forma de formulados, los genes que codifican para algunas proteínas de Bt también se expresan en cultivos transgénicos (cultivos Bt). La principal característica de estas proteínas es su habilidad para formar poros en las células intestinales de aquellos insectos que son susceptibles a ellas. Dado que su mecanismo de acción es altamente específico y a la vez complejo, numerosos estudios se han llevado a cabo para determinar los diferentes pasos que lo conforman. El conocimiento del modo de acción de las proteínas de Bt supone un elemento clave para diseñar estrategias que retrasen la aparición de insectos resistentes en campo, asegurando su uso de una forma prolongada.

PALABRAS CLAVE: entomopatógeno; proteínas insecticidas; unión a receptor; lisis celular; control integrado de plagas.

INTRODUCCIÓN

El uso de insecticidas químicos sigue siendo hoy en día mayor que el uso de insecticidas biológicos. Sin embargo, durante los últimos años la preocupación por los daños ocasionados al medio ambiente y los efectos tóxicos derivados del uso excesivo de los insecticidas de tipo químico han favorecido el aumento del uso de insecticidas biológicos o el uso combinado de ambos, también conocido como control integrado de plagas. Una de las ventajas de emplear microorganismos entomopatógenos (por ejemplo: baculovirus, hongos o bacterias) es su alta especificidad de acción, ya que afectan únicamente a un número reducido de organismos (organismos diana). *Bacillus thuringiensis* (Bt) es una bacteria entomopatógena que tiene la habilidad de producir una amplia variedad de proteínas insecticidas activas para distintos órdenes de insectos (Palma *et al.*, 2014). Durante la fase de esporulación produce una o varias inclusiones cristalinas que contienen las denominadas proteínas "Cry" y "Cyt" (del inglés *crystal y cytolytic*). Mientras que, durante la fase vegetativa, la bacteria produce unas proteínas insecticidas denominadas proteínas Vip (del inglés *vegetative insecticidal proteins*). Desde el punto de vista de control de plagas, las principales toxinas utilizadas son las proteínas formadoras de poro Cry y Vip por su alta especificidad de acción. Por otra parte, el mecanismo molecular de acción de las proteínas citolíticas Cyt ha sido poco estudiado, pese a su uso en el control de dípteros, por lo que no se han incluido en esta revisión. Con fines biotecnológicos, la mezcla de esporas y cristales de diferentes aislados de Bt se ha comercializado en diferentes productos para el control de larvas de lepidópteros, escarabajos y mosquitos (Roh *et al.*, 2007). Por último, genes que codifican para algunas proteínas Cry o Vip3A también se han introducido en cultivos, generándose así los cultivos transgénicos (o cultivos Bt) (Sanchis, 2011).

El estudio del modo de acción de Bt se ha hecho necesario para comprender cómo se puede desarrollar resistencia frente a estas proteínas, además de permitir la introducción de mejoras en los productos ya existentes. En este trabajo revisamos los mecanismos de acción de las proteínas formadoras de poro de Bt más relevantes (presentes en productos o plantas Bt), ya que se han estudiado en mayor profundidad debido a su interés comercial.

1. INGESTIÓN

El primer paso en el modo de acción de Bt supone la ingestión por parte de las especies susceptibles a su acción (**Figura 1**, paso 1). Cabe destacar que, a diferencia de otros tratamientos insecticidas, tanto Bt como sus proteínas, bien sean presentes en formulados o por su expresión en plantas transgénicas, actúan exclusivamente previa ingestión del insecto, y nunca por contacto en superficie.

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Article

Reduced Membrane-Bound Alkaline Phosphatase Does Not Affect Binding of Vip3Aa in a *Heliothis virescens* Resistant Colony

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Abstract: The Vip3Aa insecticidal protein from *Bacillus thuringiensis* (Bt) is produced by specific transgenic corn and cotton varieties for efficient control of target lepidopteran pests. The main threat to this technology is the evolution of resistance in targeted insect pests and understanding the mechanistic basis of resistance is crucial to deploy the most appropriate strategies for resistance management. In this work, we tested whether alteration of membrane receptors in the insect midgut might explain the >2000-fold Vip3Aa resistance phenotype in a laboratory-selected colony of *Heliothis virescens* (Vip-Sel). Binding of ^{125}I-labeled Vip3Aa to brush border membrane vesicles (BBMV) from 3rd instar larvae from Vip-Sel was not significantly different from binding in the reference susceptible colony. Interestingly, BBMV from Vip-Sel larvae showed dramatically reduced levels of membrane-bound alkaline phosphatase (mALP) activity, which was further confirmed by a strong downregulation of the membrane-bound alkaline phosphatase 1 (*HvmALP1*) gene. However, the involvement of HvmALP1 as a receptor for the Vip3Aa protein was not supported by results from ligand blotting and viability assays with insect cells expressing *HvmALP1*.

