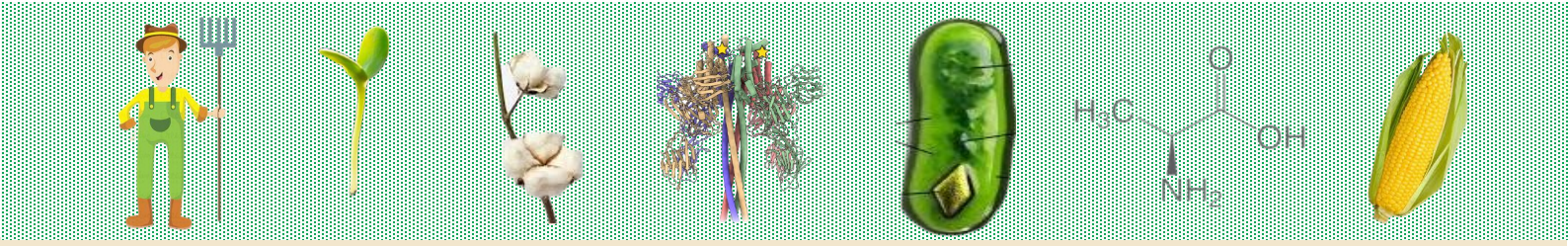

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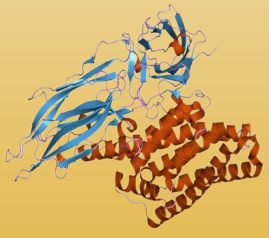
PhD Thesis

July 2021

Yudong Quan



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Informa:
Que Yudong Quan Llicenciat en Protecció de Plantes, ha realitzat sota la seva direcció el treball d'investigació recollit en esta memoria que porta per títol "Studies on the insecticidal mechanism of *Bacillus thuringiensis* Vip3A and Cry proteins", per tal d'optar al Grau de Doctor per la Universitat de València.

I per fer constància, d'acord amb la legislació vigent, signa la present a Burjassot, a 26 de Juliol de 2021.

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Informa:
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INDEX OF CONTENTS

Resumen

El control de plagas y patógenos ha tenido un efecto importante en la mejora del rendimiento de los sistemas agrícolas a nivel mundial. Diferentes tipos de insecticidas químicos se han usado extensivamente durante mucho tiempo para el control de plagas de insectos. Debido a la aparición de resistencias, problemas de contaminación de aguas y problemas de salud humana causados por dichos insecticidas de síntesis, la agricultura moderna necesita una estrategia de gestión integrada de plagas más saludable, respetuosa con el medio ambiente y sostenible. El uso de *Bacillus thuringiensis* (*Bt*) y sus proteínas insecticidas para el control de plagas es una de las estrategias biotecnológicas más importantes hasta la fecha. Además, los genes que codifican sus proteínas insecticidas han sido transferidos a plantas, las cuales están siendo utilizadas comercialmente, desde 1996 en gran parte del mundo para el control eficiente de numerosas plagas de insectos. En los últimos años, una nueva subclase de proteínas insecticidas secretables (Vip3) producida durante el crecimiento vegetativo de *Bt* se ha considerado para la aplicación combinada con las convencionalmente empleadas proteínas Cry, cuya aplicación se ve amenazada por la aparición de poblaciones de insectos resistentes. Las proteínas Vip3 no tienen homología de secuencia con las proteínas Cry y son tóxicas para insectos lepidópteros, sin embargo, su modo de acción todavía no se conoce completamente. En este proyecto de tesis, con el objetivo de mejorar su aplicación en el control biotecnológico de plagas y la comprensión del modo de acción de las proteínas Vip3, se estudiaron diversos aspectos de su actividad insecticida (espectro de acción, resistencia cruzada e interacción con otras proteínas), y se realizó un estudio de los residuos clave para el mantenimiento de la estructura tridimensional y la toxicidad de la proteína Vip3Af mediante mutagénesis dirigida. También analizamos la posible implicación de la unión a receptores en la aparición de resistencia utilizando una cepa resistente que había sido seleccionada con Vip3Aa.

En primer lugar, se investigó la toxicidad de 10 toxinas Bt (Cry1Ab, Cry1Ac, Cry1Ah, Cry1Fa, Cry2Aa, Cry2b, Cry1Ie, Vip3Aa19, Vip3Aa16 y Vip3Ca) frente a *Mythimna separata* (plaga agrícola muy destructiva en Asia y Australia), así como su aplicación combinada mediante bioensayos llevados a cabo en laboratorio. Los resultados mostraron que la concentración letal media LC_{50} (Cry1Ac/Vip3Aa19/Vip3Ca < Cry1Ab/Cry2Aa/Vip3Aa16 < Cry2Ab/Cry1Fa/Cry1Ah < Cry1Ie) osciló entre 1,6 y 78,6 µg/g (toxina/dieta). Además, sólo se detectaron efectos sinérgicos para las combinaciones entre la proteína Vip3Aa16 y proteínas Cry1. Las combinaciones de Vip3Aa16 con Cry1Fa y Vip3Aa16 con Cry1Ie mostraron un aumento de toxicidad (o factor sinérgico) de 6,3 a 9,2 veces más de lo esperado. También se probó la toxicidad de dos proteínas Vip3 (Vip3Aa y Vip3Ca) en diferentes insectos resistentes a Cry1A y Dipel (*Helicoverpa armigera, Trichoplusia ni, Ostrinia furnacalis* y *Plodia interpunctella*), resistentes a Cry2A (*H. armigera* y *T. ni*) y resistentes a Vip3 (*H. armigera*). No se detectó resistencia cruzada para las proteínas Vip3 en insectos resistentes a proteínas Cry1A, Cry2A y Dipel, pero sí se detectó resistencia cruzada a Vip3Ca en la cepa de *H. armigera* resitente a Vip3Aa y Cry2b. Además, la proteína Vip3Aa provocó una inhibición moderada del crecimiento en *O. furnacalis* (sólo a la concentración máxima de ensayo (100 µg/g)), mientras que la proteína Vip3Ca mostró ser altamente tóxica frente a esta especie. Por otra parte, una cepa de *M. separata* resistente a Vip3Aa se obtuvo y se caracterizó la toxicidad ejercida por las proteínas Cry1Ab y Cry1F, confirmando la ausencia de resistencia cruzada entre las proteínas Cry1 y Vip3Aa.

Estos resultados concuerdan con los de estudios previos que muestran la falta de resistencia cruzada entre las proteínas Vip3 y Cry en diferentes especies, por el contrario, se encontró una fuerte resistencia cruzada entre Vip3Ca y Vip3Aa en *H. armigera* (una cepa resistente seleccionada con

Vip3Aa). Varios estudios demostraron que los sitios de unión juegan un papel crítico en el mecanismo de resistencia a Cry, pero Vip3Aa no comparte sitios de unión con las proteínas Cry1 o Cry2. Nuestros resultados sobre la falta de resistencia cruzada entre las proteínas Vip3 y Cry están de acuerdo con las diferencias en el modo de acción de las mismas. Además, a diferencia de la proteína Vip3Aa, Vip3Ca fue muy activa para las larvas de *O. furnacalis* (tanto susceptibles como resistentes a Cry1Ab), lo que permitió descubrir que Vip3Ca es una toxina potencial en el control de *O. furnacalis*.

Las proteínas Vip3A no comparten secuencia ni homología estructural con las proteínas Cry, son de interés práctico debido a su eficaz actividad insecticida contra una amplia gama de insectos lepidópteros, e incluso muestran una potente actividad frente a algunas plagas agronómicamente importantes que tienen poca o ninguna susceptibilidad a las proteínas Cry. Ello las convierte en candidatos potenciales para complementar las proteínas Cry en cultivos *Bt* para ampliar el espectro insecticida y con fines del manejo de la resistencia. Recientemente, el modo de acción propuesto de las proteínas Vip3 consiste en que, una vez ingeridas (protoxinas de Vip3) por las larvas de lepidópteros, éstas son procesadas por las proteasas intestinales dando lugar a un corte en la proteína, atraviesan la membrana peritrófica, reconocen y se unen a los receptores en la membrana epitelial del intestino medio y, mediante una serie de pasos todavía desconocidos, dan lugar a poros en la membrana, lo cual produce la parálisis y degeneración completa de las células del epitelio intestinal y finalmente a la muerte del insecto. Sin embargo, algunos detalles están todavía por dilucidar.

Con el objetivo de arrojar luz sobre los dominios funcionales y estructurales putativos de las proteínas Vip3, hemos utilizado mutantes de alanina Vip3Af seleccionados por su pérdida de toxicidad, identificados a partir de un trabajo previo. La mayoría de estos mutantes se distribuyen en tres clusters a lo largo de la proteína (N-terminal, medio y C-terminal) y muestran patrones proteolíticos alterados tras la digestión con tripsina. En este estudio, analizamos el efecto de mutaciones seleccionadas sobre la estructura tridimensional de la proteína mediante cromatografía de filtración en gel, tanto para la protoxina sin activar, como después de tratada con tripsina. Los cromatogramas de dichos mutantes mostraron que Vip3Af tipo salvaje (wt) era un tetrámero de pH 7,4 a 10,0, y no se vio afectado por el tratamiento con tripsina (a pH 9,0). Posteriormente, basándonos en los patrones de digestión de proteasas, su efecto sobre la formación de oligómeros y los sitios de escisión teóricos, junto con el de trabajos anteriores, pudimos generar un mapa de la proteína Vip3Af con cinco dominios: dominio I rangos de aminoácidos (aa) 12-198, dominio II aa199–313, dominio III aa314–526, dominio IV aa527–668 y dominio V aa669–788. La comparación de los distintos mutantes en cuanto a su capacidad de formar tetrámeros indicaron que el dominio I-III es necesario para la oligomerización, mientras que el dominio V no lo es y el papel del dominio IV en este aspecto del modo de acción de la proteína no está claro.

En un estudio posterior, con la finalidad de producir variantes de la proteína Vip3Af con actividad insecticida incrementada, se generaron 12 nuevos mutantes de la proteína Vip3Af por mutagénesis dirigida en residuos puntuales clave para la integridad del oligómero y mutaciones puntuales en el extremo N-terminal. Nueve de estos mutantes (M34L, K284Q, E483D, E483H, W552H, W552F, N682K-G689S, G689S, G689E) se clonaron y expresaron con éxito. Las mutaciones M34L y K284Q se eligieron a partir de un análisis mediante comparación de secuencias de proteínas entre diferentes proteínas Vip3, mientras que el resto se eligió en función de los residuos clave que previamente se vio que afectaban significativamente la actividad insecticida. Los nuevos mutantes se probaron contra tres insectos plaga (*S. frugiperda, Spodoptera littoralis* y *Grapholita molesta*). Los resultados revelaron que el mutante M34L (Met34 a Leu34) aumentó significativamente su toxicidad frente a *S. littoralis*,

aunque no hubo cambios significativos con *S. frugiperda* o *G. molesta*. El resto de mutantes no mejoraron (K284Q, E483D, W552F) o incluso disminuyeron (E483H, W552H, N682K-G689S, G689S, G689E) su capacidad tóxica frente a estas especies plaga. La estabilidad de estos mutantes se estudió mediante la realización de perfiles de tripsinización *in vitro* en SDS–PAGE. Los resultados indicaron que, para no afectar a la estructura de la proteína, la posición 483 requiere la presencia de un residuo ácido, mientras que la posición 552 requiere un residuo aromático. Para el resto de posiciones estudiadas no se encontró la necesidad de un tipo de residuo concreto. Met^{34} es un residuo altamente conservado entre la mayoría de las proteínas Vip3 y se encuentra en una región hidrófoba de la hélice α1 que se acopla al núcleo del tetrámero en una cavidad formada por hélices α del mismo monómero. El cambio de Met^{34} a Leu^{34} no alteró el patrón proteolítico, lo que indica que el plegamiento de la proteína no se vio afectado. Pero el aumento significativo de actividad en *S. littoralis* (M34L) indicó que el aminoácido leucina tiene una preferencia helicoidal más alta que la metionina y tiene un valor de hidrofobicidad más alto que contribuye a una mayor estabilización de la hélice α. Con la creciente demanda de una agricultura más sostenible y el tiempo y los costos económicos que suponen el desarrollo de nuevas sustancias activas, la ingeniería de proteínas es una estrategia muy conveniente.

El procesamiento proteolítico es un paso bien establecido en el modo de acción insecticida de las proteínas Vip3. Sin embargo, estudios preliminares del análisis de los productos proteolíticos por SDS-PAGE dieron lugar a resultados engañosos en cuanto a que parecía que la proteína se degradara completamente. La observación de que, a mayor tiempo de incubación con tripsina, menor degradación, hizo pensar en un artefacto de la técnica. Para comprobar si la degradación era meramente aparente o era real, analizamos la estabilidad de los fragmentos proteolíticos (proteína Vip3Af activada) digeridos por tripsina comercial y jugo de intestino medio de *S. frugiperda in vitro*, en ausencia y en presencia de varios inhibidores de proteasas. Los resultados revelaron que se observaban patrones de degradación falsos debido a que la preparación de la muestra antes de cargar en el gel de electroforesis hacía que la proteína cambiara de conformación (se "abriera") antes que la tripsina o las proteasas del jugo intestinal se inactivaran completamente, lo que daba lugar a la exposición de más sitios de corte que en la conformación nativa. El uso de uno de los inhibidores de proteasas, junto con la cromatografía de filtración en gel, revelaron que, en condiciones nativas (incubado con inhibidor antes de calentar para SDS-PAGE), la Vip3Af es estable frente a proteasas y se mantiene como tetrámero tanto en su forma de protoxina como de proteína activada. Esto explicaba por qué, a mayor tiempo de incubación con tripsina (uno o dos días frente a media hora), se obtenían menos degradación de la Vip3Af: la tripsina se había autodigerido antes de calentar la muestra en presencia de SDS. La identificación de los fragmentos proteolíticos reveló un sitio de escisión principal (aa 198/199) que da lugar a dos fragmentos de aproximadamente 20 kDa y 65 kDa (activación) que aún permanecen fuertemente unidos (formando parte del tetrámero) y que no se procesan más incluso a altas concentraciones de proteasa. De manera similar, previamente se había encontrado este efecto en la Vip3Aa, confirmando que ello es una particularidad común en las proteínas de la familia Vip3. Así mismo, y mediante esta misma técnica, se pudieron encontrar sitios de tripsinización secundarios en la proteína Vip3Aa. La identidad de estos fragmentos se confirmó en dos sitios de escisión secundarios adicionales en el fragmento de 65 kDa que dan lugar a dos fragmentos menores de aprox. 45 kDa y 33 kDa.

Aunque está bien aceptado que la actividad de Vip3 requiere la unión a receptores de membrana en el intestino medio de los insectos diana, se sabe muy poco sobre cómo las proteínas Vip3 interactúan con los receptores de membrana y qué dominios Vip3 están involucrados. La comprensión del papel de la unión específica a los receptores de membrana es fundamental para determinar su

especificidad. Como uno de los objetivos de esta tesis nos propusimos encontrar las condiciones idóneas para analizar la unión de ^{125}I-Vip3Af a vesículas de membrana de borde en cepillo (BBMV) de larvas de insectos susceptibles a dicha toxina. Las especies escogidas fueron *S. frugiperda* y *Spodoptera exigua*. Intentos previos en nuestro grupo de investigación fueron infructuosos en cuanto a poder demostrar la unión específica de la Vip3Af a BBMV de estas especies, incluso utilizando las mismas condiciones que se habían puesto a punto para la Vip3Aa. Finalmente pudimos comprobar que la presencia de NaCl interfería en la unión de manera que, eliminando esta sal en el tampón usado en la reacción de unión ligando-receptor, dio lugar a la obtención de las condiciones apropiadas para demostrar la unión específica de la toxina Vip3Af a las BBMV de dichas especies. La unión específica se demostró en ensayos de competencia de la toxina marcada con concentraciones crecientes de la misma toxina no marcada (competidor homólogo). Una vez establecidas estas condiciones, pudimos realizar competencias heterólogas (usando como competidor una toxina distinta a la marcada). Los resultados revelaron que Cry1Ac y Cry1F no competían por los sitios de unión de Vip3Af. Sin embargo, Vip3Aa compitió completamente con la Vip3Af marcada, lo que indica que ambas comparten los mismos sitios de unión en la membrana. Usando Vip3Ca como competidor heterólogo observamos que, aunque había competencia por los sitios de Vip3Af, ésta no era completa, es decir, Vip3Ca no bloqueaba completamente la unión de Vip3Af a las BBMV, aunque sí parcialmente. Esto significa que comparte ciertos sitios pero no todos los utilizados por Vip3Af. Los estudios de unión por competencias heterólogas son una herramienta potente que proporciona información sobre el potencial de resistencia cruzada entre las toxinas *Bt*, ya que la alteración de los sitios de unión es el principal mecanismo de resistencia a las proteínas Cry.

Todos los estudios realizados con insectos resistentes a las proteínas Cry o Vip3A han demostrado una falta de resistencia cruzada significativa entre estas dos familias de proteínas, lo que está respaldado por el hecho de que no comparten sitios de unión. Nuestros resultados con *S. exigua* y *S. frugiperda* apoyan la ausencia de sitios de unión compartidos entre las proteínas Cry1 y Vip3. El hecho de haber encontrado sitios comunes para las proteínas Vip3 respalda el hecho de que se haya encontrado resistencia cruzada entre Vip3Aa y Vip3Ca.

Mediante el uso de mutantes Ala de Vip3Af inestables a la tripsina, hemos podido generar y purificar moléculas truncadas de Vip3Af con una composición de dominios diferente, aun así manteniendo la estructura tetramérica. Esta estructura se mantiene siempre y cuando se mantengan los dominios I a III. La falta de dominios IV-V no afecta a que se pueda mantener dicha estructura. A partir de tres mutantes Ala de Vip3Af (W552A, I699A y F229A) pudimos generar las moléculas DI-III y DI-IV, que mantienen la estructura tetramérica, y las moléculas DIV y DIV-V. Los dominios IV y V contienen los módulos de unión de carbohidratos y no pueden formar estructuras tetraméricas en ausencia del resto de la proteína. Usando estas moléculas Vip3Af truncadas (DI-III, DI-IV, DIV-V y DIV), producidas por tratamiento con tripsina, encontramos que solo aquellas moléculas que contienen los dominios I a III (DI-III y DI-IV) pudieron competir con la proteína ^{125}I-Vip3Af activada y que las moléculas que solo contenían el dominio IV o los dominios IV y V (DIV y DIV-V) no pudieron competir con Vip3Af. Estos resultados indican que la estructura tetramérica es crucial para poder unirse a las BBMV. Posteriormente realizamos estudios similares de competencias usando la proteína truncada ^{125}I-DI-III. Solo aquellas moléculas que retienen los dominios I a III fueron capaces de competir con la molécula ^{125}I-DI-III por la unión de BBMV. Del mismo modo, y al igual que con ^{125}I-Vip3Af, las proteínas nativas Vip3Af y Vip3Aa desplazaron completamente la unión de la molécula truncada de ^{125}I-DI-III, mientras que Vip3Ca compitió solo parcialmente. También hemos analizado el

papel funcional de las moléculas truncadas midiendo su toxicidad para las células Sf21. De acuerdo con los datos de unión, solo la proteína Vip3Af activada con tripsina y las moléculas truncadas que mantienen los dominios I a III eran tóxicas para estas células, lo que indica que la unión observada a las células era funcional. Nuestros resultados están de acuerdo con los publicados con fragmentos de Vip3Aa clonados usando ensayos de unión celular basados en fluorescencia con células Sf9 (aunque no demostraron su funcionalidad). Los resultados de la unión *in vitro* a BBMV y a las células Sf21, junto con el análisis funcional (toxicidad para las células Sf21), junto con los resultados de otros autores, indican claramente que los dominios I a III son fundamentales para mantener el núcleo funcional de las proteínas Vip3. El dominio III contiene tres láminas antiparalelas que forman un pliegue de prisma β sorprendentemente similar al que se encuentra en las proteínas Cry. Por lo tanto, es un candidato fuerte para ser el dominio que interactúa con el receptor de membrana, ya sea solo o en combinación con el dominio II o los dominios I + II. Estos resultados proporcionan evidencia del papel crítico de los dominios N-terminales (dominios I a III) en el modo de acción de Vip3Af y probablemente todas las proteínas Vip3.

Aunque los dominios variables C-terminales IV y V no parecen desempeñar un papel en la unión *in vitro* y en la toxicidad de las células de cultivo Sf21, se ha demostrado ampliamente que las mutaciones en esos dominios disminuyen drásticamente la actividad insecticida de la proteína Vip3A. El que su presencia sea necesaria para la toxicidad *in vivo* podría deberse a que los dominios IV y V puedan tener la función de acercar la toxina a la membrana uniéndose a abundantes moléculas glicosiladas (que pueden no estar presentes en las células Sf21), de forma similar a la función de la aminopeptidasa N con la proteína Cry. También es probable que su función *in vivo* sea aumentar la estabilidad de la estructura tetramérica frente a proteasas del lumen.

El estudio de la capacidad de las poblaciones de insectos para desarrollar resistencia es importante para preservar el uso de productos insecticidas *Bt* y de cultivos *Bt*. La información sobre las bases genéticas y bioquímicas de la resistencia nos proporciona herramientas para poder hacer frente a esta potencial amenaza. A falta de poblaciones resistentes de campo, es necesario utilizar poblaciones resistentes obtenidas en laboratorio para poder estudiar los mecanismos moleculares implicados en la resistencia. En el presente estudio se sometieron tres subpoblaciones de *M. separata*, recolectadas en campo, a selección en laboratorio con proteínas Vip3Aa, Cry1Ab y Cry1F. Estas proteínas están siendo actualmente utilizadas en cultivos comerciales de maíz *Bt*, el cual presenta una alta protección frente a insectos plaga del maíz. Sorprendentemente, la cepa de *M. separata* resistente a Vip3Aa (> 3061 veces) se obtuvo rápidamente después de 8 o 9 generaciones de selección en laboratorio. Sin embargo, no se obtuvo resistencia notable seleccionando con Cry1Ab o Cry1F en la misma población y durante el mismo número de generaciones. En un estudio realizado por otros investigadores, también se encontró una respuesta rápida similar a la selección de Vip3Aa en *H. virescens*, alcanzando un nivel de resistencia > 2300 veces mayor en la décima generación. Es importante hacer notar que esta rápida evolución de la selección en condiciones de laboratorio contrasta con los resultados obtenidos con las proteínas Cry1, tanto en nuestro trabajo como por otros autores: una la población de *O. furnacalis* adquirió un nivel de resistencia a Cry1Ab de alrededor de 100 veces sólo después de 35 generaciones de selección; de manera similar, una población de *O. nubilalis* desarrolló una resistencia de más de 3000 veces a Cry1F después de 35 generaciones de selección. Esta diferencia en respuesta a la selección, además de reflejar una frecuencia mucho mayor de alelos de resistencia para Vip3Aa, puede sugerir diferencias en los mecanismos de resistencia a las proteínas Vip3Aa y Cry1, lo cual queda en evidencia cuando se estudia la unión de Vip3A a BBMV de insectos resistentes.

El análisis de la unión de ^{125}I-Vip3Aa a BBMV de larvas de *M. separata* tanto de insectos susceptibles y resistentes no reveló ninguna diferencia de unión, ya sea cualitativa o cuantitativa. Los resultados sugieren que la unión alterada a los receptores de la membrana del intestino medio no es el principal mecanismo de resistencia a la proteína Vip3Aa. Numerosos estudios han demostrado que la alteración de los receptores de membrana es un mecanismo evolutivo común que confiere altos niveles de resistencia a las proteínas Cry, pero nunca se ha establecido su relación con la resistencia a Vip3A. Las diferencias de unión cualitativas o cuantitativas entre insectos susceptibles y resistentes están en línea con los resultados anteriores con otras cepas resistentes a Vip3Aa de otras especies de insectos para las que no se encontraron diferencias de unión a Vip3Aa. Otros estudios han encontrado una activación más lenta de Vip3Aa por el jugo del intestino medio de las larvas de *H. armigera* en insectos resistentes a Vip3Aa comparados con los insectos susceptibles, aunque estas pequeñas diferencias no parecen ser la razón principal de los altos niveles de resistencia en dicha cepa. En otro estudio, larvas de *H. virescens* resistentes a Vip3Aa mostraron niveles drásticamente reducidos de fosfatasa alcalina unida a la membrana, pero no se pudo demostrar su participación en la resistencia. Por lo tanto, a diferencia de las proteínas Cry, la unión alterada a los receptores de membrana no parece ser el principal mecanismo de resistencia a las proteínas Vip3Aa, y deben explorarse otros mecanismos, tales como cambios en otros pasos en el modo de acción de Vip3Aa, ya sea antes (como la activación por proteasas o el secuestro de la toxina por la matriz peritrófica) o después (formación de poros, transducción de señales, apoptosis, ruptura de mitocondrias, etc.) de la unión a la membrana.