Keywords: *Bacillus thuringiensis*; insecticidal proteins; insect resistance; tobacco budworm

Key Contribution: The biochemical characterization of a Vip3Aa-resistant colony of *H. virescens* shows that binding to receptors in the midgut is not affected and discards the role of mALP as a Vip3Aa receptor. This study suggests that Vip3A resistance may occur through mechanisms other than those commonly found for Cry proteins.

1. Introduction

The polyphagous pest *Heliothis virescens* (L.) (Lepidoptera: Noctuidae) is well known for producing substantial economic losses, particularly in cotton production, due to its ability to evolve resistance to different synthetic control products such as methyl parathion or pyrethroids [1,2]. As an alternative approach, genetically modified crops expressing Cry and Vip3A insecticidal protein genes from *Bacillus thuringiensis* (Bt crops) were introduced in 1996 for the control of this and other pests. However,

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REVIEW

Response Mechanisms of Invertebrates to *Bacillus thuringiensis* and Its Pesticidal Proteins

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Daniel Pinos and Ascensión Andrés-Garrido contributed equally to this article. Author order was determined by drawing straws.

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SUMMARY Extensive use of chemical insecticides adversely affects both environment and human health. One of the most popular biological pest control alternatives is bioinsecticides based on *Bacillus thuringiensis*. This entomopathogenic bacterium produces different protein types which are toxic to several insect, mite, and nematode species. Currently, insecticidal proteins belonging to the Cry and Vip3 groups are widely used to control insect pests both in formulated sprays and in transgenic crops. However, the benefits of *B. thuringiensis*-based products are threatened by insect resistance evolution. Numerous studies have highlighted that mutations in genes coding for surrogate receptors are responsible for conferring resistance to *B. thuringiensis*. Nevertheless, other mechanisms may also contribute to the reduction of the effectiveness of *B. thuringiensis*-based products for managing insect pests and even to the acquisition of resistance. Here, we review the relevant literature reporting how invertebrates (mainly insects and *Caenorhabditis elegans*) respond to exposure to *B. thuringiensis* as either whole bacteria, spores, and/or its pesticidal proteins.

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Research Article

Hetero-oligomerization of *Bacillus thuringiensis* Cry1A proteins enhance binding to the ABCC2 transporter of *Spodoptera exigua*

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The ATP binding cassette (ABC) transporters are membrane proteins that can act as putative receptors for Cry proteins from *Bacillus thuringiensis* (Bt) in the midgut of different insects. For the beet armyworm, *Spodoptera exigua*, ABCC2 and ABCC3 have been found to interact with Cry1A proteins, the main insecticidal proteins used in Bt crops, as well as Bt-based pesticides. The ABCC2 has shown to have specific binding towards Cry1Ac and is involved in the toxic process of Cry1A proteins, but the role of this transporter and how it relates with the Cry1A proteins is still unknown. Here, we have characterized the interactions between the SeABCC2 and the main proteins that bind to the receptor. By labeling the Cry1Aa protein, we have found that virtually all of the binding is in an oligomeric state, a conformation that allowed higher levels of specific binding that could not be achieved by the monomeric protein on its own. Furthermore, we have observed that Cry1A proteins can hetero-oligomerize in the presence of the transporter, which is reflected in an increase in binding and toxicity to SeABCC2-expressing cells. This synergism can be one of the reasons why *B. thuringiensis* co-expresses different Cry1 proteins that can apparently have similar binding preferences. The results from *in vitro* competition and *ex vivo* competition showed that Cry1Aa, Cry1Ab and Cry1Ac share functional binding sites. By using Cry1Ab–Cry1Ac chimeras, the presence of domain I from Cry1A proteins was revealed to be critical for oligomer formation.