Estos resultados sugieren que los alelos para la resistencia a Vip3Aa parecen ser relativamente comunes y, por lo tanto, el uso de proteínas Vip3A solas, sin combinarlas con proteínas Cry, no es una estrategia adecuada para la implementación a largo plazo de esta tecnología en el control de plagas. La acción sinérgica encontrada para algunas combinaciones de proteínas Vip3Aa y Cry1 también favorece el uso combinado de estos dos tipos de proteínas insecticidas para un uso mejor y más prolongado de la tecnología de cultivos *Bt*.

En conclusión, en esta tesis analizamos el potencial insecticida de varias proteínas Vip3 y Cry, e intentamos ver la mejor estrategia de su uso combinado (o estrategia piramidal) en cultivos *Bt*. Los resultados de este estudio serán una referencia importante para subsiguientes desarrollos y aplicaciones de formulaciones de productos basados en *Bt*, así como los cultivos *Bt*. Además, los resultados de la identificación del mapa de cinco dominios de la proteína Vip3Af son importantes para entender la relación estructura-función de las proteínas Vip3. Los dominios I a III se han identificado como críticos para mantener el tetrámero y la unión específica a BBMV, que son fundamentales para arrojar luz sobre el modo de acción de las proteínas Vip3. Estos resultados han contribuido a la elucidación de su estructura 3D. Además, la mutación M34L podría incrementar la actividad insecticida, y la identificación de que el residuo 483 ha de ser un residuo ácido, mientras que el 552 un residuo aromático, nos abren la puerta a mejorar la actividad de las proteínas Vip3 mediante ingeniería genética teniendo en cuenta estas limitaciones. Los estudios de unión ligando-receptor, especialmente los de competencias heterólogas, han permitido elaborar un “modelo de receptores” que pone de manifiesto la no utilización conjunta de sitios de unión por las proteínas Cry1 y Vip3. El estudio de la resistencia cruzada llevado a cabo con cepas resistentes está del todo de acuerdo con nuestro “modelo de receptores”, en cuanto que no se ha encontrado resistencia cruzada entre proteínas Cry y Vip3. En general, los resultados de esta tesis contribuirán de manera importante al futuro uso y aplicación de las proteínas Vip3 para el beneficio de la agricultura y el medio ambiente.

Abstract

The improvement in the performances of global agricultural or food systems significantly affects by the control of pests and pathogens. The more healthy, environmental and sustainable integrated pest management (IPM) strategies are demanded in the modern agriculture. One of the most important strategy or biotechnology rely on *Bacillus thuringiensis* (*Bt*) and its secreted insecticidal proteins has been the most economically successful use to control pests to date. Recently, a new subfamily of vegetative insecticidal proteins (Vip3) produced during the vegetative growth phase of *Bt* was considered as the combined use with the other Bt proteins, especially that have been reported evolving distinct resistant (Cry toxins) with long-term commercial use. Despite Vip3 proteins have been revealed that no sequence or homology similarity with Cry proteins and are toxic to a wide variety of Lepidoptera, its mode of action is yet not completely elucidated. To better apply and understand the Vip3 proteins, in this doctoral we investigate the potential interaction of combination use (insecticidal spectrum, cross resistance, interaction), then we analyze the critical residues on the structure and toxicity through mutagenesis, and use these critical mutations to shed the light on the mode of action of Vip3 proteins.

Firstly we investigate ten Bt toxins (Cry1Ab, Cry1Ac, Cry1Ah, Cry1Fa, Cry2Aa, Cry2Ab, Cry1Ie, Vip3Aa19, Vip3Aa16, and Vip3Ca) toxicities and their combination of use at lab against *Mythimna separata*, which is a destructive pest of agricultural crops in East Asia. The bioassays results revealed that LC_{50} (lethal concentration for 50% mortality) values (Cry1Ac/Vip3Aa19/Vip3Ca < Cry1Ab/Cry2Aa/Vip3Aa16 < Cry2Ab/Cry1Fa/Cry1Ah < Cry1Ie) ranged from 1.6 to 78.6 μg/g (toxin/diet) for those toxins. In addition, the interactions between Vip3 and Cry proteins in this study indicated the synergism was tested only in the combination group Vip3Aa16 and Cry1 toxins, that the significant groups (Vip3Aa16 and Cry1Fa, Vip3Aa16 and Cry1Ie) showed around 6.3 to 9.2 fold (or synergetic factors) than expected. We also tested the toxicities of Vip3 (Vip3Aa and Vip3Ca) proteins against different Cry1A-, Cry2A-, Dipel- and Vip3-resistant insect species. Comparing the toxicities of Vip3 proteins between resistant Cry1A proteins, Dipel (*Helicoverpa armigera, Trichoplusia ni, Ostrinia furnacalis* and *Plodia interpunctella*) or Cry2Ab (*H. armigera* and *T. ni*) and susceptible strains, there were not cross-resistant detected in these insects. In contrast, the strong cross-resistance to the Vip3Ca protein was observed in Vip3Aa (or Vip3Aa/Cry2Ab) resistant *H. armigera* colonies. Moreover, Vip3Aa protein was tested almost lost the toxicity in *O. furnacalis* (only showed moderate growth inhibition at the highest concentration tested (100 μg/g)), but Vip3Ca protein was highly toxic. Besides, a colony of Vip3Aa resistant *M. separata* was obtained and also tested the susceptibility to Cry1Ab and Cry1F, it also suggested no cross resistance between Cry1 and Vip3Aa proteins.

To shed light on the structure of Vip3 proteins, we analyzed the trypsin fragmentation of several critical mutants (or patterns) of the Vip3Af protein obtained through alanine scanning mutagenesis. Based on protease digestion patterns, their effect on oligomer formation, and theoretical cleavage sites, we generated a map of the Vip3Af protein with five domains: domain I ranges amino acids (aa) 12–198, domain II aa199–313, domain III aa314–526, domain IV aa527–668, and domain V aa669–788. The effect of some mutations on the ability to form a tetrameric molecule revealed that domains I–III are required for tetramerization, while domain V is not, domain IV is not clear.

In addition, 12 mutants of the Vip3Af protein were generated by site-directed mutagenesis, which are from critical residues to constitute the integrity of oligomer and unique mutation (at N-terminal) only observed in some Vip3 proteins. Finally ten of these mutants were successfully expressed and

tested for stability and toxicity against three insect pests (*Spodoptera frugiperda*, *Spodoptera littoralis* and *Grapholita molesta*). Regarding toxicity, only the mutant M34L (change of Met^{34} to Lys^{34}) significantly increased the toxicity in *S. littoralis*, whereas the other mutants (or substitutions) did not improve, or even decreased. The profiles of these site- directed mutagenesis upon trypsin treatmen in the SDS-PAGE (stability) and toxicity showed that, residue 483 required an acidic residue, and residue 552 an aromatic residue, the others are still not clear.

The mode of action of Vip3 proteins is still debatable currently. To clear the details, we firstly analyzed the stability of proteolytic fragments (activated Vip3Af protein) digested by commercial trypsin and *S. frugiperda* midgut juice *in vitro*. The results revealed a misleading degradation patterns of Vip3Af was observed with trypsin or midgut juice in the SDS-PAGE. However, the gel filtration chromatography indicated that, under native conditions (incubated with inhibitor before heat for SDS-PAGE), Vip3Af is stable and as a tetramer for protoxin or activation. The identification of the proteolytic fragments suggested a cleavage site (aa 198/199) renders two fragments of approximately 20 kDa and 65 kDa (activation) which still strongly remain together (as tetramer) and that are no further processed even at high protease concentrations.

The understanding of the role of specific binding to membrane receptors is so critical to determine their specificity. In this part, we have set up a new binding condition of ^{125}I-Vip3Af to *S. frugiperda* and *Spodoptera exigua* brush border membrane vesicles (BBMV), and the specific binding was observed to BBMVs. Heterologous competitions revealed that, Cry1Ac and Cry1F did not compete for Vip3Af binding sites; Vip3Aa shares the same binding sites with Vip3Af, but that Vip3Ca merely shares partially. Using the truncated Vip3Af molecules (DI-III, DI-IV, DIV-V, and DIV) by trypsin treatment of selected critical alanine-mutants as competitors (compete with ^{125}I-Vip3Af), the results showed that only those molecules containing domains I to III (DI-III and DI-IV) were able to compete with the trypsin-activated Vip3Af protein for binding, and that molecules only containing either domain IV or domains IV and V (DIV and DIV-V) were unable to compete with Vip3Af. These results were further confirmed with competition experiments using ^{125}I-DI-III. In addition, cell viability assays showed that the truncated proteins DI-III and DI-IV were as toxic to Sf21 cells as the activated Vip3Af (or DI-V), DIV-V is totally lost the toxic (at the concentration C=100 ug/ml) suggesting that domains IV and V are not necessary for the toxicity to Sf21 cells. But the domains IV and V is necessary to the toxicity *in vivo*, and the function need further research.

Finally, a field population of *M. separata* was collected and subjected to laboratory selection with either Vip3Aa, Cry1Ab or Cry1F proteins. And only the Vip3Aa resistant (>3061-fold) *M. separata* strain was rapidly obtained after 8 or 9 generations, no remarkable Cry1Ab or Cry1F resistance was observed at the same selected time. Analysis of the difference of labeled ^{125}I-Vip3Aa binding to BBMVs from larvae from the susceptible and resistance insects revealed no any qualitative or quantitative binding difference. It suggests that altered binding to midgut membrane receptors is not the main mechanism of resistance to Vip3Aa protein.

The results we obtained are helpful to the applied strategy of Bt toxins and give to a better understanding of the protein structure and function of Vip3A proteins, which will be guided for the strategies in pest management or resistance management. In addition, the identification of the binding domains of Vip3A is so critical to shed light on the mode of action of Vip3 proteins.

Introduction

The pests and pathogens are widely recognized as significant obstacles to regular and reliable food systems, from overall production to physical availability, distribution, economic access, stability of production, quality and nutritive value (Strange et al., 2005; Savary et al., 2006; Esker et al., 2012; Savary et al., 2017). Globally, an estimated 20–40% of crop is lost to pests and pathogens (FAO, 2009), and pests represent a significant portion of this loss, both by direct damage and indirectly through the transmission of plant disease (Oerke, 2006; Cerda, 2017). The improvement in the performances of global agricultural or food systems significantly affects by the control of pests and pathogens (Savary et al., 2019) in modern agriculture. Reducing the burden of insect crop pests is a key priority, particularly since an estimated 70–100% increasing demand in global food production will be required by 2050 to feed the burgeoning human population (Deutsch et al., 2018).

Considering the history of the evolution of modern pest management (Fig. 1) (Sharma et al., 2020), since 1930s, the inorganic compounds have been used as pesticides to control the pests as the solution to reduce pest losses and enhance food production. Later, other type of pesticides were developed, such as DDT and other organic compounds (Wolff et al., 2007; World Health Organizatio [WHO], 2011; Debost-Legrand et al., 2016). Due to the intensification of agriculture and the long-term application of pesticides, scientists and farmers realized that some of the chemical insecticides used to control insect pests were extremely toxic to non-target organisms. In fact, chemical insecticides are recalcitrant, breaking down only slowly, leading to soil and water pollution, and were deleterious to the health of humans and animals, inducing important human diseases, such as cancer and immune system disorders (Kole, et al, 2001; Attathom, 2002; Pimentel, 2005; WHO, 2012, 2017; La Merrill et al., 2013; Obukhov et al., 2015). In addition, many insects also have developed resistance to different chemical pesticides, resulting in inefficient insect control programs (Devine and Furlong, 2007). The demand for novel strategies to control insect crop pests is particularly acute because the growing social consensus that should be met by sustainable intensification, increased productivity without ecological degradation have been more concerned and implemented in the modern agriculture. The new healthier and more environmental friendly biopesticides have been considered reduced risk pesticides at the end of 20th centry (http://www.marketsandmarkets.com/) (Leahy et al., 2014). And the further IRM (insect resistance management) strategies also relied on these safer and efficient biopesticides.

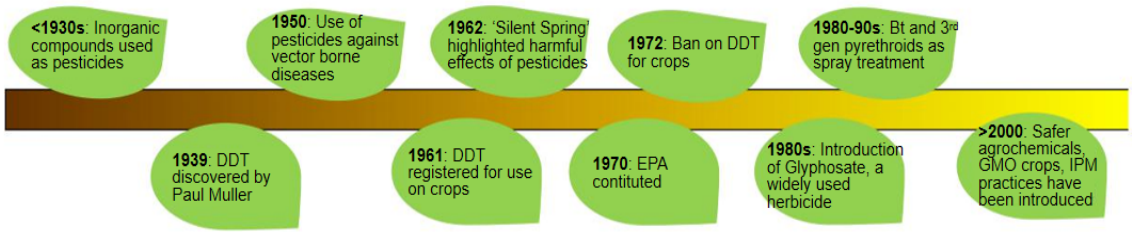


Figure 1. Evolution of modern pest management. A timeline of pesticide uses since early 1930s. Important discoveries are indicated at with their year. Harmful effects of few pesticides over the years have been described herein (Sharma et al., 2020).

1.1 Biopesticides

Biopesticides are developed from naturally occurring living organisms such as animals, plants, and microorganisms (bacteria, fungi, and viruses) that can control serious plant-damaging insect pests

(Glare et al., 2012). Biopesticides are classified into three different categories: (1) plant incorporated protectants, (2) microbial pesticides, and (3) biochemical pesticides (Chandler et al., 2008; Rajput et al., 2020). The efficacy of biopesticides can be similar to efficacy of conventional pesticides (chemical pesticides) in some crops like fruits, vegetables, nuts, and flowers (Kumar, 2019a). Microbial biopesticides are the fastest growing product segment of the global biocontrol industry, and nowadays there are hundreds of registered products enlisted in EPA (Lacey et al., 2015; EPA, 2017; Arthurs and Dara, 2019).

Microbial biopesticides are based on naturally occurring bacteria, fungi (including some protozoa, and yeasts) and viruses. They can be used by spraying or drenching, directly to the roots in the form of a tablet, coating of seeds or treating the roots before sowing (Montesinos and Bonaterra, 2009). In sustainable farming, microbial pesticides are the most favored, as the excessive use of chemicals degenerates not only the structure but also deteriorate the health of the soil (Kumar et al., 2019a, b; Singh and Yadav, 2020). Nowadays, the most widely microorganism used to develop microbial biopesticides is the insect pathogenic bacterium *Bacillus thuringiensis* (Lacey et al., 2015) (Fig. 2).

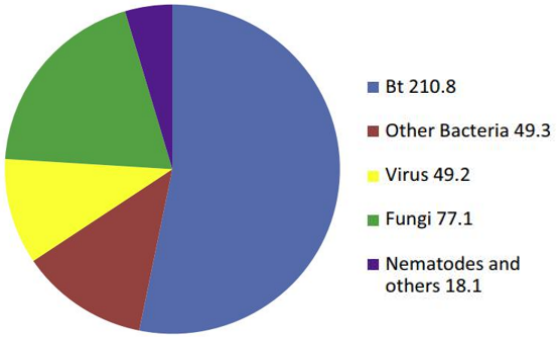


Figure 2. Types of microbial biopesiticide products used worldwide (Lacey et al., 2015).

1.2 *Bacillus thuringiensis*

Bacillus thuringiensis (Berliner) (Bt), an aerobic gram-positive bacterium, was discovered by Ishiwatta in 1902 in reared *Bombyx mori* and reisolated by Berliner, in 1911, from *Ephestia kuehniella* (De Barjac and Bonnefoi, 1986; Cannon, 1995) (Fig. 3). *B. thuringiensis* is an ubiquitous bacterium, which allows it to be found in a variety of sites, such as: soil, insects and their habitats, stored products, plants, forest, and aquatic environments, since it would remain latent in the adverse environment (Azevedo et al., 2000; Fiuza, 2001). *B. thuringiensis* life cycle includes two distinct phases: vegetative growth and sporulation (Bulla et al., 1980, Estruch et al., 1996; Ibrahim et al., 2010) (Fig. 3E). During these two phases, it can produce different types of insecticidal proteins, which some of them have been identified, and applied in the agriculture (e.g Vip3 and Cry). Bt products such as sprayable mixtures of Bt spores and parasporal crystals, or expressed the recombinant Bt or Bt toxins in other bacteria, account for 75-95% of the microbial biopesticide market (Olson, 2015). In addition, some insecticidal Bt genes (mainly *cry* and *vip3A*) are expressed in transgenic crops or genetically modified (GM) crops as plant-incorporated protectants to pest control in agriculture (Van Rie, 2000; Shelton et al., 2002). These transgenic Bt crops are commercially developed and planted in approximately 101 million hectares worldwide, representing >53% of the global GM crops cultivated area (James, 2017).

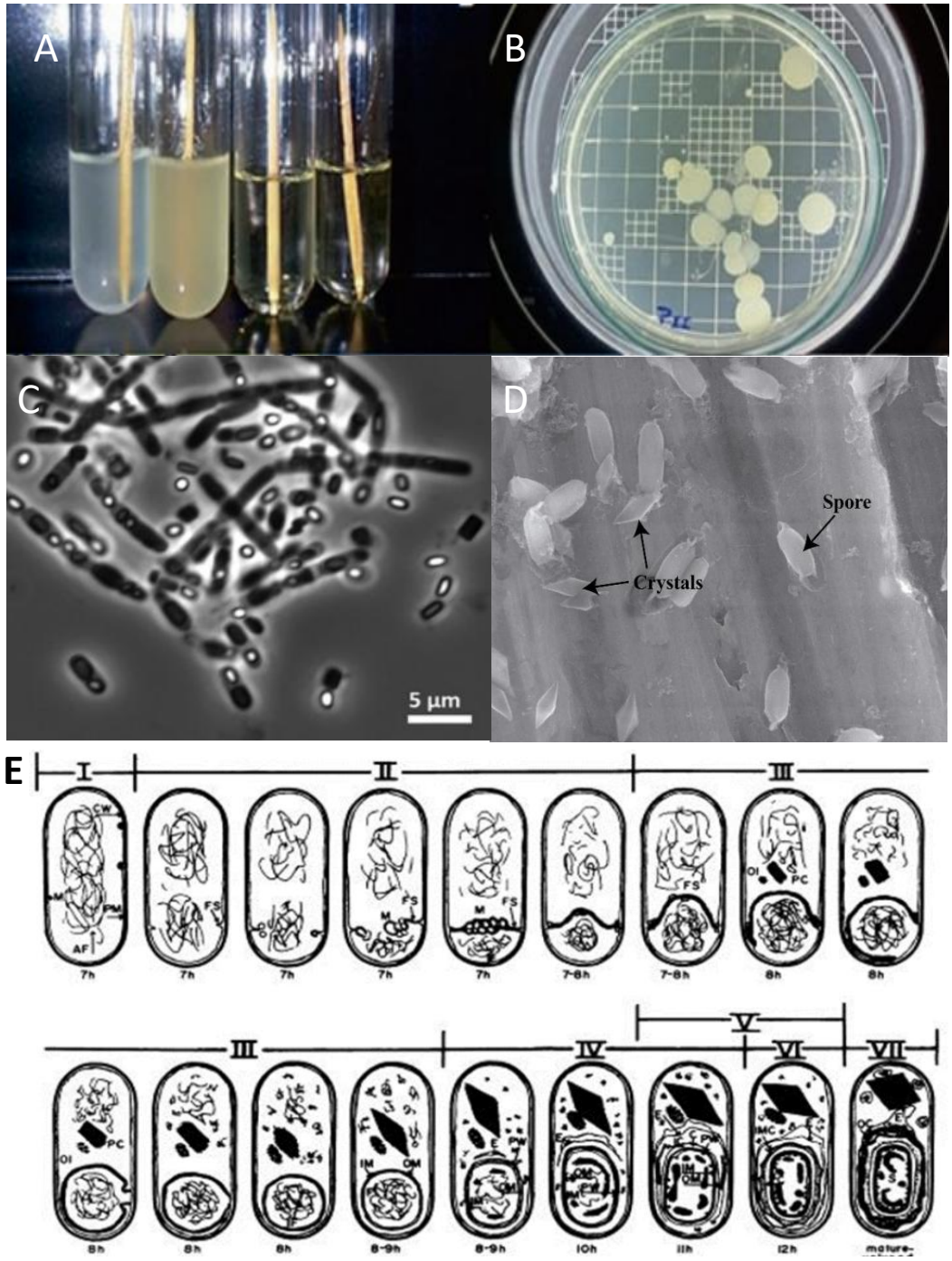


Figure 3. *Bacillus thuringiensis*: (A, B) selective culture medium, (C) phase contrast light micrograph of sporulating Bt cells (rod shaped) (Sawaya et al., 2014), (D) Protein crystals (bipyramidal) mixed with spores from Bt strain H29.3 (Palma et al., 2017), (E) Diagramatic scheme of vegetative growth and sporulation in *B. thuringiensis* (Osman et al., 2017).

1.2.1 Bt insecticidal proteins nomenclature

As described above, *B. thuringiensis* could produce various insecticidal proteins (Bulla et al., 1980; Estruch et al., 1996; Ibrahim et al., 2010). The first Bt nomenclature, classified the different Bt proteins, was according to their insecticidal activities (Hofte and Whiteley, 1989). This nomenclature was useful in systematically classifying proteins by their insecticidal activities. However, proteins with similar

sequence homology would be classified into different primary classification groups if they had different insecticidal specificities. In addition, it was necessary to obtain comprehensive bioassay data for this classfication. Later a revised nomenclature classified the proteins solely by amino acid similarity to overcome these challenges (Crickmore et al., 1998; Donovan et al., 2006), and it has been used more than 20 years. This nomenclature recommended a four-level naming system that the proteins that shared at least 45% sequence identity were placed in the same classification group (for example Vip1, Vip2, Vip3, Vip4). Inside this groups then further are split such that proteins that shared less than 78% (45-78%) identity were allocated different secondary ranks (Vip3A, Vip3B etc); the tertiary rank that shared less than 95% sequence identity (Vip3Aa, Vip3Ab etc); the fourth level that shared greater than 95% identity (Vip3Aa1, Vip3Aa2 etc) (Palma et al., 2014) (Fig. 4). With a view to this nomenclature, yet there were a number of proteins that named as being members in same groups (Cry6, Cry15 and Cry22 etc), but showed very little structure (or sequence) similarity. In addition, there is a wide variety of bacteria-derived insecticidal proteins obtained genome sequencing projects and improved procedures for protein structure determination, and this existing nomenclature heavily constrained the appreciation of their diversity. Recently, a new naming system has been proposed that reflects both sequence similarity and structural differences among the classes (Crickmore et al., 2020) (Fig. 5). In this new system, only the previous three domains “Cry” toxins (like Cry1Aa or Cry3Aa) are still called “Cry”, and merely “Vip3” (In 1998 nomenclature system) is named “Vip”, but the “Vip1” and “Vip2” have been named as “Vpa” and “Vpb” (Table 1).

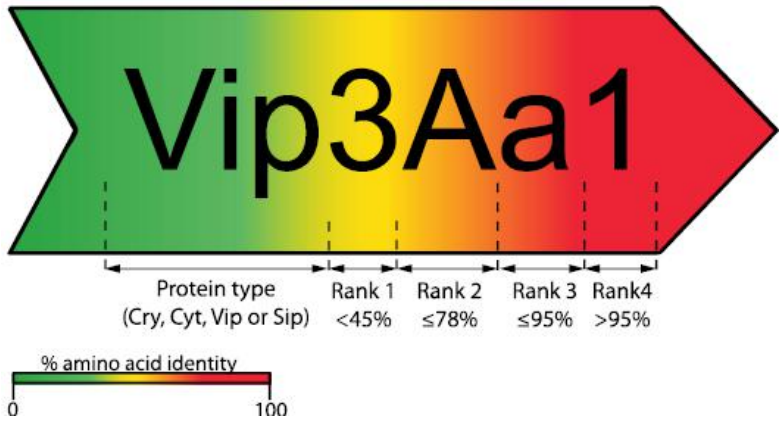


Figure 4. Schematic overview of the second nomenclature system used by the Bt Toxin nomenclature Committee for δ-endotoxins (Cry and Cyt) and secretable (Vip and Sip) toxins. In this example, numbers indicate different Vip proteins changing rank 1 depending of percentage amino acid similarity (for Vip proteins this rank may change to date among Vip1, Vip2, Vip3 and Vip4). The same rule applies for ranks 2, 3 and 4 assigning a different identification digit/letter (Palma et al., 2014).

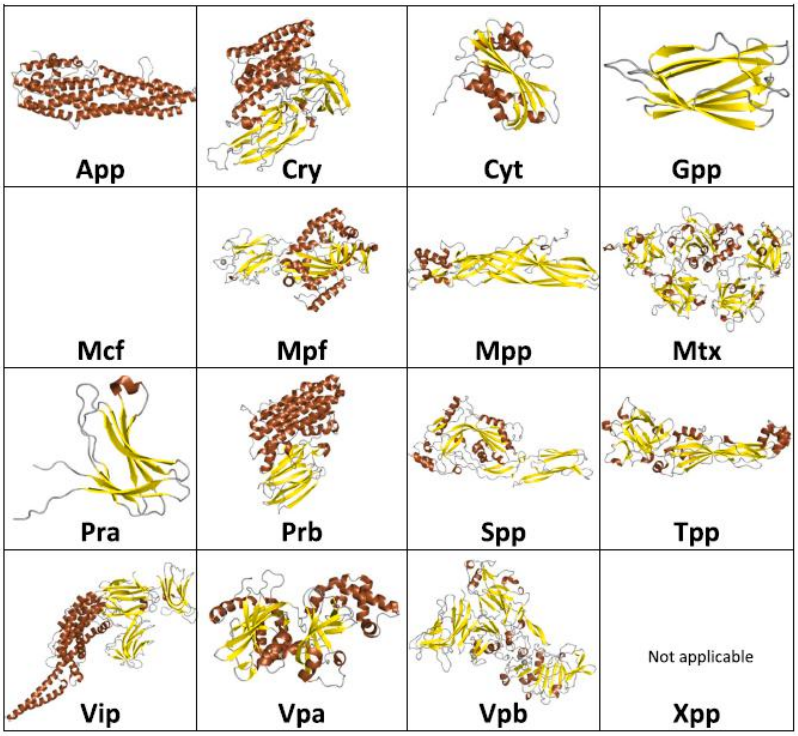


Figure 5. Representative structures, where available, of the different pesticidal protein classes (Crickmore et al., 2020).

Table 1. Classification groups within the revised 3-domain Cry and Vip protein nomenclature (Crickmore et al., 2020).

Class	Previous classification	Conserved domains	Description (PDB codes)
Cry	Cry	pfam03945, pfam00555, cd04085	Proteins originally isolated from *B. thuringiensis* **cry**stals in which the active form normally consists of three domains. Examples include Cry1Aa (1CIY) and Cry3Aa (1DLC)
Vip	Vip3	pfam12495, pfam02018	Multi-domain proteins originally identified as being **V**egetative **I**nsecticidal **P**roteins such as Vip3Bc (6V1V)
Vpa	Vip2	cd00233	Proteins related to the ADPribosyltransferase **a**ctive component of binary toxins such as **Vip**2 (1QS2) (from the Vip1 / Vip2 toxin)
Vpb	Vip1, Vip4	pfam07691, pfam03495, pfam17475, pfam17476	Proteins related to the **b**inding component of binary toxins such as **Vip**1 (6SMS), Vip4.

Note: The letters in bold stand for the source of abbreviation in class.

1.3 The three-domain Cry (3d-Cry) proteins

This family is composed by around 75 primary groups of Cry proteins (Cry1, Cry2, Cry3, etc.), containing more than 55 subgroups (Pardo-López et al., 2006). The protoxins of 3d-Cry are with two different lengths, 65 (Cry2Aa/Cry3Aa, Fig. 6) and 130 kDa (Cry1Ac, Fig. 6). For 130 kDa protoxin,

the C-terminal part would be cleaved by proteases present in the larval midgut (Fig. 6) (Fiuza et al., 2017b; de Maagd et al., 2003; Adang et al., 2014; Pigott and Ellar, 2007).

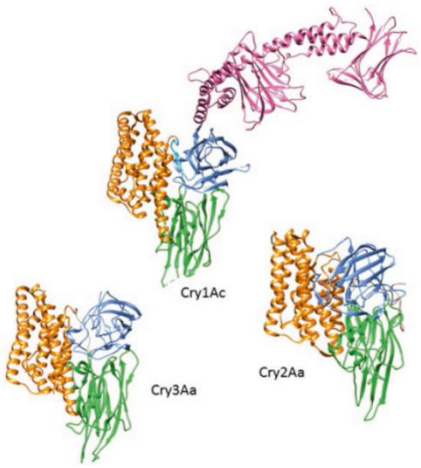


Figure 6. Structures of three-domain 65 and 130-kDa Cry proteins (Fiuza et al., 2017b).

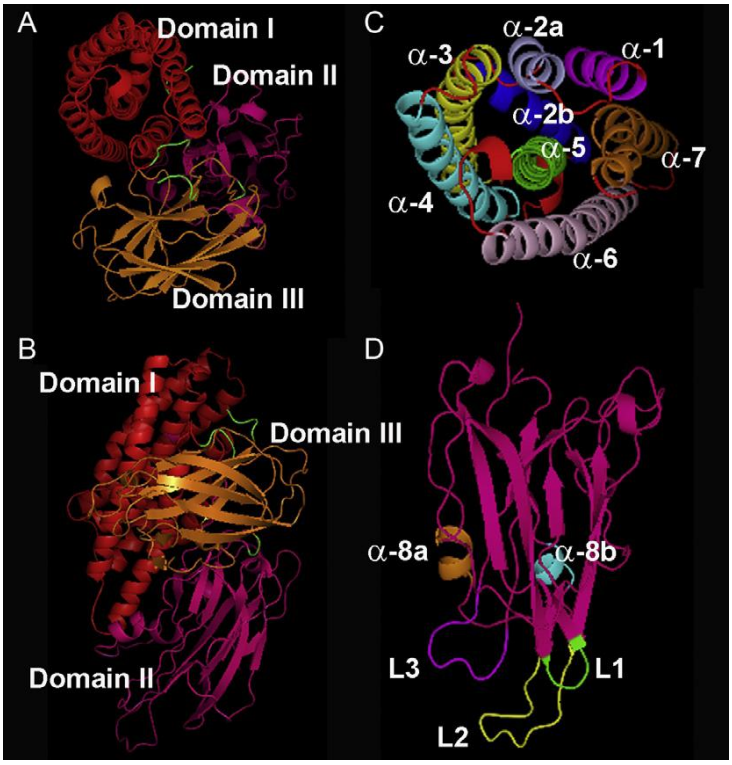


Figure 7. Views of Cry1Aa toxins fromthe Resource for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/) of (id: 1CIY) (Grochulski et al., 1995) modified using PyMOL Version 1.7 (http://www.pymol.org/). Domain amino acid sequence position information was obtained from NCBI data base (GenBank: AAP40639.1) and (Grochulski et al., 1995). Overall views of Cry1Aa toxin are shown (Panels A and B). Domain I is red, domain II is pink and domain III is orange. Domain I with the names of α-helices and domain II with named α-helices and loops are shown in Panels C and D, respectively (Grochulski, et al, 1995; Adang, et al., 2014).

1.3.1 Proteins structure and function

The three-dimensional (3D) structures of nine Cry toxins (Cry1Aa, Cry1Ac, Cry2Aa, Cry3Aa, Cry3Bb1, Cry4Aa, Cry4Ba, Cry8Ea1, and Cry5B) have been determined by X-ray crystallographic methods (PDB numbers: 1CIY, 4ARX, 1I5P, 1DLC, 1JI6, 2C9K, 1W99, 3EB7, 4D8M) (Grochulski et al., 1995; Derbyshire et al., 2001; Li et al., 2001; Morse et al., 2001; Li et al., 1991; Galitsky et al., 2001; Boonserm et al., 2006; Boonserm et al., 2005; Guo et al., 2009). These toxins show considerable differences in their amino acid sequences and insecticidal spectrum, they all share a wedge-like global shape consisting of three domains. There is a common feature that the five highly conserved blocks are present in all the 3d-Cry proteins except Cry2Aa (only 20 to 23% sequence identity with the other toxins or two of the five highly conserved blocks) (Pardo-López et al., 2013).

Domain I consists of a bundle of 7-8 α-helices with the hydrophobic α5 helix located in the center (Fig. 7) (Grochulski et al., 1995), sharing structural similarity with the pore-forming domain of alternative bacterial toxins (like diphtheria toxin and colicin A) (Parker et al., 1989). According to the umbrella model of crystal structure of Cry3Aa (Li et al., 1991) toxin, the hydrophobic helices α4 and α5 of domain I penetrates the bilayer (insert into the membrane) as an antiparallel hairpin, while the remaining helices spread on the membrane out-surface with a conformation change (Gazit et al., 1998). Additionally, mutated α4 and α5 residues that impair Cry1Aa pore formation in planar lipid bilayers (Masson et al., 1999) and in brush border membrane vesicles from midgut of larval tobacco hornworm, *Manduca sexta* (Girard et al., 2009) support a pore-lining function of helix α4 and α5 participation in channel formation (Florez et al., 2012). Site-directed mutagenesis of helices α-3 and 6 residues of Cry1Ab resulted in unable to form oligomers or pores, supporting the relevance of these structures to oligomerization (Jiménez-Juárez et al., 2007).

Domain II, which is the most diverse domain, consists of three antiparallel β-sheets packed together to form a β-prism with pseudo threefold symmetry (Li et al., 1991). The three apical loops at the base of domain II are thought to be flexible and they vary considerably in length and amino acid sequence. The domain II loops are believed to be an important recognition of midgut receptors and toxin specificity (Pigott and Ellar, 2007; Smedley and Ellar, 1996; Rajamohan et al., 1995). Previous studies show that loops 2, 3 and α-8 of Cry1A domain II are involved in receptor recognition in lepidopteran larvae (Lu et al., 1994; Rajamohan et al., 1995, 1996a, b; Lee et al., 2000, 2001). And the loop 2 and α-8 of Cry1Ab domains II are thought to recognize BtR175 cadherin of silkworm, *Bombyx mori* (Obata et al., 2009) and the BtR1 cadherin of *M. sexta* (Rajamohan et al., 1995).

Domain III, is a β-sandwich forms of two β-sheets compressed into a jelly roll topology (Fig. 7). Both sheets are composed of five strands, with the outer sheet facing the solvent and the inner sheet packing against domain II (Li et al., 1991). The sequences of domain III has been compared to a number of different proteins, and its similarity to carbohydrate-binding modules (CBMs) has been shown to be critical in determining specificity (de Maagd et al., 1999a, 2003). The loop of Cry1Ac domain III participates in recognition of *M. sexta* midgut and in binding to N-acetylgalactosamine (GalNAc) attached to aminopeptidase (de Maagd et al., 1999b), mediates binding and specificity in *Lymantria dispar*, *Heliothis virescens*, *Trichoplusia ni* and *Spodoptera* species (Lee et al., 1995; Ge et al., 1991; Herrero et al., 2004). Moreover, GalNAc binding to Cry1Ac induces a slight conformational change that enhances membrane insertion of an oligomeric prepore structure (Pardo-López et al., 2006). The chimeric proteins built by Cry3Aa domains I–II and domain III of Cry1Ab, did not compete for binding with Cry3Aa-like proteins in coleopterans (Walters et al., 2010). These results supports an important determining specificity or toxicity role for the Cry1 domain III that is functional in

lepidopterans and coleopterans.

1.3.2 Mode of action of three domain Cry proteins

The mode of action of Cry proteins has been extensively investigated and reviewed (Pigott and Ellar, 2007; Adang et al., 2014; Pinos et al., 2021). Figure 8 represents the current proposed models of Cry toxin action in the insect midgut epithelium (Adang et al., 2014).

Upon ingestion by a target insect, the parasporal crystal is solubilized in the host gut fluids and protoxin is relased. After, the protoxin is processed by gut proteases to an active core (55-65 kDa) (Pigott and Ellar, 2007; Adang et al., 2014; Pinos et al., 2021). Activated Cry proteins then have to pass through the peritrophic matrix, which contains carbohydrate moieties that may bind and retain some of the protein (Pigott and Ellar, 2007; Adang et al., 2014; Pinos et al., 2021). Once reaching the midgut brush border membrane epithelium, activated Cry proteins bind to specific proteins that are considered critical to the activity. After binding, the proteins are able to get inserted (as oligomers) into the enterocyte membrane forming a cation selective pore that results in osmotic imbalance and cell death (Endo et al., 2017). Massive enterocyte death destroys the midgut epithelial barrier and allows invasion of the hemocoel by Bt and other gut-resident bacteria, ultimately causing death of the host by septicemia (Raymond et al., 2010).

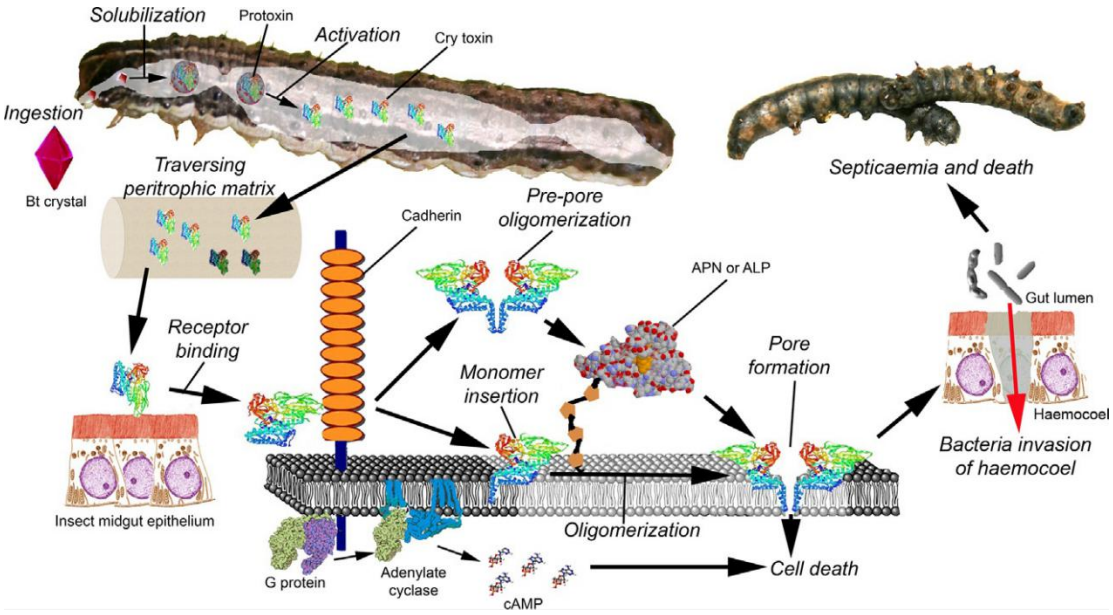


Figure 8. Schematic description of the proposed mode of action of Cry toxins in the insect midgut epithelium. After ingestion, toxin crystals are solubilized in the midgut fluids to yield protoxin which is processed to an activated Cry toxin by the extracellular enzyme. Then Cry toxin core traverses the peritrophic matrix, once reaching the midgut epithelium, binds with high affinity to receptors (cadherin), which results in activation of intracellular cell death pathways (Adang et al., 2014).

1.3.3 Putative Cry receptors

Cry toxin binding to insect midgut epithelial receptors is a major determinant of specificity (Jurat-Fuentes et al., 2021; Pinos et al., 2021). The binding parameters and the potential binding alterations in resistant insects has been shown through monitoring specific and nonspecific binding to brush border membrane vesicles (BBMV) (labeled with radioactive iodine) (Wolfersberger, 1990; Hernández and Ferré, 2005). In addition, the binding site models derived from different proteins

displacement assays have been reviewed for several relevant species to predict risks of cross-resistance (Jakka et al., 2015a). Major receptors of Cry proteins such as Aminopeptidase-N (APN), Alkaline phosphatase (ALP), Cadherins, ABC transporters are the best known and its functional relevance in the mode of action has been shown (Jurat-Fuentes et al., 2021; Pinos et al., 2021). Also, there are several proteins (like the proteins on the peritrophic matrix) or lipids that have been reported as Cry toxin-binding molecules and putative receptors (Griffitts et al., 2005; Valaitis et al., 2001; Hossain et al., 2004; Pandian et al., 2008), a possibility that needs further experimental study.

1.3.3.1 APNs and ALPs

In lepidopteran, the midgut receptors APNs and ALPs that both interacted with GalNAc (N-acetylgalactosamine) proteins during the binding to Cry1 proteins step have been identified in previous studies (Masson et al., 1995; Gill et al., 1995; Luo et al., 1997; Jenkins et al., 2000; Garczynski and Adang, 1995; Terra et al., 1996; Jurat-Fuentes and Adang, 2004; Ning et al., 2010; Martins et al., 2010; Sengupta et al., 2013). Phylogenetic analyses of lepidopteran APNs cluster these proteins into seven classes, though only some APNs have been described as putative Cry receptors in insects (Knight et al., 1994, 1995; Sangadala et al., 1994; Pigott and Ellar, 2007; Jurat-Fuentes et al., 2021; Pinos et al., 2021). It was reported that a small patch of seven amino acids of APN in cotton leafworm, *Spodoptera litura* serves as epitope for recognition by loops 2 and 3 of Cry1C domain II (Kauer et al., 2014). Mutation or reduced expression of specific APNs has been observed correlated with resistance to Cry1 toxins in *Diatraea saccharalis* (Yang et al., 2010), *Spodoptera exigua* (Herrero et al., 2005) and *Helicoverpa armigera* (Zhang et al., 2009). In addition, ALPs in lepidoptera have also been identified as the functional Cry receptors in some studies (English and Readdy, 1989; Jurat-Fuentes and Adang,2004; Jurat-Fuentes et al., 2011; Adang et al., 2014). The correlations between reduced ALP expression and resistance to Cry1 toxins in strains of *H. virescens*, *H. armigera* and *Spodoptera frugiperda* further support the role of ALPs in Cry intoxication (Jurat-Fuentes et al., 2011).

1.3.3.2 Cadherins

A cadherin called BT-R1 (Bt receptor 1) was first identified as a high affinity Cry toxin receptor in *M. sexta* larvae (Vadlamudi et al., 1995), and also related to resistance (or cross-resistance) against *Pectinophora gossypiella, H. virescens,* and *H. armigera* (Morin et al., 2003; Gahan et al., 2001; Xu et al., 2005). Later, further evidence of cadherins as Cry1A receptors was provided in *B. mori* (Nagamatsu et al., 1999), *Ostrinia nubilalis* (Flannagan et al., 2005) and *H. virescens* (Jurat-Fuentes and Adang, 2004). Mutations in a *cadherin* gene from a field Cry1Ac-resistant *H. armigera* population revealed that most of these mutations were recessive (Xu et al., 2005; Zhang et al., 2012; Zhao et al, 2010; Wang et al., 2016; Baxter et al., 2005).

1.3.3.3 ABC transporters

The ABC proteins in insects are a family of integral membrane proteins and they are normally related to the multi-drug resistance (Heckel, 2012). There is mounting evidence supporting the role of ABC transporter genes related to Cry resistance or toxicity (Sato et al., 2019). Initial evidence for a role of ABCC2 proteins in the Cry1 mode of action was provided by genetic linkage between an ABCC2 mutation and resistance to Cry1A toxins in *H. virescens* (Gahan et al., 2010). Resistance to Cry1Ac was also mapped to the ABCC2 locus in diamondback moth, *Plutella xylostella*, and *T. ni* providing further support for the function of this protein in Cry1A toxin action (Baxter et al., 2011). In *B. mori*, positional cloning in a Cry1Ab resistant strain revealed several amino acid substitutions and one

tyrosine insertion in the second extracellular loop of an ABCC2 gene (Atsumi et al., 2012). The importance of the tyrosine insertion in adquiring Cry1 resistance and functional role of the ABCC2 protein in susceptibility to Cry1Ab, Cry1Ac, Cry1Fa and even relatively unrelated Cry8Ca in *B. mori* was demonstrated by expressing the mutated and the wildtype *ABCC2* genes in cultured insect cells (Tanaka et al., 2013). Other type of ABC proteins, ABCB1 (related to P-glycoprotein), was reported as a Cry3Aa functional receptor in the *Chrysomela tremula* (coleopteran), and it was also shown that a mutation in this protein conferred resistance against Cry3Aa (Pauchet et al., 2016). Another ABC transporter, ABCA2 for Cry2Ab was identified as receptor in *H. armigera* (Wang et al., 2017). Also, field and laboratory-evolved resistance to Cry2Ab in *P. gossypiella* was linked to ABCA2 (Mathew et al., 2018), and an essential role of ABCA2 in Cry2Ab toxicity in *T. ni* was demonstrated by CRISPR/Cas9 knockouts (Yang et al., 2019).

1.4 Vip3 proteins

Vegetative insecticidal proteins (Vip) are proteins secreted by Bt during their vegetative growth stage and can be detected in culture supernatants from 15 h postinoculation to beyond sporulation (Estruch et al., 1996; Mesrati et al., 2005a, b). Many new *vip3* genes of Bt isolates have been identified by using different strategies (Sattar et al., 2008; Hernández-Rodríguez et al., 2009; Murawska et al., 2013; Liu et al., 2007; Franco-Rivera et al., 2004; Baranek et al., 2015; Loguercio et al., 2002; Bhalla et al., 2005; Rang et al., 2005; Abulreesh et al., 2012; Asokan et al., 2012). To date, around 111 Vip3 proteins have been reported and named in the Bt toxin database, 66 Vip3Aa, 2 Vip3Ab, 1 Vip3Ac, 6 Vip3Ad, 1 Vip3Ae, 4 Vip3Af, 15 Vip3Ag, 2 Vip3Ah, 1 Vip3Ai, 2 Vip3Aj, 2 Vip3Ba, 3 Vip3Bb, 1 Vip3Bc and 4 Vip3Ca (Fig. 9) (Crickmore et al., 2018 (http://www.btnomenclature.info)). Genes coding for Vip3 proteins are 2.4 kb in length, and they are normally carried on large plasmids (Mesrati et al., 2005a, b, Wu et al., 2004), although in some cases, they have been proposed to be located in the bacterial chromosome (Franco-Rivera et al., 2004; Chakrabarty et al., 2020). Vip3 proteins are efficient insecticidal toxins against a wide range of Lepidoptera (Chakroun et al., 2016a; Chakrabarty et al., 2020). Also, Vip3 proteins have no sequence homology with Cry proteins, and do not share common binding sites in target insects (Lee et al., 2003; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014).