Introduction

The insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) have been largely used to control different pests in agricultural crops. The most common Bt proteins to control lepidopterans both in Bt crops and in formulated sprays are the Cry1A proteins, being these one of the best characterized group of proteins [1,2]. The proposed mode of action of Cry1A proteins starts in the midgut of the larva after the ingestion in the protoxin form. Protoxins are solubilized and partially digested by midgut enzymes to render the active form. Then, the activated proteins, as monomers, undergo a complex sequence of binding events with several midgut proteins that lead to the cleavage of helix α1 (located in Domain I) causing the oligomerization of monomers [3]. Next, the oligomer binds to different putative receptors and causes pores in midgut cells that cause the disruption of the membranes, leading to septicemia and insect death [4].

The ATP binding cassette (ABC) transporters are a family of primary-active transporters with an increasing number of members that have been shown to be used as receptors by Cry proteins, mainly Cry1, Cry2, Cry3 and Cry8 proteins [5]. The approaches used to characterize the role of these ABC transporters with Cry proteins have been diverse, from the expression of the transporters in insect or human cell lines to their silencing or knock-out in live insects [6–8]. In *Spodoptera* spp., the ABCC2 and the ABCC3 transporters have been characterized as putative receptors for certain Cry1A proteins [6–12]. Martínez-Solís et al. [6] investigated the role of SeABCC2 in the mode of action of Cry1Aa,

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Article

Alteration of a Cry1A Shared Binding Site in a Cry1Ab-Selected Colony of *Ostrinia furnacalis*

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Abstract: The Asian corn borer, *Ostrinia furnacalis* (Guenée, 1854), is a highly damaging pest in Asia and the Pacific islands, and larvae feed mainly from corn crops. To determine the suitability of Bt-corn technology for the future control of this pest, understanding the potential to develop resistance to Cry1Ab and the basis of cross-resistance to other Cry1 proteins is of great interest. Here, we have explored the binding of Cry1A proteins to brush border membrane vesicles from two *O. furnacalis* colonies, one susceptible (ACB-BtS) and one laboratory-selected with Cry1Ab (ACB-AbR). The insects developed resistance to Cry1Ab and showed cross-resistance to Cry1Aa, Cry1Ac, and Cry1F. Binding assays with radiolabeled Cry1Ab and brush border membrane vesicles from susceptible insects showed that Cry1A proteins shared binding sites, though the results were not conclusive for Cry1F. The results were confirmed using radiolabeled Cry1Aa. The resistant insects showed a reduction of the specific binding of both Cry1Ab and Cry1Aa, suggesting that part of the binding sites were lost or altered. Competition binding assays showed full competition between Cry1Ab and Cry1Aa proteins in the susceptible colony but only partial competition in resistant insects, confirming the alteration of some, but not all, binding sites for these two proteins. The binding site model for Cry1A proteins in *O. furnacalis* is in agreement with the occurrence of multiple membrane receptors for these proteins.

Keywords: Asian corn borer; *Bacillus thuringiensis*; Cry1 toxins; binding site model; pyramid strategy

Key Contribution: We have shown that Cry1A proteins share binding sites in the larva midgut of *O. furnacalis*. The alteration of a common binding site may confer cross-resistance to these proteins.

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1. Introduction

Bacillus thuringiensis (Bt) is known to produce many insecticidal proteins that, either as Bt-based pesticides or expressed in genetically modified crops, can effectively control different insect pests [1]. One highly effective tool to control stem borers is Bt corn, which co-expresses different Bt proteins, mainly from the Cry1 family [2]. The adoption of Bt corn expressing Cry1Ab has quickly expanded globally since it has been demonstrated to control the European corn borer, *Ostrinia nubilalis*, and other pests [3]. Bt corn is also considered a promising technology to control the Asian corn borer, *Ostrinia furnacalis*, a highly damaging insect that affects mainly this crop [4,5].

The mode of action of Bt insecticidal proteins involves, among other steps, binding to membrane molecules in the midgut (referred to as "receptors"). This step is not only responsible for the specificity of the toxic action, but it is also the main step responsible for developing high levels of resistance by alteration of the membrane receptor [6]. Competition binding studies, in which the inhibition of binding of a labeled protein by different proteins is determined, have provided models showing whether Bt proteins bind to more than one site and whether different proteins share binding sites. These binding site models have