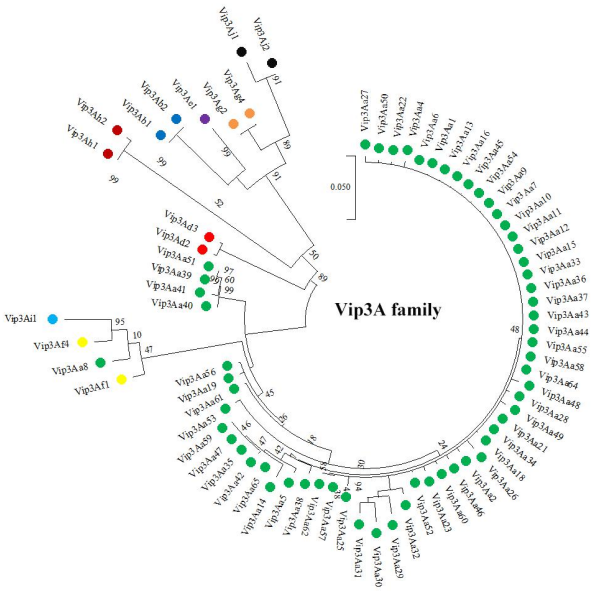


Figure 9. Phylogenetic relationship among the Vip3A proteins. The colour stands for different Vip3A proteins.

(Chakrabarty et al., 2020).

1.4.1 Proteins structure and function

The Vip3 proteins usually have a molecular mass of approximately 89 kDa having 787 to 789 amino acids. Two fragments of around 20 and 65 kDa, which remain strongly associated, are generated through trypsin-like enzymes (in midgut juice) that cleave the molecule at residue 198/199 (Kunthic et al., 2017a, b; Bel et al., 2017; Banyuls et al., 2018a). Later the 3D (three dimensional) structure of Vip3 proteins also showed the consistent results with the previous studies (Fig 10).

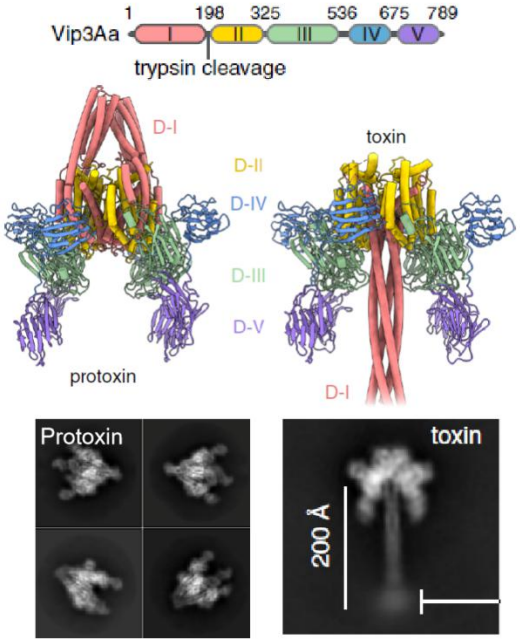


Figure 10. Structure of Vip3Aa from *Bacillus thuringiensis* (Núñez-Ramírez et al., 2020).

The highly conserved N-terminal region of Vip3 has an important role regulating the tetrameric conformation and insecticidal functions (Shao et al., 2020). The exchange of the N-terminal (20 kDa) sequence of Vip3 proteins Vip3Ab1 and Vip3Bc1 revealed that although the proteolytic activation is similar, the insecticidal activity is totally lost (Zack et al., 2017). What's more, deletion of the first 27 N-terminal amino acids from Vip3Aa rendered the protein inactive. This lack of activity was attributed to a total loss of protein solubility (Selvapandiyan et al., 2001). The deletion of the first 39 N-terminal amino acids from Vip3Aa affected the toxicity toward the two susceptible insect species *S. litura*, and *Chilo partellus* (Selvapandiyan et al., 2001). However, elimination of the first 200 residues from the N-terminal sequence of Vip3Aa toxin augmented its insecticidal activity 2 to 3 fold against *H. armigera, A. ipsilon, Spodoptera littoralis* and *Scirpophaga incertulas* (Gayen et al., 2012). A N-terminal truncation of Vip3Aa14 toxin up to 33 amino acid residues did not alter its toxicity against *S. litura*, *P. xylostella* and *Earias vitella* (Bhalla et al., 2005).

The highly variable C-terminus of Vip3 proteins has been generally accepted that is relevant to the insecticidal activity, even to the target specificity (Wu et al., 2007; Núñez-Ramírez et al., 2020). The deletion, substitution, or exchange of amino acids (at the C-terminal) of Vip3 proteins suggested strong effects in the loss of the insecticidal activity in *S. frugiperda, S. exigua, S. littoralis,* and *Agrotis segetum* (Selvapandiyan et al., 2001; Li et al., 2007; Gayen et al., 2012; Banyuls et al., 2018a; Zhang et al., 2018). In addition, a carbohydrate binding motif (CBM_4_9 superfamily, pfam02018) is present in all the Vip3 proteins with the exception of Vip3Ba, has been proposed to be related to the specific

binding of Vip3A proteins (Chakroun et al., 2016a; Jiang et al., 2020; Núñez-Ramírez et al., 2020). In agreement with this idea, Boonyos et al. showed that the cloned CBM sequence of Vip3Aa could inhibit the toxicity of full-length Vip3Aa in *S. exigua* (Boonyos et al., 2020). In addition, it is observed that the C terminus was critical for stability or activity of Vip3 proteins, as their deletion or replacement increase the susceptibility to proteases (Estruch and Yu, 2001; Li et al., 2007; Selvapandiyan et al., 2001; Gayen et al., 2012; Soonsanga et al., 2019).

1.4.2 Mode of action of Vip3 proteins

The mode of action of the Vip3 proteins has been studied since their discovery in 1996 (Estruch et al., 1996). Figure 11 represents the mode of action of Vip3 proteins currently proposed in different studies (Jiang et al., 2018a, b; Núñez-Ramírez et al., 2020). It is generally observed that after ingestion, the Vip3A protoxin is activated by midgut proteases (trypsin- or chymotrypsin-like) rendering two fragments of ca. 20 and 65 kDa (Abdelkefi-Mesrati et al., 2011; Yu et al., 1997; Lee et al., 2003; Liu et al., 2011; Caccia et al., 2014; Banyuls et al., 2018b). The activated Vip3A protein crosses the peritrophic membrane to interact with putative receptors in the apical membrane of the epithelial cells. A set of structural transformation events (the primary cleavage site is required) is triggered possibly by conjunction with some factors, that releases the N-terminus segment and leads to the formation of a four-helix coiled coil (as stalk), which is sufficiently long to reach and permeate the lipid bilayer. This “four-helix coiled coil” could become ordered in contact with the lipid bilayer (or penetrate the membrane) to form a four-helix bundle similar to the transporting protons and divalent metals channel (like Rocker transporter or the influenza M2 channel), that induced pore formation, ultimately cell death by lytic osmosis. Also, a process that could be further stimulated by other factors like the interaction of the membrane receptors with the C terminal region of Vip3, which could also increase the concentration of the toxin at the membrane (Núñez-Ramírez et al., 2020; Byrne et al., 2021). However, the debatable processes that the engagement to receptor (or receptors recognition), penetrating the membrane, and the channel are further required to establish the detail mechanism.

In addition, it has been reported that the full-length Vip3Aa (protoxin) can directly bind to the scavenger receptor class C proteins (SR-C) and fibroblast growth factor receptor-like proteins (FGFR) on the surface of Sf9 cells without the proteolysis (or activation), and then enter the cell with endocytosis mediated by receptor proteins, thereby inducing apoptosis (Jiang et al., 2018a, b; Jiang et al., 2020).

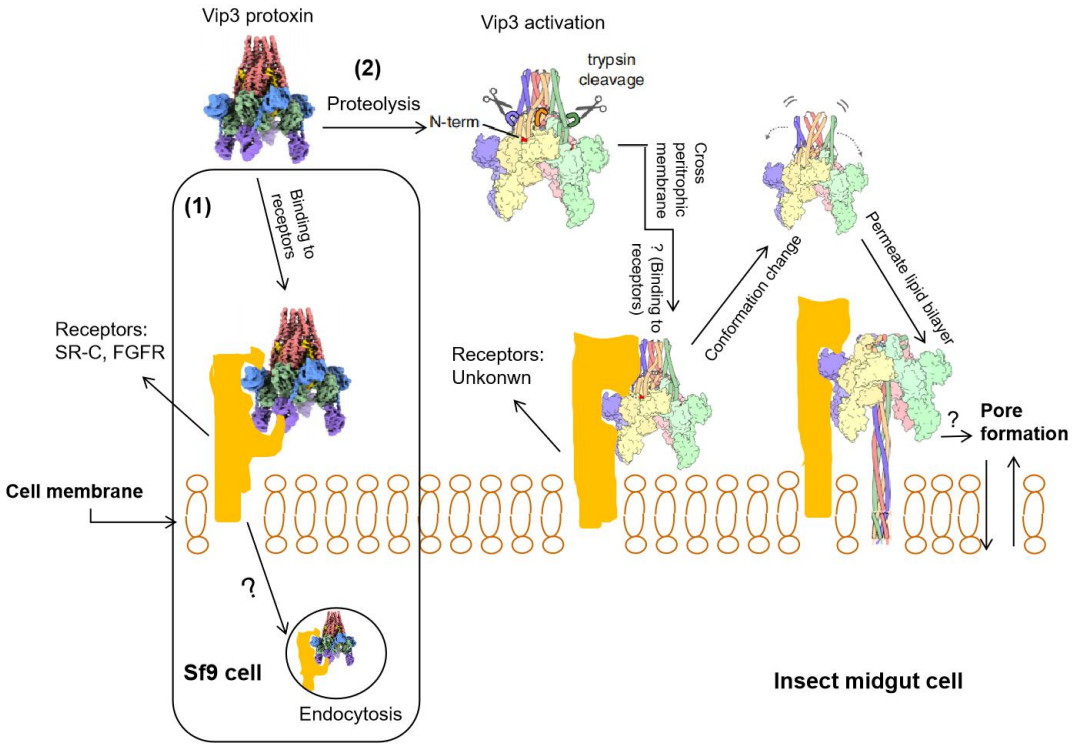


Figure 11. Proposed mechanism of toxicity for Vip3 proteins. Vip3 accomplishes its insecticidal function in two ways: **(1)** apoptosis induced by Vip3 protoxin in Sf9 cell and **(2)** midgut cell perforation mediated by activated Vip3 toxin (Jiang et al., 2018a, b; Chakrabarty et al., 2020; Núñez-Ramírez et al., 2020). According to current results, **(1)** Vip3 protoxin probably directly binds to receptor proteins (include scavenger receptor class C like proteins (SR-C), fibroblast growth factor receptor (FGFR)) on the surface of Sf9 cells without the activation process, and then enter the cell with endocytosis mediated by receptor proteins, thereby inducing apoptosis. **(2)** But *in vivo* (or *in vitro* (with BBMV)), the main pathway was proposed that, once ingestion, Vip3A protoxin would be immediately activated by the midgut proteases to two strongly associated fragments about 20 and 65 kDa. Then, activated Vip3A protein crosses the peritrophic membrane to interact with putative receptors in the apical membrane of the epithelial cells. After a set of structural transformation events, the bound tetramers induce pore formation, ultimately cell death by lytic osmosis (Núñez-Ramírez et al., 2020; Byrne et al., 2021).

1.4.2.1 Binding to larval midgut epithelium

The studies performed in *S. frugiperda* larvae by immunohistochemical analysis show that Vip3A binds in the brush border membrane of the midgut epithelial cells (Chakroun and Ferré, 2014) (Fig. 12). Specific binding to brush border membrane vesicles (BBMV) or cells has been shown by using labeled Vip3A proteins in *S. frugiperda*, *Spodoptera albula*, *Spodoptera cosmioides*, and *Spodoptera eridania*, *A. ipsilon*, *H. virescens*, *Helicoverpa zea*, *H. armigera*, *Ephestia kuehniella*, and the extensive midgut damage, with lysed and swollen cells leaking cellular material to the lumen, was induced by ingestion of Vip3Aa or Vip3Ca (Lee et al., 2006; Sena et al., 2009; Abdelkefi-Mesrati et al., 2011; Ben Hamadou-Charfi et al., 2013; Bergamasco et al., 2013; Liu et al., 2011; Chakroun and Ferré, 2014; Chakroun et al., 2016a; Kahn et al., 2018). However, Vip3Aa also binds specifically to BBMV of *O. nubilalis*, which is a nonsusceptible species to Vip3A proteins (Lee et al., 2003). These evidence indicated that the step binding to receptor may be considered critical, but not sufficient, to determine activity.

The binding profile of Vip3A to *S. frugiperda* BBMV reveals that both the 62-65 kDa and 20 kDa

fragments of trypsin-activated ^{125}I-labeled Vip3A bound to BBMV, which these fragments remain strongly associated detected in previous study (Chakroun and Ferré, 2014). Domains III or domain II-III of Vip3Aa has been considered as the possible binding domain based on the observation that truncated peptides containing these domains could bind to the Sf9 cell (Jiang et al., 2020; Nimsanor et al., 2020). In addition, the cloned CBM (domain IV) of Vip3Aa also could inhibit the toxicity of full-length Vip3Aa in *S. exigua* indicated the potential binding relevance of domain IV (Boonyos et al., 2020). Further studies are required to shed light on this process.

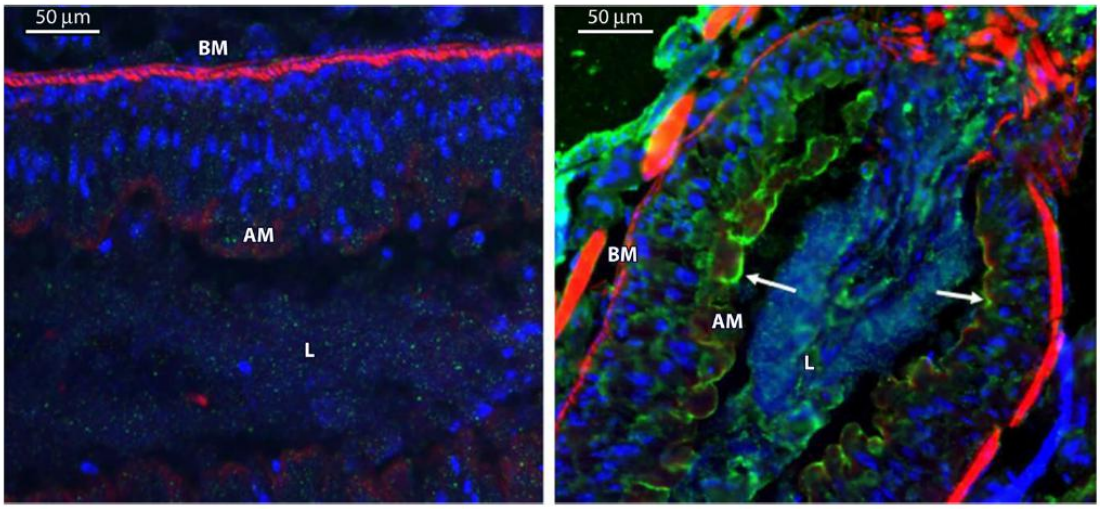


Figure 12. Immunolocalization of Vip3Aa in midgut tissue sections after ingestion by *S. frugiperda* larvae. (Left) Control larvae. (Right) Larvae that ingested Vip3Aa. Nuclei were stained blue, and the apical and basal membranes were stained red. Binding of Vip3Aa to the apical membrane is shown in green. BM, basal membrane; AM, apical membrane; L, gut lumen. (Chakroun and Ferré, 2014)

Competition binding assays showed the absence of shared binding sites between Vip3A and some Cry proteins (Cry1Ac, Cry1Ab, Cry1Fa, Cry2Ae and Cry2Ab) (Ben Hamadou-Charfi et al., 2013; Chakroun and Ferré, 2014; Lee et al., 2006; Liu et al., 2011; Sena et al., 2009; Gouffon et al., 2011). It has been shown that only Cry1Ia in *S. eridania* shares binding sites with Vip3Aa (Bergamasco et al., 2013). Among Vip3 proteins, Vip3Aa proteins shared all the binding sites with other Vip3A proteins in *Spodoptera* spp, and with Vip3Ca in *H. armigera* (Chakroun and Ferré, 2014; Kahn et al., 2018).

Nowadays, several studies have been focused on the interaction between Vip3A proteins and their putative receptors. Ligand blot analyses revealed that Vip3Aa recognized 80- and 110-kDa proteins in *M. sexta* (Lee et al., 2003), a 65-kDa protein in *Prays oleae* and *A. segetum* (Abdelkefi-Mesrati et al., 2009; Ben Hamadou-Charfi et al., 2013), and the 55 and 100 kDa proteins from the midgut membrane of *S. littoralis* (Abdelkefi-Mesrati et al., 2011). In addition, a 65 kDa protein that could bind to both Vip3Aa and Cry1Ia was detected in the *Spodoptera* species (Bergamasco et al., 2013). However, few studies have identified these putative recpetors. A Vip3Aa-binding protein (gene accession no. KX858809) from BBMV of *A. ipsilon* was identified and analyzed, and it had no homology with any gene in the gene bank (Osman et al., 2019). Knockdown of S2 ribosomal protein expression resulted in reduced mortality of Vip3A toxin to Sf21 cell and *S. litura* larvae (Singh et al., 2010). In addition, the scavenger receptor class C proteins and fibroblast growth factor receptor-like proteins from member of Sf9 cells has been shown to mediate full-length Vip3Aa endocytosis, which is associated with

cytotoxicity (Jiang et al., 2018a, b; Jiang et al., 2020).

1.4.2.2 Post-binding events

The activated Vip3Aa protein binds to the apical membrane of brush border epithelial cells and then it has been shown that it is able to induce pores in the midgut (or BBMV) (Liu et al., 2011), and planar lipid bilayers (Lee et al., 2003). Extensive midgut damage with lysed and swollen cells leaking cellular material to the lumen was also observed *in vivo* (Lee et al., 2003). In addition, sublethal doses of either Vip3Aa and Vip3Ca trigger apoptosis in *S. exigua* larvae (Hernández-Martínez et al., 2017). Cell death via apoptosis was also observed when cultured insect cells were exposed to Vip3Aa (Jiang et al., 2016; Hou et al., 2020).

1.5 Insecticidal activity of Bt proteins

Bt proteins have been reported active against different species, such as insects Coleoptera, Lepidoptera, Hemiptera, Diptera, Hymenoptera, Mallophaga and Blattodea, also against some nematodes, acari (mites and ticks), snails or the protozoan, even kill human-cancer cells (Fig. 13) (Fiuza et al., 2017a; Van Frankenhuyzen, 2009, 2013; Palma et al., 2014). Plentiful pesticides based on recombinant Bt or Bt toxins expressed in other bacteria have been commercialized in the world (Karabörklü et al., 2018).

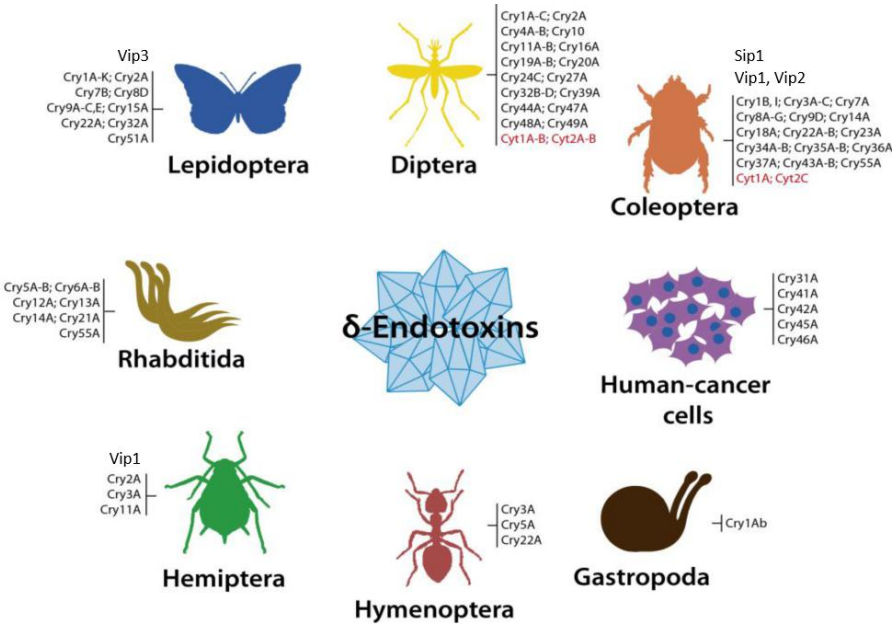


Figure 13. Overview of the insecticidal spectrum of the Bt produced proteins (Palma et al., 2014)

The insecticidal activity of Vip3 proteins has been reported in numerous lepidoptera insects. Most of the information of current studies are from the subfamily of Vip3A proteins, that are toxic in *A. ipsilon, Anticarsia gemmatalis, B. mori, Cydia pomonella, Cydia molesta, Dendrolimus pini, H. armigerda, Helicoverpa punctigera, H. virescens, H. zea, Ostrinia furnacalis, Phthorimaea oprculella, P. xylostella, Mamestra brassicae, Mythimna separate, S. albula, S. exigua, S. frugiperda, S. ericlania, S. incertulas,* and *S. littoralis* (Chakroun et al., 2016a; Chakrabarty et al., 2020). It is worth mentioning that Vip3A proteins are very active against insect species of the genus *Agrotis*, which are known to be tolerant to Cry proteins, and also against the genus *Spodoptera*, which display low susceptibility to Cry proteins, but some species such as *O. nubilalis* susceptible to Cry proteins, are marginally or not susceptible to any Vip3A proteins (Estruch et al., 1996; Doss et al., 2002; Yu et al., 1997, 2012;

Hernández-Martínez et al., 2013; Gomis-Cebolla et al., 2017). Vip3B and Vip3C proteins differ from Vip3A proteins in their activity spectrum, but the scarce body of results on these two proteins hasprecluded their field application so far.

The interactions between Vip3A proteins and Cry proteins were also evaluated to determine if it would be worth to use them in combination. Synergism and antagonism have been observed between Vip3Aa and different Cry proteins against several insect pests (Table 2). However, the mechanisms underlying synergism and antagonism are still unknown so far.

Table 2. The interactions between Vip3A and Cry proteins in toxicity.

Proteins	Synergism (in species)	Antagonism (in species)	References
Vip3A and Cry1Ia	*S. frugiperda*, *S. albula* and *S. cosmioides*	*S. eridania*	Bergamasco et al., 2013
Vip3Aa, Vip3Ae or Vip3Af and Cry1Ca	/	*H. virescens*	Lemes et al., 2014
Vip3Af and Cry1Ac or Cry1Aa	/	*H. virescens*	Lemes et al., 2014
Vip3Aa and Cry1Ca	*Diatraea saccharalis*	*S. frugiperda*	Lemes et al., 2014
Vip3Aa16 and Cry1	*Mythimna separata*	/	Yang et al., 2018b
Vip3Aa and Cry9Aa	*C. suppressalis*	/	Wang et al., 2018b
Vip3Aa and Cry1Ab; Vip3Aa and Cry2Ab; Cry1Ab, Cry2Ab and Vip3Aa	*S. frugiperda*	/	Figueiredo et al., 2019

Chimeric genes, obtained by genetic engineering that combine parts of different Vip3 proteins or between Vip3A and Cry proteins, have been constructed to obtain new proteins with novel or improved properties (Table 3). For example, the chimera Vip3AcAa has been created by combining 600 amino acids from the N-terminus of Vip3Ac and 180 amino acids of the C terminus of Vip3Aa, and it resulted in toxicity against *O. nubilalis* and increased toxicity against *S. frugiperda*, *H. zea*, and *B. mori* (Fang et al., 2017). However, other chimeras (fusion of *vip3Aa* and *cry genes*) did not improve their insecticidal activity (of Vip3Aa and Cry toxins) (Table 2). In this case, an incorrect folding or low solubilization was proposed for the Vip3-Cry chimeras, that led to the decrease or loss of activity (Saraswathy et al., 2008; Song et al., 2008).

Table 3. The genetic engineering Vip3A proteins and their effect on insecticidal activity.

Chimeras	Modification	Description	Effect(s)	Reference
Vip3AcAa	Domain swapping	Chimera of Vip3Ac N terminus (600 aa) and Vip3Aa C terminus (189 aa)	Gain of toxicity against *O. nubilalis*; IA against *S. frugiperda*, *H. zea*, and *B. mori*	Fang et al., 2017
Vip3AaAc	Domain swapping	Chimera of Vip3Aa1 N terminus (610 aa) and Vip3Ac C terminus (179 aa)	DA against *S. frugiperda* and *H. zea*; LA against *B. mori*	Fang et al., 2017
Vip3Ab1-740	Sequence swapping	Combination of 612 N-terminal amino acids of VipAb1 with 177 C-terminal amino acids of Vip3Ai1	Gained lethality against *S. eridania* and maintained lethality against *S. frugiperda*,	Sopko et al., 2019

			H.zea and *P. includens*	
Vip3Aa14	Protein fusion	Chimera of Vip3Aa14 and Cry1Ac	As effective as Cry1Ac against *H. armigera* and *P. xylostella* but DA compared to Vip3Aa against *S. litura*	Saraswathy et al., 2008
Vip3Aa7	Gene promoter change and protein fusion	Chimera of Cry1C promoter with truncated Vip3Aa7 (39 aa deleted at N terminus) and Cry1C C-terminal region	Higher yield of Vip3Aa7, Vip relocation in Bt inclusion bodies but DA against *P. xylostella*, *H. armigera*, and *S. exigua*	Song et al., 2008
Vip3Aa7	Protein fusion	Chimera of Vip3Aa7 and Cry9Ca N-terminus	IA against *P. xylostella*	Dong et al., 2012
Vip3Aa16	Protein fusion	Fusion of Vip3Aa16 with the toxic core sequence of the Cry1Ac	Increased the mortality of the first instar larvae of *E. kuehniella*	Sellami et al., 2018

1.6 Resistance

1.6.1 Cry-resistance

Cry proteins as the most widely used and best-known insecticidal proteins, have been used to control a wide range of lepidopteran, coleopteran, blackfly, and mosquito species (Karabörklü et al., 2018) for more than 20 years, through sprayable mixtures of Bt spores and parasporal crystals, or transferring *cry* genes to plants. However, with long-time use of Cry proteins in pest control, target pests also respond actively and sometimes they become resistant. Resisitance has been observed in some cases of field populations (for example *H. armigera*, *H. punctigera, P. xylostella, P. gossypiella, T. ni, Diabrotica virgifera virgifera, C. tremula, H. zea, and S. frugiperda, ...*) (Jurat-Fuentes et al., 2021; Xiao and Wu, 2019). Table 4 summarizes the main Cry resistance mechanisms (or features) which have been recently reported. In general, the resistance features mainly comprised three types: variations in toxin activation, alteration of toxin binding (or receptor) and immune response.

Table 4. Published Bt-resistance mechanisms in target pests.

Species	Source	Resistance mechanism	Toxin or event	Reference
Bombyx mori	lab	ABCC2 variation in amino acid residues around 770DYWL773 of ECL4	Cry1Aa	Tanaka et al., 2017
Chilo suppressalis	lab	ALP downregulation	Cry1A, Cry2A, Cry1C	Qiu et al., 2018
Chrysomela tremula	field	ABCB1	Cry3Aa	Pauchet et al., 2016
Helicoverpa armigera	field	cadherin premature stop codon	Cry1Ac	Xu et al., 2005
	field	cadherin point mutation leads to cadherin mislocalization	Cry1Ac	Xiao et al., 2017
	lab	APN1 deletion mutation	Cry1Ac	Zhang et al., 2009
	filed	ALP N-glycosidase digestion	Cry1Ac	Ning et al., 2010
	field	ABCA2 three independent indel mutations	Cry2Ab	Tay et al., 2015
	lab	ABCC2 insertion of 73 bp in cDNA leads to 6-bp deletion at splicing site	Cry1Ac	Xiao et al., 2014
	lab	protease altered protease profile leads to improper processing of the protoxin	Cry1Ac	Rajagopal et al., 2009
	lab	trypsin mutations in promoter region	Cry1Ac	Liu et al., 2014
	field	Mutations in tetraspanin	Cry1Ac	Tay et al., 2015
Helicoverpa	field	ABCA2deletion of 14 bp leads	Cry2Ab	Tay et al., 2015

punctigera		to loss of tpm2 transporter motif in NBF2		
	field	cadherin	Cry1Ac	Walsh et al., 2018
	lab	cadherin retrotransposon-mediated insertion	Cry1Ac	Gahan et al., 2001
Heliothis virescens	lab	cadherin single-nucleotide mutation	Cry1Ac	Xie et al., 2005
	lab	ALP downregulation	Cry1Ac	Jurat-Fuentes and Adang, 2004
	lab	ABCC2 inactivating mutation	Cry1Ac	Gahan etal., 2010
Ostrinia furnacalis	lab	cadherin downregulation and mutation	Cry1Ac	Jin et al., 2014
	lab	ABCG1 knockdown	Cry1Ab, Cry1Ac	Zhang et al., 2017
	lab	cadherin premature termination codons and/or large deletions	Cry1Ab	Bel et al., 2009
Ostrinia nubilalis	lab	APN downregulation	Cry1Ab	Coates et al., 2013
	lab	ABCC2 mutation	Cry1Fa	Coates et al., 2015
	lab	Aminopeptidase-P like gene mutation	Cry1Ab	Khajuria et al., 2011
	field	cadherin three mutant alleles in toxin-binding region	Cry1Ac	Morin et al., 2003
Pectinophora gossypiella	field	cadherin deletion of 207 bp and loss of transmembrane domain	Cry1Ac	Wang et al., 2018a
	field	cadherin premature stop codon, deletion of at least 99 bp or both	Cry1Ac	Fabrick et al., 2014
	field	cadherin insertion of intact CR1 retrotransposon	Cry1Ac	Fabrick et al., 2011
	field	ALP downregulation mediated by MAPK pathway	Cry1Ac	Guo et al., 2015
Plutella xylostella		ABCC2 mutation	Cry1Ac	Baxter et al., 2011
	field	ABCG1 downregulation mediated by MAPK pathway	Cry1Ac	Guo et al., 2015
Spodoptera exigua	lab	APN downregulation	Cry1Ca	Herrero et al., 2005
	lab	ALP2 knockdown	Cry2Aa	Yuan et al., 2017
	lab	ABCC2 mutation	Cry1Ac, Cry1Ca	Park et al., 2014
Spodoptera frugiperda	field	ABCC2	Cry1Fa/TC1507	Boaventura et al., 2020
	lab	ALP downregulation	Cry1Fa	Jakka et al., 2015b
Trichoplusia ni	field	APN1 downregulation	Cry1Ac	Tiewsiri and Wang, 2011
	greenhouse	ABCA2	Cry2Ab/MON15985	Yang et al., 2019

1.6.2 Vip3-resistance

Genes for Vip3A proteins have already been expressed in cotton and maize (Bt crops) to control different insect pests. The extensive use of these crops threatens the long-term application by the potential evolution of resistance. Currently no mechanistic data on field-evolved resistance population to Vip3 proteins has been reported (Jurat-Fuentes et al., 2021). However, a high frequency of Vip3Aa alleles were detected in field populations of *H. armigera* (at 2.7% frequency) and *H. punctigera* (0.8%) in Australian using F2 screens method, though it did not increase over the following four seasons (Mahon et al., 2012; Chakroun et al., 2016b). The Vip3Aa-resistance alleles, were detected recessive and probably conferred at a single autosomal gene (Mahon et al., 2012; Kahn et al., 2018). The F2 screens for major Vip3Aa resistance in *H. zea* from Texas of United States in 2019 have estimated 0.65% frequency, and a resistance strain >588 fold to a susceptible population has been selected at laboratory (Yang et al., 2020). An estimated Vip3Aa resistance allele frequency in field *S. frugiperda* population from Louisiana, USA was 0.48% in 2016 (Yang et al., 2018a). In 2018, the frequency of

major resistance alleles against Vip3Aa51 in field *S. frugiperda* population in Texas was estimated 0.78% using F2 screening method, and a resistance strain >395 fold to susceptible population was rapidly obtained (Yang et al., 2019). In Brazil, a Vip3Aa20 resistant strain of *S. frugiperda* was screened from a field population (0.09% frequency of resistance alleles) and the resistance ratio was >3200-fold, also did not confer cross-resistance to Cry1Fa, Cry2Ab, or Cry2Ae, and the further analysis showed that the resistance allele was probably belong to an autosomal single recessive gene (Bernardi et al., 2015, 2018). In addition, a recessive polygenic resistance in *H. virescens* strain was about 2,040-fold after 12 generations selection, with no cross-resistance to Cry1Ab or Cry1Ac (Pickett et al., 2017). Although it is about 7-fold increasing of LC_{50} to Cry1Ab observed in the Vip3Aa resisitance *H. virescens* strain, that couldn't form the stable heritable feature (less than 10-fold) (Kurtz, 2010; Pickett et al., 2017). Lack of cross-resistance was generally accepted between Vip3 and Cry proteins in the most studies.

In addition, Vip3-resistant mechanisms have been studied using the laboratory strains. The results showed proteolytic activity (midgut juice) in Vip3A-resistant *S. litura* or *H. armigera* strain was significantly reduced, but the profile of activation was still similar to that of the susceptible strain (Barkhade and Thakare, 2010; Chakroun et al., 2016b). Besides, dramatically reduced levels of membrane bound alkaline phosphatase (mALP) activity and down regulation of the membrane-bound alkaline phosphatase 1 (*HvmALP1*) gene from a Vip3Aa resistant *H. virescens* strain was detected (Pinos et al., 2020). However, the experiment of binding to BBMV indicated the potential mutations of resistant individual may be not related to binding or receptors alterations (Chakroun et al., 2016b; Pinos et al., 2020).

1.7 Strategies to overcome resistance

The refuge strategy is the primary approach used to delay Bt-resistance in the United States and elsewhere (Tabashnik et al., 2013; Carrière et al., 2015). However, social and economic factors that result in less than 100% adoption of regional 'unstructured' refuges, and risk of economic damage to refuges or lack of compensation in part of refuge requirements, remains a major drawback of this (or high-dose) strategy (Hurley et al., 2002). Then a new strategy of pyramid is expected to delay resistance longer than single-toxin (Tabashnik et al, 2013; Roush, 1998; Onstad and Meinke, 2010). The new pyramided transgenes (into Bt crops), that expressed multi-toxin toxin, provide at least two key advantages over the conventional high-dose/refuge strategy: (1) two-transgene (or more) plants may provide better control of the entire pest complex, because species that are less susceptible to one toxin may still obtain a high-dose from the other; (2) a smaller refuge (less economic damage) with plants expressing two transgenes are required that a 5-10% refuge are equivalent to 30–40% refuge when two transgenes are deployed sequentially (Roush, 1997). These new pyramided (multi-toxin) crops have been as the second generation of Bt crops that more effectively control pests and reduce crop damage in the future (Fig. 14) (Carrière et al., 2015).

To obtain the maximum benefits of the pyramided Bt genes, the encoded proteins do not have to share binding sites to avoid development of cross-resistance (Roush, 1997; Tabashnik et al., 2013). Recently, a good option that use of novel Cry and Vip3 proteins has been implemented in Bt crops to broaden the insecticidal spectrum and resistance management, in several agro-biotech companies, such as Bayer CropScience, Syngenta, and Dow Agrosciences (Table 5). Along with some traditional integrated pest management methods (like nature enemies) other actions (like crop rotation), are important ingredients in the IRM (insect resistance management) strategies (Fig. 14) (Bates et al., 2005).

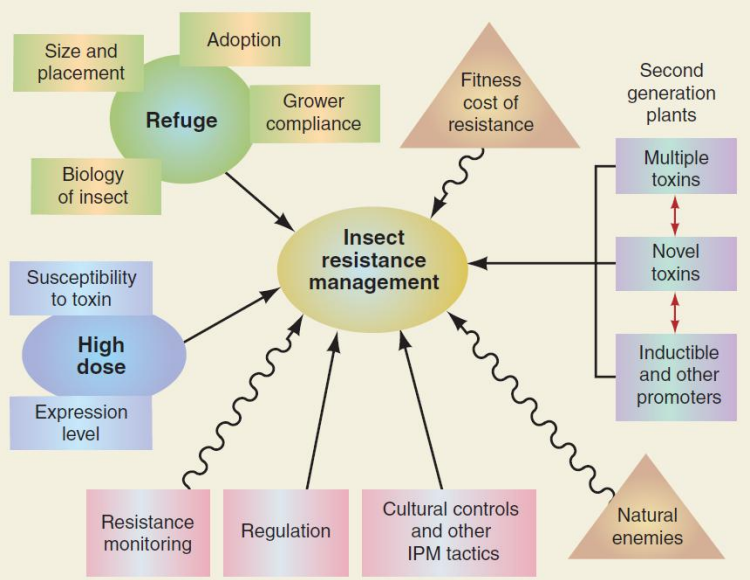


Figure 14. Factors affecting the efficacy of IRM strategies for insect-resistant transgenic crops (Bates et al., 2005).

1.8 Combination of *vip3* and *cry* genes in Bt crops

The combined use of *cry* and *vip3Aa* genes has been a clear interest of Dow Agrosciences, Bayer CropScience, and Syngenta, which have already been transferred in cotton and maize to control pests (Burkness et al., 2010). Several GM products have been approved in the world, such as VIPCOT™, Agrisure™ Viptera (Table 5). The efficacy of combining cultivar (like VipCot™ developed by Syngenta, *cry1Ab+vip3Aa*) showed significant increase of the toxicity against some target pests (such as *H. zea*, *H. virescens*) as compared with the conventional ones (only expressed Cry toxins). In addition, multiple genes (more than two genes) have been already combined in the Bt crops. The sustainable use of Vip3 and Cry proteins (or genes) in commercial Bt crops has been an important candidate in pest and resistance management strategies.

Table 5. The commercially available Bt-crops expressing Vip3A proteins.

Crops	Bt genes	Trade name	Developer, country and year of first approval
Cotton	*vip3Aa19*	VIPCOT™	Syngenta, USA, 2008
	cry1Ab+vip3Aa	VIPCOT™	Syngenta, Costa Rica, 2009
	cry1Ac+cry1F+vip3Aa	Widestrik™ × Roundup Ready Flex™ × VIPCOT™ cotton	Dow AgroSciences LLC, Japan, 2013
	cry1Ac+cry2Ab2+vip3Aa	Bollgard® III	Monsanto Company, Australia, 2014
	cry1Ac+cry2Ae+vip3Aa	Glytol™ × Twinlink™ × VIPCOT™ cotton	Bayer CropScience, Australia, 2016
Maize	*vip3Aa20*	Agrisure™ Viptera	Syngenta, USA, 2008
	cry1Ab+ vip3Aa20	Agrisure® Viptera™ 4	Syngenta, Japan, 2010
	cry1Ab (truncated) + *vip3Aa20*	Agrisure® Viptera™ 2100	Syngenta, Japan, 2010

cry1Ab+cry1Fa2+vip3Aa20	Agrisure™ Viptera 3220	Syngenta, Japan, 2010
ecry3.1Ab+cry1Ab+cry1Fa2 +vip3Aa20	Agrisure® Duracade™ 5222	Syngenta, Japan, 2013
cry2Ab2+cry1Fa2+vip3Aa20	Power Core™ × MIR162 × Enlist™	Dow AgroSciences LLC, Japan, 2016

Note:http://www.isaaa.org/gmapprovaldatabase/gmtrait/default.asp?TraitID=6&GMTrait=Lepidopteran%20 insect%20resistance. (Chakrabarty et al., 2020).

Objectives

The main goal of this thesis is to shed some light on the structure and insecticidal mechanism of Vip3A proteins by the critical amino acids site-mutants (or ala-mutants) identified from previous work, and their relationship with other insecticidal Cry1 toxins in lepidopteran pests of agriculture. Considering the Vip3A proteins have been already used as biopesticide or cloned in genetically modified crops, a better understanding of the mode of action of Vip3A proteins is urgent and critical to guide the safety risk assessment and application. In addition, a greater development of the binding site models help to optimize the combinations of diverse Vip3A and Cry toxins for increasing the combined pest spectrum and delaying the increasing Cry-resistance.

To achieve this goal, we base on the critical amino acids sites of Vip3Af previously obtained by previous work, the following specific objectives are established.

Chapter 1. Interactions of the combinations of Vip3 and Cry proteins.

1.1 Insecticidal activity and synergistic combinations of ten different Bt toxins against *Mythimna separata* (Walker).
1.2 Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins.

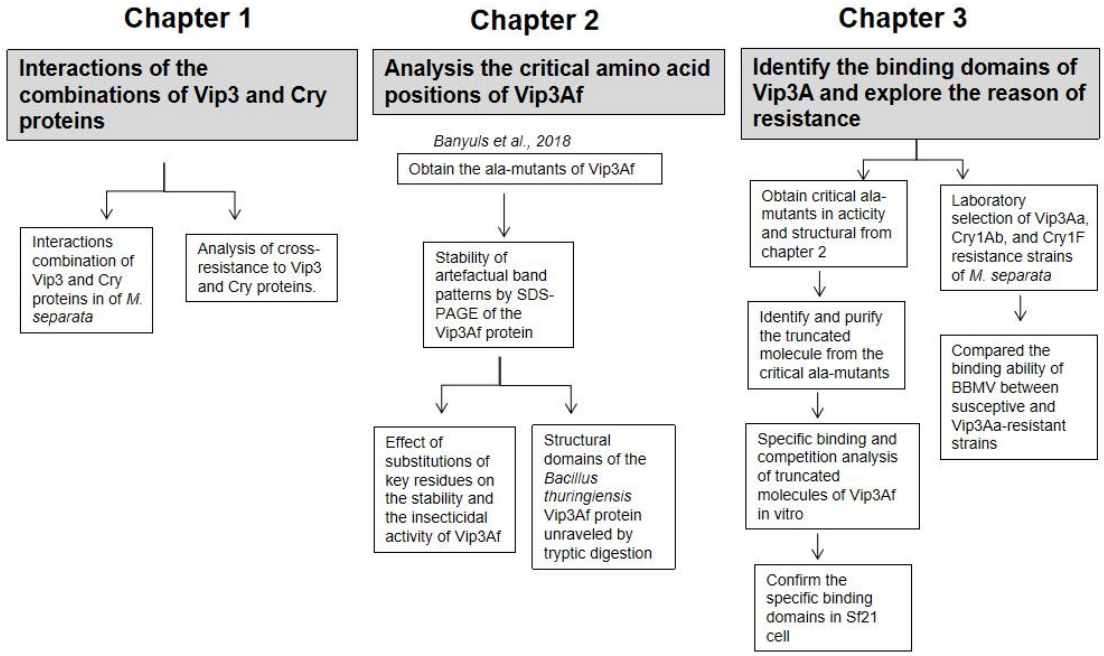
Chapter 2. Identify the critical amino acid positions of Vip3Af for the insecticidal activity and structure.

2.1 Structural domains of the *Bacillus thuringiensis* Vip3Af protein unraveled by tryptic digestion of alanine mutants.
2.2 Effect of substitutions of key residues on the stability and the insecticidal activity of Vip3Af from *Bacillus thuringiensis*.

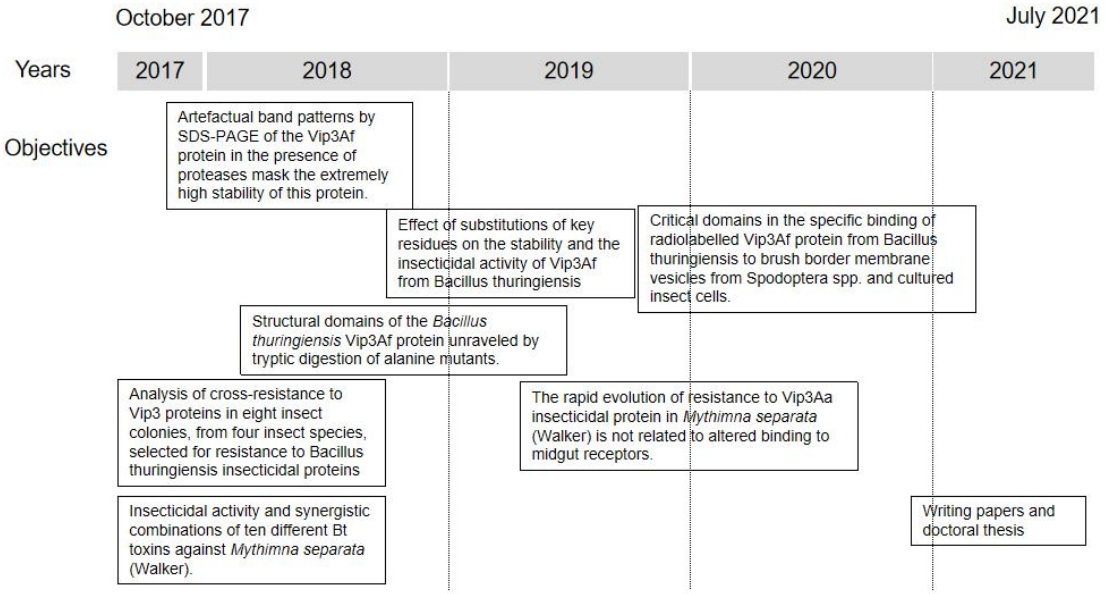
Chapter 3. Analyze the mode of action of Vip3A, and explore the reason of the resistance.

3.1 Artefactual band patterns by SDS-PAGE of the Vip3Af protein in the presence of proteases mask the extremely high stability of this protein.
3.2 Specific binding of radioabeled Vip3Af insecticidal protein from Bt to brush border membrane vesicles from *Spodoptera* spp.
3.2 The rapid evolution of resistance to Vip3Aa insecticidal protein in *Mythimna separata* (Walker) is not related to alter binding to midgut receptors.

2.1 Workflow



2.2 Timeline



2.3 Contributions

Objectives	Contributions			
	Idea/ design	Experiment by YQ[1]	Writing draft	Modification
1.1 Insecticidal activity and synergistic combinations of ten different Bt toxins against *Mythimna separata* (Walker)	KH[2]	Bioassays for Vip3Aa16, Vip3Ca, Cry1, and their combination use	YJ[3], YQ	KH, YJ
1.2 Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins	JF[4], JGC[5]	Together with YW[6], did bioassays in *Ostrinia furnacalis*	JGC, JF	JF
2.1 Structural domains of the *Bacillus thuringiensis* Vip3Af protein unraveled by tryptic digestion of alanine mutants	JF, YQ	Together with JF accomplish all the work	YQ, JF	JF
2.2 Effect of substitutions of key residues on the stability and the insecticidal activity of Vip3Af from *Bacillus thuringiensis*	NB[7], JF	Bioassays for the mutants	NB, JF	JF
3.1 Artefactual band patterns by SDS-PAGE of the Vip3Af protein in the presence of proteases mask the extremely high stability of this protein	NB, JF	Chromatogram of Vip3Af	NB, JF	JF
3.2 Specific binding of radioabeled Vip3Af insecticidal protein from Bt to brush border membrane vesicles from *Spodoptera* spp.	JF, PHM[8], YQ	Binding condition, truncated molecules purification, binding assays in vitro. The cell viability tested and binding in Sf21 cells with PHM and MLB[9]	YQ, JF, PHM, MLB	JF, PHM
3.3 The rapid evolution of resistance to Vip3Aa insecticidal protein in *Mythimna separata* (Walker) is not related to altered binding to midgut receptors	KH, JF	Engaged the 1-3 generation of selection and performed binding analysis	YQ, JF, PHM	JF, PHM, KH

Note: [1] YQ: Yudong Quan; [2] KH, Kanglai He; [3] YJ, Yangjing; [4] JF, Juan Ferré; [5] JGC, Joaquín Gomis-Cebolla; [6] YW, Yueqin Wang; [7] Nuria Banyuls; [8] PHM: Patricia Hernández-Martínez; [9] MLB, Maria Lázaro-Berenguer.

Chapter 1. Interactions of the combinations of Vip3 and Cry proteins

1.1 Insecticidal activity and synergistic combinations of ten different Bt toxins against *Mythimna separata* (Walker).

Results are included in:

Yang J, Quan Y, Sivaprasath P, Shabbir MS, Wang Z, Ferré J, He K. 2018. Insecticidal activity and synergistic combinations of ten different Bt toxins against *Mythimna separata* (Walker). Toxins 10, 454. https://doi.org/10.3390/toxins10110454.

1. Introduction

Bacillus thuringiensis (Bt) is a gram-positive bacterium that produces pesticidal crystal proteins which are active against insects, nematodes and other invertebrates (Palma et al., 2014; Wei et al., 2003; Iatsenko et al., 2014). Since Hannay discovered the crystalline inclusion in sporulating cells of Bt (Hannay, 1953), the crystal proteins have been studied extensively to control the lepidopteran pest (Palma et al., 2014; Frankenhuyzen, 2013; Schnepf et al., 1998). Because of the lack of toxicity to vertebrates and most other nontarget organisms, Bt toxins are environmentally friendly, either applied as biopesticides, or in transgenic crops (Palma et al., 2014; Frankenhuyzen, 2013; Schnepf et al., 1998). Currently, transgenic crops producing Bt toxins (Bt crops) that kill major target pests have been globally adopted and are grown in more than 70 million hectares annually since 2010, having reached over 100 million hectares in 2017 (James, 2017). Despite the high number of Bt toxins discovered to date, only a few Bt toxins, such as Cry1Ab, Cry1Ac, Cry1F, Cry2Ab, Cry3, Cry1C, and Vip3A,are found in commercial Bt crops (ISAAA, 2018, http://www.isaaa.org/gmapprovaldatabase/default.asp). Although they show a high efficacy against some pests, each one of these toxins has its own insecticidal spectrum of toxicity and, therefore, only a few of them are appropriate to control a given pest (Hernández-Martínez et al., 2013; Ruiz de Escudero et al., 2014; Walters et al., 2008; Walters et al., 2010; Chakroun et al., 2016a; Estruch et al., 1996). The evolution of insect resistance is the primary threat to the continuous success of Bt crops (Tabashnik, 1994; Ferré and Rie, 2002). To deal with the above two issues, strategies of stacking (combining toxins that have different insect targets) and/or pyramiding (combining toxins that have the same insect target) two or more insecticidal traits in Bt plants have been deployed, with excellent results (James, 2017). These strategies have been shown to be very effective in achieving a broader spectrum for insect control and combating resistance (Adamczyk et al., 2008; Roush, 1998; Zhao et al., 2003; Carrière et al., 2015; Carrière et al., 2016; Zhao et al., 2005; Tabashnik et al., 2009). Therefore, the characterization of which set of Bt toxins is useful in the control of a given pest is of paramount importance to predict the efficacy of Bt crops on an insect pest that has not been tested previously, and even to predict the chances of developing resistance based on previous information on cross-resistance episodes in other related species.

Both Cry and Vip3 toxins follow similar steps in their mode of action. They are synthesized as protoxins, which must be cleaved by midgut proteases to be converted into active toxins after intake by the larvae (Andrews et al., 1985; Caccia et al., 2014). Activated toxins insert into the epithelial cell membrane via binding to the specific brush border receptors and elicit the formation of pores, which eventually lead to the destruction of midgut epithelial cells (Knowles, 1994; Bravo et al., 2004; Bravo et al., 2007; Gómez et al., 2007; Pardo-López et al., 2013; Gomis-Cebolla et al., 2017). These events ultimately lead to the death of larvae by septicemia (Yu et al., 1997; Caccia et al., 2016). Despite Cry and Vip3 toxins following similar steps in the mode of action, they differ in that they do not compete for binding sites (Lee et al., 2006; Sena et al., 2009; Abdelkefi-Mesrati et al., 2009; Chakroun and Ferré, 2014; Gouffon et al., 2011). A combination of Vip3Aa/Cry1Ac in cotton, Vip3Aa/Cry1Ab in corn, Vip3Aa/Cry1Ac/Cry1Fa in cotton, and Vip3Aa/ Cry1Ab/Cry1Fa in corn have been applied for combating resistance to the first-generation Bt cotton and corn that express Cry1Ac and Cry1Ab toxins, respectively (Adamczyk et al., 2008; Zhao et al., 2003; Carrière et al., 2015; Chen et al., 2017). The distinct mode of action between Cry and Vip3 proteins makes them good candidates to be combined in Bt crops to delay resistance evolution and broaden the insecticidal spectrum of the crop.

The oriental armyworm (OAW), *Mythimna separata* (Walker), (Lepidoptera: Noctuidae), is a well-known long-distance migratory and major agricultural pest in Asia and Australia (Burgess, 1987; Drake and Gatehouse, 1995; Sharma et al., 2002; Chang et al., 2015; EPPO, 2018; Kouassi et al., 2009; Sharma and Davies, 1983; Jiang et al., 2014). Practically, control of OAW relies on chemical insecticide spray (Wang et al., 2018; Song et al., 2017). The adoption of Bt maize would provide new and alternative tools for limiting the damage caused by this pest. A laboratory study showed that *Mythimna unipuncta* could evolve resistance to Cry1Ab (González-Cabrera et al., 2013). In addition, the fall armyworm (FAW), *Spodoptera frugiperda* (another species of the same family and a long-distance migratory moth in America), which also can feed on maize (Buntin et al., 2001), has been reported to have developed resistance to Cry1Fa in Bt maize (Storer et al., 2010; Monnerat et al., 2015; Omoto et al., 2016). Therefore, research on the efficacy and interaction effects of Bt toxins on OAW are urgent and needed in ecological safety, as well as in building a biological control method for this pest.

In this work, we evaluated the efficacy of individual Cry (Cry1Ab, Cry1Ac, Cry1Ah, Cry1Fa, Cry1Ie, Cry2Aa, and Cry2Ab) and Vip3 proteins (Vip3Aa19, Vip3A16, and Vip3Ca), with an interest in maize crop protection. In addition, we studied the possible synergistic effects of combinations of Cry and Vip3 proteins.

2. Materials and Methods

2.1 Insect Strains

Eggs of OAW were obtained from Keyun Biology Company. The eggs were transferred to Zip-lock bags (#10) and reared under laboratory conditions (28 ± 1 ◦C, L:D = 16:8 h, RH = 70%~80%). Neonates were used for diet bioassay within 6 h after hatching.

Diet Formulation

Artificial diet was formulated according to Wang and Zheng (Wang et al., 1983) with slight modifications. Ingredients: 250 g of powdered rat feed, 300 g of powdered corn leaf, soybean flour 40 g, corn flour 50 g, wheat germ 30 g, casein 20 g, yeast 40 g, glucose 20 g, fructose 20 g, sugar 30 g, Weber's salt mixture 10 g, ascorbic acid 6 g, cholesterol 2 g, mixed vitamins 1 g, sorbic acid 6 g, erythromycin 1.2 g, and thiabendazole 2 g. These powders were mixed together, vacuum packed, and stored at 4 ◦C until use.

2.2 Bt Toxins

Trypsin-activated Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab, and Cry1Fa toxins were purchased from Envirologix (Portland, OR, USA). Cry1Ie and Cry1Ah are chromatographically purified recombinant proteins expressed in *Escherichia coli* and *Bacillus thuringiensis* (Biot 1 Ah), which were provided by the Chinese Academy of Agricultural Sciences, Biotech group. Vip3Aa16 and Vip3Ca were provided by the Department of Genetics, University of Valencia (Valencia, Spain). Vip3Aa19 was provided by the Da Bei Nong Group.

2.3 Bioassays

2.3.1 Bt Toxin Bioassay

Ten toxins were used to check their toxicity against OAW neonates. Initially, the Bt toxins stock solutions (1 mg/mL) were prepared by dissolving the toxins in sodium carbonate buffer (50 mM, pH =

10) separately in their respective vials. In a 50 mL beaker, the protoxins were mixed thoroughly with the diet (3.5 g) and distilled water (6.5 mL). The Bt toxicity screening was performed by increasing the concentration gradually according to Shabbir et al., 2018 [71]. This mixture was divided equally into the cells of a 48-well plate. One OAW neonate was placed on the surface of the diet in each well, using a fine brush. These plates were sealed with sealing film and a small hole was punctured on each well and these plates were placed in the rearing room (28 ± 1 ◦C, L:D = 16:8 h, RH = 70%~80%). The survival rate and larval weight were recorded after 7 days. Larva weighed <0.2 mg and was beyond second instar, so it was considered dead.

2.3.2. Assessment of Synergism between Bt Toxins

The synergetic effect between Bt toxins was assessed using combinations of the Cry and Vip3 families. In choosing the toxin ratios, the Cry1Ab is exemplified in order to bring the expected LC_{50} values of the toxin mixture to an appropriate range. A ratio of 0.7/0.3, or close to it, was chosen for ease of comparison and calculation. The diet bioassays for different combinations of Bt toxins werecarried out with the same method as the one mentioned above for single toxins. Three replications were performed for each combination.

2.4 Statistical Analysis

PoloPlus (v 1.0, LeOra Software, Parma, MO, USA) was employed to estimate the 50% lethal concentration (LC_{50}) with 95% fiducial limits (FL) and the slope for bioassays by probit analysis. Tests for expected and observed mortalities were evaluated using the method described by Tabashnik (Tabashnik, 1992). Differences between observed and expected LC_{50} values were analyzed by the U-test, that is, the LC_{50} ratio test (Wheeler et al., 2006). The difference between the observed and expected mortalities was analyzed by conducting a $\chi 2$ -test. Both U-test and $\chi 2$ -test were run through the SAS 9.4 software (North Carolina State University, Raleigh, NC, USA).

3. Results

3.1. Susceptibility of *Mythimna separata* to Bt Toxins

The toxicities of different Bt toxins against OAW varied considerably (Table 1). The LC_{50} values ranged from 1.6 to 78.6 µg/g. Vip3Aa19 was the toxin with the lowest LC_{50} value (1.6 µg/g), though not significantly different from Vip3Ca and Cry1Ac. However, considering the LC_{50} and LC_{95} values together, Vip3Aa19 and Vip3Ca were the most potent toxins, followed by Cry1Ab, Cry1Ac, Cry2Aa, and Vip3Aa16. The rest of the toxins tested (Cry1Ah, Cry1Fa, Cry1Ie, and Cry2Ab) had significantly less potency, either because of having high LC_{50} values or by having a low regression slope. Cry1Ie was significantly the less toxic protein, with an LC_{50} value of 78.6 µg/g.

Table 1. Effect of Cry and Vip3 family protoxins against *Mythimna separata* neonates.

Toxin	n	LC_{50}(95%FL) µg/g*	LC_{95}(95%FL) µg/g*	Slope ± SE	χ^2	*df* (χ^2)
Cry1Ab	480	6.4(4.2, 9.0) c	326.5(158.3, 1034.2) a	0.96 ± 0.12 bc	3.3	5
Cry1Ac	480	3.7(2.1, 5.7) cd	255.0(121.0, 893.7) a	0.89 ± 0.12 bc	1.6	5
Cry2Aa	528	6.2(3.9, 9.8) c	725.6(229.4, 5792.6) a	0.80 ± 0.12 c	4.2	6
Cry2Ab	768	22.3(15.3, 32.3) b	>1000	0.82 ± 0.06 c	20.8	14
Cry1Fa	672	14.4(5.7, 24.6) b	>1000	0.22 ± 0.04 c	12.8	10
Cry1Ie	672	78.6(47.3, 160.3) a	>1000	0.86 ± 0.11 c	13.7	9
Cry1Ah	576	18.7(13.1, 25.9) b	>1000	0.26 ± 0.03 c	9.9	8
Vip3Aa16	528	7.4(2.7, 19.3) c	351.8(74.4, 939.9) a	0.98 ± 0.14 bc	17.5	6
Vip3Aa19	384	1.6(0.55, 3.53) d	35.0(17.0, 148.0) b	1.24 ± 0.15 b	11.2	6
Vip3Ca	480	3.4(2.5, 4.6) cd	27.1(17.4, 54.9) b	1.83 ± 0.19 a	5.2	5

n, Number of larvae tested. 95%FL, 95% fiducial limits. *, Values followed by the same lowercase letter in the same column indicate no significant difference at $p \geq 0.05$. SE, Standard error.

3.2. Effect of Bt Toxins Combinations against *Mythimna separata*

In the search for possible synergistic interactions between Cry and Vip3 toxins, different toxin combinations were tested against the OAW neonates and results are shown in Table 2. There were four combinations showing a statistically significant interaction, all of them with Vip3Aa16 (marked with an asterisk in Table 2). For these synergistic interactions, the synergistic factor (*SF)* ranged from 2.2 to 9.2. No significant antagonistic effects were found.

Table 2. Susceptibility of *Mythimna separata* neonate larvae to combinations of Cry and Vip protoxins.

Toxins	Ratio	n	Slope ± SE	LC_{50}(95%FL) µg/g		χ^2	*df* (χ^2)	*SF*	*P*
				Observed	Expected				
Cry1Ab/Vip3Aa16	0.71:0.29	480	0.9 ± 0.1	3.1 (1.8, 4.5)	6.6 (3.6, 10.7)	2.8	5	2.2	0.03
Cry1Ab/Vip3Ca	0.71:0.29	480	1.0 ± 0.1	2.6 (0.4, 5.5)	5.1 (3.5, 7.1)	15.1	5	2.0	0.29
Cry1Fa/Vip3Aa16	0.71:0.29	480	1.0 ± 0.1	1.8 (0.3, 3.9)	11.3 (4.3, 22.8)	9.1	5	6.3	0.01
Cry1Fa/Vip3Ca	0.71:0.29	480	1.2 ± 0.1	11.7 (5.7, 21.8)	7.5 (4.1, 10.9)	11.3	5	0.6	0.29
Cry1Ie/Vip3Aa16	0.67:0.33	480	0.6 ± 0.1	2.0 (0.57, 3.9)	18.6 (7.2, 46.7)	1.3	5	9.2	<0.01
Cry1Ie/Vip3Ca	0.67:0.33	480	0.9 ± 0.1	6.2 (1.4, 13.9)	9.5 (6.7, 12.9)	13.6	5	1.5	0.48
Cry1Ah/Vip3Aa16	0.68:0.32	480	1.5 ± 0.1	5.12 (3.4, 7.1)	12.6 (5.9, 23.4)	1.7	5	2.5	0.02
Cry1Ah/Vip3Ca	0.68:0.32	480	1.6 ± 0.2	8.12 (5.7, 10.7)	7.8 (5.5, 10.4)	0.9	4	1.0	0.84
Cry2Aa/Vip3Aa16	0.50:0.50	480	1.4 ± 0.2	4.07 (2.4, 6.2)	4.4 (3.0, 6.2)	7.8	5	1.1	0.78

n, Number of larvae tested. SE, Standard error. 95%FL, 95% fiducial limits. *SF*, Calculated as the expected LC_{50} divided by the observed LC_{50}. *P*, Probability value based on U-test.

3.3. Determination of the Mortality Rate

The interaction of some combination of toxins was tested by the mortality rate at a fixed toxin concentration instead of the LC_{50} values. Of the four combinations tested, only the Cry2Aa/Vip3Ca combination showed a significant result (synergism), with an observed mortality of 69.8% vs. the expected 50% (Table 3).

Table 3. Mortality of *Mythimna separata* neonate larvae to combinations of Cry and Vip protoxins.

Toxins	Proportion	Concentration (ug/g)	n	Mortality ± SE (%)		*P*
				Observed	Expected	
Cry1Ac/Vip3Aa16	0.56/0.44	2.5	144	55.6 ± 0.9	50	0.69
Cry1Ac/Vip3Ca	0.56/0.44	1.8	144	48.3 ± 0.2	50	0.83
Cry2Aa/Vip3Aa16	0.50/0.50	3.4	144	57.8 ± 0.1	50	0.35
Cry2Aa/Vip3Ca	0.50/0.50	2.2	144	69.8 ± 0.3	50	0.05

n, Number of larvae tested. SE, Standard error. *p*, Probability value based on χ^2 -test.

4. Discussion

It is known that insects have varying degrees of susceptibility to different Bt toxins, and the assessment is necessary for defining susceptibility before the implementation of commercial cultivation of Bt crops. In this study, the toxicity (LC_{50}) and synergistic effects of Cry and Vip3 toxins were assessed against OAW. The LC_{50} values obtained for OAW ranged from 1.6 to 78.6 μg/g for Cry and Vip toxins (Table 1). In previous reports, FAW and *Ostrinia nubilalis* showed higher susceptibility to Cry1Fa (Omoto et al., 2016), which is even more effective than Cry1Ab and Cry1Ac against *Helicoverpa armigera* or *Plutella xylostella* (Granero et al., 1996; Siebert et al., 2008). Co-expressing Cry1F with Cry1Ac in cotton, and Cry1Ab in maize, can broaden the number of targets species of Bt cotton and Bt maize (Chen et al., 2017; Siebert et al., 2008). However, in this study, Cry1Fa was less efficient than Cry1Ac and Cry1Ab. This indicates differences in susceptibility to those toxins between OAW and FAW. This suggests that pyramiding Cry1F and Cry1Ab in maize may not increase the efficacy against OAW control.

The LC_{50} and LC_{95} values of Vip3Aa19 and Vip3Ca did not show any significant difference. However, the slope showed a significant difference (Table 1), thus indicating that the toxicity of Vip3Ca was greater than that of Vip3Aa19. Although the value of Vip3Ca was not significantly different from those of Cry1Ab, Cry1Ac, and Cry2Aa at the LC_{50} level, it was significantly more active at the level of LC_{95}. Vip3Ca showed remarkable efficacy against the OAW, according to both the LC_{50} and LC_{95} values, which clearly indicates that Vip3Ca could be recommended in controlling the OAW. Similarly, high slope values of Vip3A proteins were also reported in *Heliothis virescens*, which can be interpreted as that the activity of Vip3A proteins need a particular threshold concentration to be toxic in the insect midgut (Lemes et al., 2014). In a recent study, it was reported that Vip3Ca was as effective as Cry1Ab against *Ostrinia furnacalis*, but Vip3Aa was less toxic (Gomis-Cebolla et al., 2018). In addition, Vip3Ca was also found to overcome resistance to Cry1Ab in Cry1Ab-resistant *O. furnacalis*, indicating that Cry1Ab and Vip3Ca may have different binding sites. This suggests that co-expressing Cry1Ab and Vip3Ca may be a useful component in OAW and *O. furnacalis* pest management programs, as well as pest resistance management programs.

The interaction effect between proteins is an important issue in the selection of toxins to use in pest control and insect resistance management. A number of investigations have confirmed that synergism and antagonism may occur between Vip3 and Cry proteins (Siebert et al., 2008; Lemes et al., 2014; Gomis-Cebolla et al., 2018; Graser et al., 2017). Bergamasco et al. reported the species specificity effect of protein interaction between Cry1Ia and Vip3Aa (Bergamasco et al., 2013; Iatsenko et al., 2014; Wang et al., 2018). A synergistic effect was found in FAW, *Spodoptera albula*, and *Spodoptera cosmioides*, but an antagonistic effect was found in *Spodoptera eridania*. In the present study, the highest synergistic effect was observed in the combination of Cry1Ie and Vip3Aa16. Cry1Ie

is highly toxic to *O. furnacalis* (Song et al., 2003) and effective in preventing the development of resistance in *H. armigera* targeted by Bt maize (Zhang et al., 2013). This suggests the potential use of combination of Cry1Ie and Vip3Aa16 in maize for a broader target species control and favoring the pest resistance management.

Cry1F expressing maize TC1507 is toxic to *O. nubilalis* (Siegfried et al., 2006), a relative species to *O. furnacalis*. Cry1F expressing maize has the potential to control *O. furnacalis*, as evidenced from results from both laboratory studies with pure protein (Wang et al., 2016) and field trials with TC1507 maize (unpublished data). In the present study, we found Cry1Fa and Vip3Aa16 interacted synergistically, with an *SF* of 6.3. This indicates that a pyramided maize plant expressing Cry1Fa and Vip3Aa16 would be a new strategy in an overall pest management program for both *O. furnacalis* and OAW. Meanwhile, synergistic effects were also observed in the combinations of Vip3Aa16 with either Cry1Ab (2.2-fold) or Cry1Ah (2.5-fold), although they were relatively low. Interestingly, we did not find any synergistic effect in the combinations of Vip3Ca with Cry1 type toxins (Tables 2 and 3), although previous studies suggested that the mode of action of Vip3Aa was similar to that of Vip3Ca (Gomis-Cebolla et al., 2017; Tabashnik, 1992; Liao et al., 2002); the difference in synergistic properties may unravel differences in their mode of action at the biochemical level.

5. Conclusions

This study demonstrated the toxicity of seven Cry and three Vip3 protoxins to OAW. Our results reveal that, among the Bt toxins tested, Vip3Aa19 and Vip3Ca have the highest toxicity, followed by Cry1Ab, Cry1Ac, Cry2Aa, and Vip3Aa16, whereas Cry1Fa, Cry1Ah, Cry2Ab, and Cry1Ie possess the lowest larvicidal activity. Combinations of Vip3Aa16 with Cry1 toxins as pyramids showed a significant synergistic activity, while combinations of Vip3Ca with Cry1 toxins did not show any synergism. The results obtained provide precise information for projecting new combinations of Bt genes in transgenic crops for a broader target spectrum and a reliable component of pest resistance management programs.

Chapter 1. Interactions of the combinations of Vip3 and Cry proteins

1.2 Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins.

Results are included in:

Gomis-Cebolla J, Wang Y, Quan Y, He K, Walsh T, James B, Downes S, Kain W, Wang P, Leonard K, Morgan T, Oppert B, Ferré J. 2018. Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins. J Invertebr Pathol 155, 64–70. https://doi.org/10.1016/j.jip.2018.05.004

1. Introduction

Vip3 insecticidal proteins are synthesized by *Bacillus thuringiensis* (Bt) during the vegetative growth phase and are active against lepidopteran insects (Chakroun et al., 2016a; Estruch et al., 1996). Vip3 proteins are classified into three protein subfamilies based on their amino acid sequence identity: Vip3A, Vip3B, and Vip3C (Crickmore et al., 2013). Most studies on the insecticidal activity of Vip3 proteins have been performed on the Vip3A protein subfamily, in particular, with the Vip3Aa protein. Vip3Ca was discovered more recently and show some toxic effect against some lepidopteran species (Palma et al., 2012; Gomis-Cebolla et al., 2017). In contrast to Vip3Ca, Vip3A proteins have a broad insecticidal spectrum against lepidopteran pests (Chakroun et al., 2016a). The fact that the insecticidal spectrum and the mode of action of the Vip3 proteins differ from that of the Cry1 andCry2 proteins, makes Vip3 proteins good candidates to be used in combinations with Cry proteins in Insect Resistance Management (IRM) programs.

The mode of action of Vip3 proteins (Vip3A and Vip3C) shares some similarities to that of the Cry proteins in that they are synthesized in the form of protoxins, which are further processed by midgut proteases rendering the active toxin (Estruch et al., 1996; Yu et al., 1997; Lee et al., 2003; Chakroun et al., 2012; Caccia et al., 2014; Gomis-Cebolla et al., 2017). The activated toxins bind to specific receptors in the midgut membrane leading to the disruption of the midgut epithelial cells and eventual death of the larva. The Vip3 receptors are not shared by Cry proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014; Gomis-Cebolla et al., 2017). However, it has been recently shown that the Vip3Aa and Vip3Ca proteins compete for shared binding sites (Gomis-Cebolla et al.,2017).

In agriculture worldwide, repeated applications of Bt sprays and widespread adoption of Bt-crops (transgenic crops protected from insects by the expression of cry or/and vip3 genes) have led to resistance (Ferré and Van Rie, 2002; Ferré et al., 2008, Tabashnik, 2015; Tabashnik et al., 2009). Therefore, in this arms race against insects, it is necessary to keep exploring the potential of new insecticidal proteins for pest control and, at the same time, to test for their compatibility in combinations with other proteins in terms of cross-resistance. Although cross-resistance studies have been performed with Cry1- and Cry2-resistant colonies against Vip3Aa (Jackson et al., 2007; Fang et al., 2007; Anilkumar et al., 2008; Vélez et al., 2013; Huang et al., 2014; Qian et al., 2015; Welch et al., 2015; Horikoshi et al., 2016), and with Vip3Aa-resistant colonies against Cry1A proteins (Mahon et al., 2012; Chackroun et al., 2016b; Pickett et al., 2017), cross-resistance to Vip3Ca has never been tested.

In this study, we tested the susceptibility of Cry1-, Cry2- and Dipelresistant colonies from four insect species (*Trichoplusia ni*, *Plodia interpunctella*, *Helicoverpa armigera* and *Ostrinia furnacalis*) to the Vip3Aa and Vip3Ca proteins and compared the results to the non-selected controls. In addition, we tested two Vip3Aa-resistant colonies from *H. armigera* for cross-resistance to Vip3Ca.

2. Materials and methods

2.1. Insect colonies

2.1.1. Insect rearing of *T. ni* strains

Three *T. ni* strains were used to examine their response to Vip3Aa and Vip3Ca. The *T. ni* Cornell laboratory strain (Wang et al., 2007) was used as the susceptible control strain. The two resistant strains were a Cry1Ac-resistant strain, GLEN-Cry1Ac-BCS (Wang et al., 2007), and a Cry2Ab-resistant strain,

GLEN-Cry2Ab-BCS (Song et al., 2015). Both of the resistant *T. ni* strains were near-isogenic to the susceptible Cornell strain and the resistance is fixed (i.e., they were homozygous for the resistance genes). The *T. ni* colonies were maintained on artificial diet without exposure to Bt toxins (Bell et al., 1981).

2.1.2. Insect rearing of *H. armigera* strains

Five *H. armigera* strains were used to determine their response to Vip3Ca. The *H. armigera* susceptible colony, GR, was used as a control (Mahon et al., 2007). The *H. armigera* homozygous resistant colonies ISOC8, (Cry1Ac), Sp15 (Cry2Ab resistant) and Sp85 (Vip3A resistant) were established from lab selection (ISOC8) and positive F2 tests in 2002 (Sp15) and 2010, (Sp85) respectively. The *H. armigera* Cry2Ab/ Vip3A resistant strain was established by reciprocal crosses placing male pupae from one strain with female pupae from the other in cages (Walsh et al., 2014). All of the resistant strains were repeatedly outcrossed to a susceptible colony and reselected with the appropriate toxin(s). The Cry2Ab resistant line Sp15 carries an ABCA2 transporter mutation that confers the phenotype (Tay et al., 2015), but the mechanism of resistance is unknown for ISOC8 (Cry1Ac) and Sp85 (Vip3A). The rearing methods used to maintain *H. armigera* were modified from those described by Teakle and Jensen (1985).

2.1.3. Insect rearing of *P. interpunctella* strains

Two *P. interpunctella* strains were used to characterize their response to Vip3Aa. The *P. interpunctella* susceptible colony, EP, was obtained from a grain storage bin and has been maintained in the laboratory on cracked wheat diet (Oppert et al., 2010). The resistant colony EPDpl500 was selected from the parental EP, with 500 mg Dipel (Bt subspecies kurstaki, strain HD-1) per kg diet, gradually increasing the dose to 10,000 mg/kg, the maintenance dose for this resistant colony.

2.1.4. Insect rearing of *O. furnacalis* strains

Two strains of *O. furnacalis*, a Bt susceptible strain and a Cry1Abresistant strain were established in the laboratory. The Bt susceptible strain was collected from the field and had been reared using standard rearing techniques without exposure to any insecticide before bioassays were conducted (Song et al., 1999). The Cry1Ab-resistant strain was selected from the Bt susceptible strain by exposure to trypsin-activated Cry1Ab. The Cry1Ab-resistant strain was initially exposed throughout larval development to Cry1Ab in the artificial diet (2.5 ng of toxin/g diet). The toxin concentration was increased in succeeding generations to target 40–70% mortality in the exposed insects. After 51 generations, larvae were reared on diet containing 400 ng of toxin/g diet. The Cry1Ab-selected colony had developed > 100-fold resistance to Cry1Ab after 35 generations (Xu et al., 2010).

2.2. Source and expression of Vip3 proteins for cross-resistance assays

Vip3Aa (NCBI accession No AAW65132) was overexpressed in recombinant Escherichia coli BL21 carrying the vip3Aa16 gene (Abdelkefi- Mersati et al., 2009). The Vip3Ca protein (NCBI accession No AEE98106) was prepared from recombinant E. coli WK6 carrying the vip3Ca2 gene (Palma et al., 2012). The Vip3Aa protein was expressed following the conditions described by Chakroun et al., 2012. For the Vip3Ca protein, a single colony was inoculated in 7 ml of LB medium containing 100 µg/ml ampicillin and grown overnight at 37 °C and 180 rpm. A 1/100 dilution of the culture in 700 ml LB medium containing 100 µg/ml ampicillin was further incubated at 37 °C and 180 rpm. The culture was induced with 1 mM IPTG at an OD of 0.7 and it was grown overnight at 37 °C

and 200 rpm. Cells were collected at 6000 g for 15 min at 4 °C. The pellet was weighed and suspended in 3 ml lysis buffer (PBS, pH 8.0, containing 3 mg/ml lysozyme, 10 µg/ml DNase, and 100 µM PMSF) per gram of pellet. The sample was incubated at 37 °C for 30 min and then sonicated on ice applying two 1 min pulses at 70 W at a constant duty cycle, separated by a 10-s cooling period on ice. Then, the insoluble material was separated by centrifugation at 16,000 g for 15 min at 4 °C and the soluble cellular fraction sequentially filtered through sterile 0.45 µm and 0.22 µm cellulose acetate filters.

2.3. Purification of Vip3 proteins for cross-resistance assays

Vip3 proteins used for dose-response assays for the *T. ni*, *H. armigera* and *O. furnacalis* colonies were purified by isoelectric point precipitation (IPP) (Chackroun et al., 2012; Gomis-Cebolla et al., 2017). The pH of the lysate was lowered with acetic acid to pH 5.5 for Vip3Aa and pH 5.95 for Vip3Ca. The pellets were recovered by centrifugation at 16,000 g for 10 min and then dissolved in 20 mM Tris, 150 mM NaCl, pH 9, and dialyzed against the same buffer overnight. The Vip3Aa protein used for dose-response assays for the *P. interpunctella* colonies was purified by immobilized metal ion absorption chromatography (IMAC) on a Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+} (Fig. 1A) (Chackroun et al., 2012). The purified proteins were frozen at −80 °C and then lyophilized. The concentration of the Vip3 proteins purified by IPP was estimated by densitometry after SDS-PAGE separation. The concentration of the Vip3Aa purified by Hi-Trap chelating HP column was measured by the method of Bradford (Bradford et al., 1976). In both methods, bovine serum albumin (BSA) was used as standard. The purity of the Vip3 proteins was analyzed by SDS-PAGE (Fig. 1).

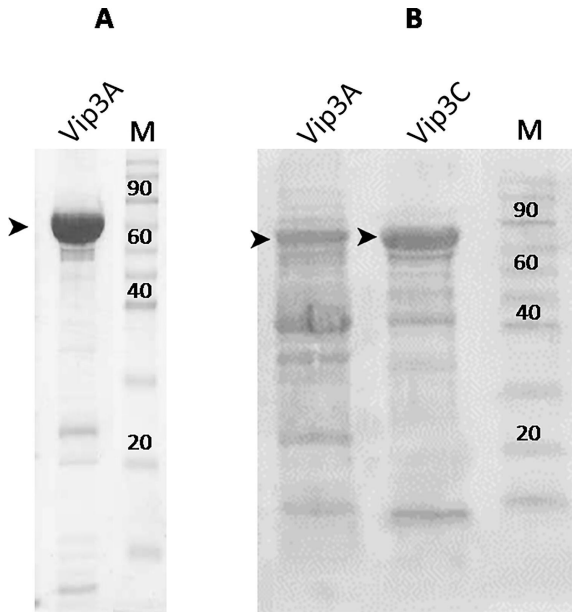


Figure 1. SDS-PAGE of Vip3Aa and Vip3Ca protoxins after partial purification from E. coli extracts. (a) Vip3Aa purified by Hi-Trap chelating HP column charged with Ni^{+2}. (b) Vip3Aa and Vip3Ca purified by isoelectric point precipitation. M: Molecular Weight Markers ("PINK PlusPrestained Protein Ladder", from Genedirex). The arrowhead indicates the band corresponding to the Vip3 protein.

2.4. Insect toxicity assays

2.4.1. Dose-response assays for the susceptible and resistant *T. ni* strains

Examination of *T. ni* strains for their susceptibilities to Vip3Aa and Vip3Ca were conducted using the surface contamination method (Kain et al., 2004). Briefly, an aliquot of 200 µl of Vip3Aa or Vip3Ca solution was spread on the surface of diet in 30-ml cups (surface area is approximately 7 cm^2) and 10 neonate larvae were placed in each cup that was covered with a lid and placed at 27 °C. Eight concentrations of Vip3Aa or Vip3Ca in 2-fold serial dilutions from 800 to 6.25 µg/ml and a non-toxin control were used for each bioassay. Five replicate cups of larvae (50 larvae in total for each dose) were included for each concentration of toxin. Larval mortality and growth inhibition (larval developmental stage remaining in the first instar) were recorded after 4 days of rearing on diet. The bioassay data were subjected to Probit analysis using the software POLO, LeOra Software to obtain the LC_{50} and IC_{50} of the Vip proteins (Russell et al., 1977).

2.4.2. Dose-response assays for the susceptible and resistant *H. armigera* strains

The susceptibility of *H. armigera* strains to Vip3Aa and Vip3Ca was conducted using the surface contamination method as follows. Approximately 300 µl of standard diet was added to straight sided 96 well trays providing 0.567 cm^2 of surface area. Once the diet had cooled, 20 µl of a solution containing an appropriate concentration of toxin was added and allowed to air dry. One neonate was placed in each well before it was sealed with a perforated heat-sensitive lid. Trays were incubated at 25 °C, 60% RH, and 14:10 h light: dark for 8 days. Six concentrations of Vip3Ca in 2-fold serial dilutions from 20 to 0.625 µg/ cm^2 and a non-toxin control were used for each bioassay. A minimum of three replicate bioassays of larvae (16 larvae in total for each dose) were included for each concentration of toxin. The numbers of dead and alive larvae were counted and the data were subjected to Probit analysis using the software POLO, LeOra Software to obtain the LC_{50} of the Vip proteins (Russell et al., 1977). Moreover, the instars of surviving larvae were recorded to obtain the larval development index (LDI) of surviving larvae.

$$LDI = \frac{[(\mathrm{n}L1 \times 1) + (\mathrm{n}L2 \times 2) + (\mathrm{n}L3 \times 3) + (\mathrm{n}L4 \times 4) + (\mathrm{n}L5 \times 5)]}{N}$$

where nL1, nL2, nL3, nL4 and nL5 are the numbers of alive larvae in the respective larvae development stage and N refer the total number of alive larvae. We consider as statistically significant all those LDI values where the 95% confidence intervals of the mean did not overlap with another LDI value for the susceptible and resistant population (Cry1Ac-, Cry2Ab-, Vip3A- and Vip3/Cry2Ab-resistant) of *H. armigera*. Confirmation of resistance in the Cry1-, Cry2-, Vip3-, and Vip3/Cry2Abresistant insects at the time of Vip3 bioassays was obtained from single dose assays at a discriminant dose (Table S1).

2.4.3. Dose-response assays for the susceptible and resistant *P. interpunctella* strains

The *P. interpunctella* strains used in the current study are EP (Dipelsusceptible) from which EP-Dpl500 was selected with Dipel, and the parent strain for the Dipel-resistant strain which was selected and maintained on 10,000 mg/kg Dipel (the resistant strain used in this study). The Dpl500 strain was moderately resistant to Cry1F (18-fold) and Cry1Ab (over 100-fold), and very resistant to Cry1Ac (> 3000-fold) and Dipel (with no mortality observed at the highest dose) (Table S2). The bioassays of *P. interpunctella* strains with Vip3 toxins were conducted using 4 mm round diet disks

punched out from a flattened cereal mixture (Herrero et al., 2001). Briefly, 5 µl of 11 different doses of Vip3Aa (from 0.1 to 100 µg/4 mm diet disk for Dipel-susceptible insects, and from 2 to 100 µg/4 mm diet disk for Dipel-resistant insects) and Vip3Ca (from 2 to 100 µg/4 mm diet disk for Dipel-susceptible insects, and from 2 to 100 µg/4 mm diet disk for Dipel-resistant insects) were added to diet disks using a micropipettor, with controls of 5 µl water only for each replicate. Treated diet disks were placed in 16-well black assay trays (Frontier Agricultural Sciences, Newark, DE, USA), and eggs were added to each well (n = 16 individuals per dose, three independent biological replicates for each toxin and *P. interpunctella* strain). Trays were covered with perforated adhesive plastic sheets and incubated at 28 °C and 75% relative humidity (R.H.) in darkness. Mortality was recorded at approximately 21 d. Data was analyzed by Probit analysis using the software POLO, LeOra Software (Russell et al., 1977) to obtain the LC_{50} of the Vip proteins, reported in mg of toxin per 4 mm (15 mg) diet disk.

2.4.4. Dose-response assays for the susceptible and resistant *O. furnacalis* strains

The susceptibility of *O. furnacalis* neonates to Vip3 proteins (Vip3A and Vip3Ca) was determined in dose-response assays in agar-free semi-artificial diet (He et al., 2005). Briefly, a single neonate was randomly transferred into each well of 48-well tray and then covered with a piece of paper and the lid. Trays were held in a growth chamber for seven days at 27 °C, 80% RH and a 16:8 h photoperiod. Number of dead larvae and the weight of larvae surviving per tray were recorded after seven days of exposure. If a larva had not developed beyond the first instar and weighed ± 0.1 mg, it would be counted as dead for calculating practical mortality. Average larval weight of survivors would be used to determine the larval growth inhibition rate as a function of toxin concentration. Bioassays were repeated on two dates with total of 96 larvae per concentration and included 6–10 concentrations of purified toxin. Dilutions of Vip3 toxins were prepared in 20 mM Tris 150 mM NaCl, pH 9. The same buffer was used as a negative control. Bioassay data were subjected to Probit analysis using the software POLO, LeOra Software 1977 to obtain the LC_{50} of the Vip proteins (Russell et al., 1977). Confirmation of resistance in the Cry1Ab-resistant insects at the time of Vip3 bioassays was obtained from dose-response bioassays with Cry1Ab (Table S3).

3. Results and discussion

3.1. Cry1A and Dipel-resistant colonies

We first tested the vulnerability of susceptible and Cry1A and Dipelresistant colonies (*P. interpunctella*, *T. ni*, *H. armigera* and *O. furnacalis*) against Vip3 proteins (Table 1). Previously it was demonstrated in *T. ni* that Cry1Ac-resistant strains were not cross-resistant to Vip3A proteins (Fang et al., 2007), but Vip3Ca had never been tested. Our results show that Vip3Ca is 5–10-fold less active than Vip3Aa to this insect species and that Cry1Ac-resistance does not confer cross-resistance to either of the two Vip3 proteins. However, growth inhibition assays showed that Cry1Ac-resistant insects were slightly less affected by both Vip3Aa and Vip3Ca (IC_{50} around 2-fold higher) compared to the susceptible insects (Table 2).

P. interpunctella had never been tested for cross-resistance to Vip3 proteins. Although the LC_{50} for Vip3Aa in the Dipel-resistant strain was approximately 5-fold higher than that of the Dipel-susceptible strain, the fiduciary limits were overlapping. Conversely, the Dipel-susceptible strain was approximately 5-fold less susceptible to Vip3Ca than the Dipel-resistant strain, but again with overlapping fiduciary limits. Therefore, cross-resistance to Vip3 proteins was not observed in the two *P.*

interpunctella strains. In comparing responses of the two Vip3 toxins to Cry toxins, Dipel-susceptible insects were less susceptible to Vip3 than Cry toxins, whereas Dipel-resistant insects were more susceptible to Vip3 toxins than Dipel, Cry1F, and Cry1Ac (Table S2).

Lack of cross-resistance to Vip3Aa in Cry1Ac-resistant *H. armigera* colony has been previously shown (Mahon et al., 2012; Chackroun et al., 2016b) and, therefore, only Vip3Ca was used in our study. The LC_{50} values for the Cry1Ac-resistant versus susceptible colonies were statistically different, indicating that resistant insects were 3.3-fold more susceptible to Vip3Ca. This difference could reflect a cost in the Cry1Ac-resistant insects which makes them less fit to withstand the same doses of Vip3Ca. As with P. interpuntella, fitness costs in Cry1Acresistant *H. armigera* may induce increased susceptibility to other toxins, such as Vip3Ca. An assay with sublethal doses of Vip3Ca was performed to test whether the slightly higher mortality produced by this protein on Cry1Ac-resistant larvae was reflected in the development time. As is indicated in Table 3, no significant differences in the larval development index (LDI) were detected between susceptible and Cry1Ac-resistant population.

Regarding *O. furnacalis*, this insect species has never been tested before against Vip3 proteins. The results of *O. furnacalis* (both susceptible and Cry1Ab-resistant strains) with Vip3Aa show that this species is not susceptible to this protein (Table 1). Only at 100 µg/g diet about 50% growth inhibition was observed in the two strains. In contrast, Vip3Ca was highly active to *O. furnacalis* larvae from both strains. The LC_{50} value of Vip3Ca for the susceptible strain was not significantly different from that of Cry1Ab (LC_{50} = 0.23 µg/g, FL = 0.17–0.30) (Table S3). However, because of the different slope values of their respective regression lines (Tables 1 and S2), Vip3Ca has an LC90 value (LC90 = 0.98 µg/g, FL = 0.81–1.31) lower than that of Cry1Ab (LC90 = 2.94 µg/g, FL = 1.94–2.53), suggesting that the former is more effective to control this pest than the latter. The small difference (LC_{50} 3.12-fold and LC_{90} 3 fold) between Vip3Ca LC values of the susceptible and Cry1Ab-resistant insects is statistically different, suggesting that Cry1Ab-resistance in this strain confers minimum cross-resistance to Vip3Ca.

Table 1. Evaluation of the susceptibility to Vip3 proteins of susceptible and Cry1-, Cry2-, Dipel- and Vip3-resistant insect colonies from different insect species.

Insect species	Protein tested	Replicates	Colony	Slope ± SE[1]	LC_{50}(95%FL)[2]	Resistance[3] ratio
T. ni					ug/cm^2	
	Vip3Aa	R1[4]	Susceptible	4.0 ± 0.4	0.95 (0.84–1.09) a	-
			Cry1Ac-resistant	5.0 ± 0.6	1.41 (1.07–1.91) a	1.48
			Cry2Ab-resistant	2.7 ± 0.3	1.10 (0.51–1.77) a	1.15
		R2[4]	Susceptible	4.3 ± 0.5	1.05 (0.65–1.80)[5] a	-
			Cry1Ac-resistant	4.2 ± 0.4	2.16 (1.30–3.83)[5] a	2.05
			Cry2Ab-resistant	3.4 ± 0.4	1.63 (1.36–1.90) a	1.55
	Vip3Ca	R1[4,§]	Susceptible	2.5 ± 0.2	8.53 (5.42–15.09) b	-
			Cry1Ac-resistant	2.5 ± 0.3	10.02 (6.07–18.3) b	1.17
			Cry2Ab-resistant	1.6 ± 0.2	16.12 (8.33–82.09) b	1.88
		R2[4,§]	Susceptible	3.3 ± 0.4	3.02 (2.53–3.54) c	-
			Cry1Ac-resistant	2.9 ± 0.3	4.95 (2.82–8.33) c	1.62
			Cry2Ab-resistant	3.0 ± 0.3	4.05 (3.40–4.78) c	1.33
P. interpunctella					µg/15 mg diet disk	

	Vip3Aa	Susceptible	0.43 ± 0.09	15.9 (2.25 – 151) d	-
		Dipel-resistant	0.65 ± 0.16	78.7 (27.2–792) d	4.95
	Vip3Ca	Susceptible	0.58 ± 0.20	67.5 (5.87–394) e	-
		Dipel-resistant	0.42 ± 0.12	13.7 (1.04–76.0) e	0.20
H. armigera				µg/cm^2	
	Vip3Ca	Susceptible	2.1 ± 0.20	5.34 (4.34–6.65) f	-
		Cry1Ac-resistant	2.1 ± 0.30	1.60 (1.11–2.11) g	0.30
		Cry2Ab-resistant	1.9 ± 0.27	1.60 (1.11–2.11) g	0.44
		Vip3Aa-resistant	-	NA[6]	0
		Vip3Aa/Cry2Ab-resistant	-	NA[6]	0
O. furnacalis				µg/g	
	Vip3Aa	Susceptible	-	UD[7]	-
		Cry1Ab-resistant	-	UD[7]	0
	Vip3Ca	Susceptible	2.52 ± 0.37	0.31 (0.22–0.38)h	-
		Cry1Ab-resistant	1.67 ± 0.15	0.97 (0.74–1.22)i	3.12

[1] SE: Standard error of the slope.

[2] LC_{50} values followed by the same letter are not significantly different from their corresponding susceptible strain based on the overlap of fiducial limits (FL).

[3] Resistance Ratio was calculated dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain.

[4] The dose-response assays, R1 and R2, are biological replications. Within each biological replication, three *T. ni* strains were assayed using the same Vip solutions at the same time.

[5] FL90, instead of FL95, was calculated and presented, as the heterogeneity of the data was above the default threshold (g > 0.5) by POLO for adequate FL95 calculation.

[6] NA: Non active. The highest dose tested (20 µg/cm^2 for the Vip3-resistant and 10 µg/cm^2 for theVip3Aa/Cry2Ab-resistant) caused a mortality of 4.7% and 6.2%, respectively.

[7] UD: Unable to determine. The highest dose tested (100 µg/g for the susceptible and resistant *O. furnacalis* strains) there was not significant mortality observed. However, it showed about 50% growth inhibition.

[§] The differences observed in the Vip3 proteins were considered as variations between replicates.

3.2. Cry2Ab-resistant colonies

Prior to this study, cross-resistance to Vip3 proteins was not found in two Cry2A-resistant colonies from *Heliothis virescens* (Jackson et al., 2007). In the present study, Cry2Ab-resistant colonies of *T. ni* and *H. armigera* were tested against Vip3 proteins, and the results were compared to their susceptible controls (Table 1). Cry2Ab-resistant *T. ni* was not cross-resistant to either of the two Vip3 proteins as measured by mortality (Table 1) or growth inhibition (Table 2).

Table 2. Evaluation of the growth inhibition to Vip3Aa and Vip3Ca of susceptible and Cry1-, Cry2-resistant *T. ni* insect colonies.

Insect species	Protein tested	Replication	Colony	Growth inhibition		Resistance ratio[4]
				Slope ± SE[1]	IC_{50} (FL95)[2] µg/cm^2	
T. ni						
	Vip3Aa	R1[3]	Susceptible	5.5 ± 0.7	0.33 (0.30–0.37) a	-
			Cry1Ac-resistant	4.3 ± 0.5	0.64 (0.44 0.86) b	1.93
			Cry2Ab-resistant	4.0 ± 0.7	0.24 (0.18–0.29) a	0.72
		R2[3]	Susceptible	4.0 ± 0.5	0.30 (0.26–0.35) a	-
			Cry1Ac-resistant	3.6 ± 0.4	0.64 (0.55–0.73) b	2.10
			Cry2Ab-resistant	5.0 ± 0.9	0.27 (0.22–0.31) a	0.88
	Vip3Ca	R1[3]	Susceptible	3.4 ± 0.3	1.17 (1.00–1.35) c	-
			Cry1Ac-resistant	4.3 ± 0.5	2.20 (1.34–3.42) c	1.88
			Cry2Ab-resistant	4.2 ± 0.5	1.38 (1.21–1.57) c	1.18
		R2[3]	Susceptible	4.2 ± 0.7	0.41 (0.33–0.47) d	-
			Cry1Ac-resistant	3.8 ± 0.6	0.93 (0.75–1.09) c	2.28
			Cry2Ab-resistant	4.8 ± 0.7	0.54 (0.45–0.62) d	1.33

[1] SE: Standard error of the slope.

[2] IC values followed by the same letter are not significantly different based on the overlap of FL.

[3] The dose-response assays, R1 and R2, are biological replications. Within each biological replication, three *T. ni* strains were assayed using the same Vip solutions at the same time.

[4] Resistance Ratio was calculated dividing the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain.

Cry2Ab-resistant *H. armigera* had never been tested for cross-resistance to Vip3 proteins, though lack of cross resistance or genetic linkage with Cry2Ab-resistance loci had been reported for Vip3Aa-resistant colonies (Mahon et al., 2007; Mahon et al., 2012; Chackroun et al., 2016b). Similarly to Cry1Ac-resistant *H. armigera* colony, the Cry2Ab-resistant insects were slightly more vulnerable (2.3-fold) than susceptible ones (Table 1). As with Cry1Ac-resistant insects, sublethal doses of Vip3Ca did not drive differences in larvae development (Table 3).

Table 3. Evaluation of the larval development index to Vip3Ca of susceptible and Cry1-, Cry2-, Vip3-, and Vip3/Cry2Ab-resistant *H. armigera* insect colonies.

Dose (µg/cm^2)	LDI[1] ± CI[2]				
	Susceptible	Cry1Ac-resistant	Cry2Ab-resistant	Vip3A-resistant	Vip3A/Cry2Ab-resistant
20	1.06 ± 1.25	0.62 ± 2.00	0.00 ± 0.00	3.58 ± 2.86	-
10	1.00 ± 1.24	0.83 ± 3.59	1.09 ± 4.69	3.73 ± ND*	3.56 ± 5.53
5	2.00 ± 1.17	1.72 ± 3.96	2.10 ± 2.24	3.9 ± ND *	3.60 ± 5.15
2.50	2.70 ± 0.61	2.27 ± 1.87	2.66 ± 2.58	3.9 ± ND*	3.60 ± 5.10
1.25	2.54 ± 0.56	2.83 ± 1.84	2.87 ± 2.93	3.9 ± ND*	3.62 ± 4.77
0.60	3.05 ± 0.56	3.05 ± 1.80	3.12 ± 1.83	3.9 ± ND*	3.80 ± 2.80
Controls					

(μg/cm^2)					
0	3.60 ± 0.63	3.50 ± 1.29	3.78 ± 1.28	3.20 ± 2.48	3.65 ± 4.44
Cry1Ac[3]	0.16 ± 0.43	3.50 ± 1.29	0.00 ± 0.00	0.0 ± 0.0	0.00 ± 0.00
Cry2Ab[3]	0.17 ± 0.43	1.00 ± 0.00	3.80 ± 0.94	0.0 ± 0.0	3.60 ± 5.10
Vip3A[3]	0.67 ± 0.54	0.66 ± 2.87	1.00 ± 0.00	2.90 ± 1.20	3.40 ± 6.35

[1] Larval development index calculated as: LDI = [1 × (Number of L1 larvae)+2 × (Number of L2 larvae)+3 × (Number of L3 larvae)+4 × (Number of L4 larvae)+5 × (Number of L5 larvae)]/Total of alive larvae. Differences between LDI values from the susceptible and resistant populations were considered statistically significant if the 95% confidence intervals (CI) of the mean did not overlap.

[2] CI: Confidence interval of the mean.

[3] Discriminant dose for Cry1Ac (0.25 μg/cm^2), Cry2Ab (0.25 μg/cm^2), and Vip3Aa (20 μg/cm^2), respectively.

* ND: Not possible to calculate the 95% CI of the Vip3A-resistant *H. armigera* colony because the assay was done with one replicate.

3.3. Vip3Aa-resistant colonies

Cross-resistance within the Vip3 subfamily of proteins has not yet been established. We tested the susceptibility of two Vip3-resistant *H. armigera* colonies (one resistant to Vip3Aa alone and the other resistant toVip3Aa and Cry2Ab) against the Vip3Ca protein (Table 1). The highest Vip3Ca doses tested (20 μg/cm^2 for the Vip3Aa-resistant insects and 10 μg/cm^2 for the Vip3Aa/Cry2Ab-resistant insects) only caused a mortality of 4.7% and 6.2%, respectively. Compared to the mortality observed in the susceptible control insects and the Cry1- and Cry2-resistant insects, these results clearly indicate that resistance to Vip3Aa conferred cross-resistance to Vip3Ca. This conclusion is supported by the results in Table 3, where Vip3Ca did not cause any delay in the development of the two Vip3Aa-resistant colonies.

The results obtained regarding cross-resistance are in agreement with the differences in the mode of action of Cry and Vip3 proteins. Several studies demonstrated that Vip3Aa does not share binding sites with Cry1 or Cry2 proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014) and recent work showed that Vip3Aa and Vip3Ca share binding sites in Mamestra brassicae (Gomis-Cebolla et al., 2017). Some of the Cry-resistant insect colonies evaluated in this study had previously been examined for alteration in Cry protein binding and reduced binding was found for the Cry proteins used as the selective agent. For example, Cry1Ac toxins did not bind to gut membrane proteins in the Cry1Acresistant *T. ni* colony (Wang et al.; 2007), gut proteins from a Dipelresistant *P. interpunctella* colony had drastically reduced binding of Cry1Ab (Herrero et al., 2001), and gut proteins from the Cry2Ab-resistant *H. armigera* colony had a greatly reduced binding of Cry2Ab (Caccia et al., 2010). In the case of *O. furnacalis*, no binding assays have been reported with resistant strains. Although binding is not the sole mechanism of resistance to Bt toxins, it is the one that confers more specific and higher levels of resistance (Ferré and Van Rie, 2002; Ferré et al., 2008).

Despite the fact that Vip3Aa and Vip3Ca have been shown to share binding sites in M. brassicae (Gomis-Cebolla et al., 2017), our crossresistance result is not straightforward to explain, Vip3Aa binding apparently was not affected in the Vip3Aa-resistant *H. armigera* colony (Chackroun et al., 2016b). We can think of several scenarios to explain this apparent paradox. First, the binding of Vip3Aa to a “functional” receptor (by this we mean a membrane molecule that triggers the subsequent steps to kill the cell) can be masked by binding to other molecules in the membrane. Examples have

been reported for Cry proteins, such as the case of Cry1Ac in H. virescens, which binds to three binding sites while only binding site A is responsible for toxicity and, thus, reduced Cry1Ac binding is not detected in resistant insects (Lee et al., 1995; Jakka et al., 2015). Second, assuming that no brush border epitope has been altered in the Vip3Aa-resistant insects, the mechanism of resistance may lay in successive steps, such as membrane insertion, pore formation, or any other post-binding event such as a signal transduction leading to cell death. If this was the case, our results would indicate that Vip3Aa and Vip3Ca, besides sharing a binding epitope in the brush border membrane, share a post-binding step which would be impaired in resistant insects. Whichever the case, our cross-resistanceresults indicate these two Vip3 proteins share common steps in the mode of action.

Supplementary material

Table S1. Confirmation of resistance in the susceptible and Cry1Ac, Cry2Ab, Vip3Aa, and Vip3Aa/Cry2Ab-resistant *H. armigera* insect colonies.

Toxins	Dose (µg/cm^2)	Mortality[1] ± SE[2]				
		Susceptible	Cry1Ac-resistant	Cry2Ab-resistant	Vip3A-resistant	Vip3Aa/Cry2Ab-resistant
Buffer[3]	-	0 ± 0	6.3 ± 4.6	1.0 ± 1.5	2.1 ± 2.1	0 ± 0
Cry1Ac	0.25	100 ± 0	11.7 ± 7.9	94.8 ± 7.4	100 ± 0	95.8 ± 4.2
Cry2Ab	0.25	100 ± 0	72.4 ± 8.0	1.4 ± 2.0	100 ± 0	4.2 ± 4.2
Vip3Aa	20	100 ± 0	96.9 ± 5.4	86.2 ± 6.8	6.3 ± 6.3	10.6 ± 6.1

[1] Mortality: Number of death larvae in each treatment, expressed as percentage

[2] SE: Standard error of the mean

[3] Buffer: 20 mM Tris, 150 mM NaCl, pH 9

Table S2. Comparison of LC_{50} values of Cry and Vip3Aa protoxins for Dipel-susceptible and -resistant *Plodia interpunctella* strains.

Toxin	Susceptible[1]	Dipel-Resistant[2]
Cry1Ab	0.02	2.37
Cry1Ac	0.08	>250
Cry1F	15.3	280
Dipel	4.80	UD
VIP3Aa	15.9	78.7
Vip3Ca	67.5	13.7

[1]Data in ug applied to a 15 mg diet disk; from Oppert et al., 2010 and this study

[2]UD – unable to determine

Table S3. Confirmation of resistance in the susceptible and Cry1Ab-resistant *O. furnacalis* insect colonies

O. furnacalis strains	LC_{50} (FL_{95})	Resistance Ratio[1]	Slope ± SE[2]
	µg/g diet		
Susceptible	0.23 (0.17 - 0.30)	-	1.16 ± 0.11
Cry1Ab-resistant	183 (155 - 213)	794	2.41 ± 0.26

[1] Resistance Ratio was calculated divided the LC_{50} value of the resistant strain by the LC_{50} value of the susceptible strain respectively.

[2] SE: Standard error of the slope.

Chapter 2. Identify the critical amino acid positions of Vip3Af for the insecticidal activity and structure

2.1 Structural domains of the *Bacillus thuringiensis* Vip3Af protein unraveled by tryptic digestion of alanine mutants.

Results are included in:

Quan Y, Ferré J. 2019. Structural domains of the *Bacillus thuringiensis* Vip3Af protein unraveled by tryptic digestion of alanine mutants. Toxins 11, 368. https://doi.org/10.3390/toxins11060368.

1. Introduction

Vip3A proteins are produced during the vegetative phase of growth of *Bacillus thuringiensis* and are of practical interest because of their insecticidal activity against Lepidoptera (Chakroun et al., 2016a). Because Vip3A proteins share no sequence and structural homology with *B. thuringiensis* Cry proteins, they are considered an excellent complement to Cry proteins in crop protection and resistance management. Some commercial Bt-crops (crops protected from insect attacks by expressing insecticidal proteins from *B. thuringiensis*) combine Cry and Vip3 proteins, and this strategy of pyramiding proteins with different modes of action is expected to continue in the future (Carriere et al., 2016).

Despite the increasing interest in Vip3 proteins, their mode of action is not completely understood, and their 3D structure still remains unknown. Recently, a number of studies have provided valuable information toward the structure of these proteins. Multiple-sequence alignments of Vip3 proteins have shown that they contain between 786 and 803 amino acids (corresponding to a molecular weight of around 89 kDa), with a highly conserved N-terminal part (up to residue 334) and a highly variable C-terminal region (Chakroun et al., 2016a). Proteolytical activation in the midgut of insects eliminates a small part of the N-terminus, which, in the case of Vip3A, takes place at residue R11/12 (Zack et al., 2017; Banyuls et al., 2018a) and, in the case of Vip3Bc1, at R20 (Zack et al., 2017), followed by the cleavage of the protein at the primary cleavage site, which in Vip3Aa and Vip3Af is K198/D199 (Banyuls et al., 2018a; Bel et al., 2017). Then, two peptides, of about 19 and 65 kDa, are generated, and these remain strongly bound to each other (Zack et al., 2017; Chakroun and Ferré, 2014; Banyuls et al., 2018b). More recently, it has been shown that Vip3 proteins are found in solution as homo-tetramers, both as protoxins and after activation by proteases (Zack et al., 2017; Banyuls et al., 2018b; Palma et al., 2017; Sahin et al., 2018).

To date, a high resolution 3D structure of a Vip3 tetrameric protein is lacking, though low resolution images have been obtained (Palma et al., 2017; Kunthic et al., 2017). In an attempt to propose a 3D structure for Vip3 proteins, Vip3Af1 and Vip3Aa16 have been subjected to in silico modelling, and several domains have been proposed. For Vip3Af, five structural domains were proposed (Banyuls et al., 2018a), with domain 1 spanning from the N-terminus to residue 188, domain 2 from residue 189 to 272, domain 3 from 273 to 542, domain 4 from 543 to 715, and domain 5 from 716 to the end. For Vip3Aa16, three domains were proposed, though domain 1 was further subdivided into three domains (Sellami et al., 2018): Subdomain 1.1 spanned from the N-terminus to residue 313, subdomain 1.2.1 from 314 to 441, subdomain 1.2.2 from 442 to 532, domain 2 from 533 to 667, and domain 3 from 668 to the end. Given the high sequence similarity between the two proteins (92.7%), the discrepancy between them regarding the regions spanned by the domains just reflects inaccuracies of the modelling programs used, probably due to the low availability of reference sequences with known 3D structures.

With the aim of shedding light on the putative functional and structural domains of Vip3 proteins, we have made use of selected Vip3Af alanine mutants (Ala-mutants) from a previous work (Banyuls et al., 2018a) which drastically affect insecticidal activity. Most of these mutants are distributed in three clusters along the length of the protein and show altered proteolytic patterns upon trypsin digestion (Banyuls et al., 2018a). Banyuls et al. labeled these altered patterns as “a” to “f” (Banyuls et al., 2018a). In the present work, we have made use of these critical Ala-mutants with the rationale that the altered patterns, generated by conformational changes due to the residue substitution, may unravel structural

and functional domains. The results, based on protease digestion patterns, oligomer formation, and theoretical tryptic sites, have allowed us to propose a map of the Vip3Af protein with five domains. The information thus generated will contribute to the better understanding of the structure of Vip3 proteins and may be useful in the search of the 3D structure of this family of proteins.

2. Materials and Methods

2.1 Protein Source, Expression and Purification

The source of the 788 amino acid protein Vip3Af1(WT) (NCBI accession No. CAI43275) and that of its mutant proteins has been described in Banyuls et al. (Banyuls et al., 2018a). The mutant proteins, all with decreased insecticidal activity, differed from Vip3Af(WT) and from each other, by a single amino acid residue which had been changed to an alanine residue. For this work, we selected the mutants which decreased the toxicity: T167A, E168A, P171A, L209A, F229A, M238A, E483A, W552A, G689A, I699A, Y719, and G727. The expression and purification of Vip3Af(WT) and the mutant proteins was carried out as described before (Banyuls et al., 2018a), using 1 mL HisTrap FF columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Vip3Af proteins were eluted with a phosphate buffer (50 mM phosphate, 300 mM NaCl, pH 7.4) containing 150 mM imidazole, and 1 mL fractions were collected in tubes containing 50 µL of 0.1 M ethylenediaminetetraacetic acid (EDTA). Fractions with a high protein concentration (determined photometrically at 280 nm) were pooled and dialyzed overnight at 4 ◦C against a TNE buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.6). The purity of the preparation (10 µL) was checked by SDS-PAGE, and the protein concentration was determined by the Bradford's method. After dialysis, the proteins were stored at −20 ◦C until used.

2.2 Trypsin Treatment and SDS-PAGE Analysis of the Tryptic Fragments

The purified Vip3Af protoxins were subjected to proteolytic activation with commercial trypsin (trypsin from bovine pancreas, SIGMA T8003, Sigma-Aldrich, St. Louis, MO, USA). A mixture of protein:trypsin (5:100, *w*/*w*), in a TNE buffer was incubated at 30 ◦C for 24 h. Aliquots (10 µL) of the trypsinized proteins were subjected to 12% SDS-PAGE. Prior to electrophoresis, the samples were made 1 mM with an 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) protease inhibitor (ThermoFisher, Waltham, MA, USA), left standing for 10 min at room temperature, and then heated at 100 ◦C for 5 min with a loading buffer (0.2 M Tris-HCl pH 6.8, 1 M sucrose, 5 mM EDTA, 0.1% bromophenol blue, 2.5% SDS, and 5% β-mercaptoethanol) (2:1, sample:loading buffer). The trypsin-treated samples to be used for chromatography and bioassays were stored at −20 ◦C for less than one week.

2.3 Insect Rearing and Bioassays

Insect rearing and bioassays were carried out in a rearing chamber maintained at 25 ± 2 ◦C, 70 ± 5% relative humidity, and 16:8 h light:dark on a semi-synthetic diet based on corn flour and wheat germ that contained yeast, ascorbic acid, and nipagin. Surface contamination assays were performed with 50 µL of protein sample on 2 cm2 diameter well plates. The concentration of Vip3Af protein was 1 µg/cm2 , a concentration at which the Vip3Af(WT) kills 100% of the larvae. A Tris buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.6) was used as a blank control. Once the surface was dry, a neonate *S. frugiperda* larvae was gently placed into the well and then sealed. The number of dead and 1-instar larvae were recorded after 7 days. A larva was considered dead if it did not respond to mechanical

stimulation. The mean mortality and functional mortality (dead larvae plus larvae that had not developed beyond the first instar) were determined from two replicates of 32 insects each.

2.4 Gel Filtration Chromatography

Gel filtration chromatography was performed with an ÄKTA explorer 100 chromatography system in a Superdex 200 10/300 GL column (GE Healthcare Life Sciences, Uppsala, Sweden) at a flow rate of 0.5 mL/min of a Tris buffer (50 mM Tris-HCl, 150 mM NaCl, pH 9.0), unless otherwise indicated. To estimate the molecular weight of the peaks, the column was calibrated with the following mix of standards: 4 mg/mL ovalbumin (44 kDa), 3 mg/mL conalbumin (75 kDa), 4 mg/mL aldolase (158 kDa), 0.3 mg/mL ferritin (440 kDa), 5 mg/mL thyroglobulin (6690 kDa), and Blue Dextran 200 (exclusion limit), dissolved in water.

2.5 Identification of Tryptic Fragments

Major bands (27 and 17 kDa) from the trypsin-treated F229A mutant were identified after separation in a 2D-gel. The 17 kDa band from the trypsin-treated I699A mutant was first separated by chromatography in the Superdex 200 column and then by SDS-PAGE. The 38 kDa band from the trypsin-treated W552A mutant was first isolated by Superdex 200 chromatography and then by SDS-PAGE. For the peptide identification, protein bands were directly cut out from the gel and digested with trypsin. The peptide mass and sequence were determined by liquid chromatography and tandem mass spectrometry (LC-MS/MS) in a nanoESI qQTOF (5600 TripleTOF, ABSCIEX, Framingham, MA, USA). The mass transitions were scanned first from 350–1250 m/z and then followed by a second scan from 100–1500 m/z. The peptides sequence identified were compared to the Vip3Af1(WT) protein sequence to match the region corresponding to each SDS-PAGE proteolytic band. Expected molecular weights were calculated using the online SIB Compute pI/Mw tool (https://web.expasy.org/compute_pi).

3. Results

3.1 Effect of Residue Substitution on the Proteolytic Cleavage of Vip3Af

Changes in protein conformation may expose potential cleavage sites otherwise buried inside the protein which, when exposed to proteases, give rise to altered patterns of fragments compared with that of the wild type protein (WT). Proteolytic patterns may thus unravel structural domains in the Vip3Af protein.

We confirmed the altered proteolytic patterns obtained before with Ala-mutants (Banyuls et al., 2018a). To better define the major fragments generated by the action of trypsin on each of the mutants, we used an irreversible trypsin inhibitor to terminate the reaction and avoid further processing during SDS denaturation before gel loading (Chakroun and Ferré, 2014; Banyuls et al., 2018b). Figure 1 shows the SDS-PAGE separation of the tryptic fragments from six selected mutants (T167A, F229A, E483A, W552A, G689A, and I699A). Regarding the major fragments, Vip3Af(WT) and mutant T167A showed the 65 and 19 kDa bands (pattern “a”) as a result of the cleavage at the primary cleavage site after residue K198. The rest of the patterns lacked the 65 kDa band. Patterns “b”, “c”, and “e” contained the 19 kDa band, indicating that they altered the C-terminal part of the protein but not the N-terminal part. Patterns “d” and “f” did not contain the 19 kDa band either, indicating that the conformational change had a larger effect on the overall structure of the protein. Pattern “b” and “c” share the 35 and 19 kDa bands in common; in addition, the former showed strong bands of 38 and 10

kDa, whereas the latter showed main bands of 53, 17 (a doublet), and 15 kDa. Patterns “d” and “f” lack large fragments (larger than30 kDa); instead, they share a main band of 27 kDa; in addition, pattern “d” has a strong band of 17 kDa, and pattern “f” has a strong band of <10 kDa. Finally, pattern “e” is the same as pattern “c” but still maintains the band of 65 kDa, suggesting that mutant G689A (the only representative of pattern “e”) was relatively stable compared with those mutants with pattern “c”.

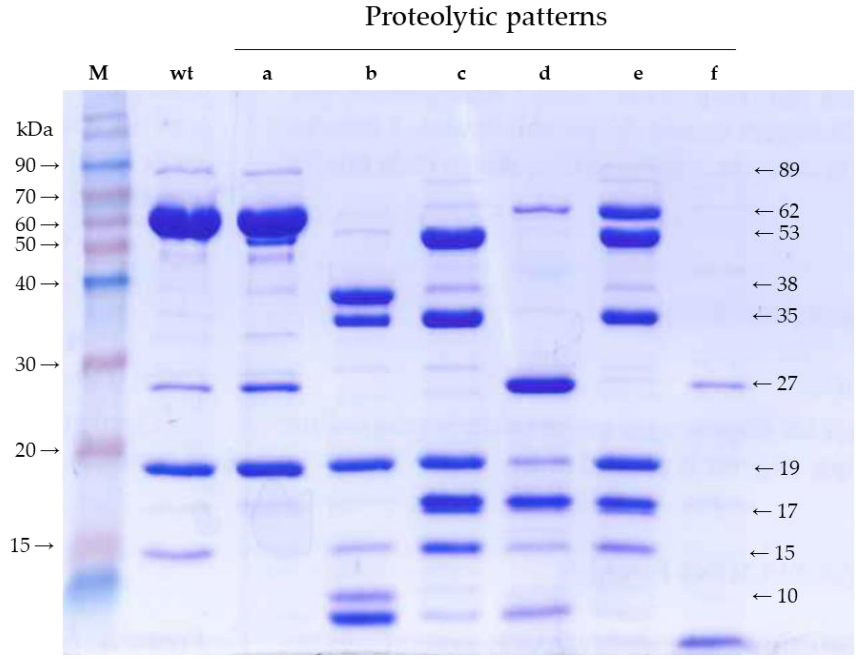


Figure 1. Trypsin digestion of Vip3Af(WT) and some of the selected mutants (one representative of each of the patterns “a” to “f”) after SDS-PAGE. The proteins were treated with 5% trypsin (*w*/*w*) at 30 ◦C for 24 h and then stopped with the addition of an irreversible trypsin protease inhibitor (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)) at room temperature for 10 min). M: Molecular weight markers. Patterns “a” to “f” were obtained from mutants T167A, W552A, I699A, F229A, G689A, and E483A, respectively.

3.2 Insecticidal Activity of the Ala-Mutants after Trypsin Treatment

The mutants selected in this study had been shown to have decreased insecticidal activity when tested as protoxins (Banyuls et al., 2018a). Here we tested the activity of the most drastic ones after in vitro treatment with trypsin (Table 1). The results were similar to the ones reported previously for the protoxin form, confirming that these mutations have a strong deleterious effect on the insecticidal activity of the protein. It is worth mentioning the differences in insecticidal activity observed among mutants with the same proteolytic pattern—between P171A and F229A (both giving rise to pattern “d”), and among I699A, Y719A, and G727A, for example (all giving rise to pattern “c”). This might be explained by either the effect of the residue substitution on intra- or intermolecular interactions or by differences in their stability to proteases. Mutant G689A, which gives pattern “e” after trypsin treatment, is the most toxic one among those tested. As mentioned above, this mutant has the same band pattern as pattern “c” with an extra 65 kDa band, reflecting its higher stability compared with mutants giving pattern “c”.

Table 1 Toxicity, against *S. frugiperda*, of trypsin-treated Vip3Af and selected mutants, with indication of the proteolytic pattern after trypsin digestion[1].

Toxins	Tryptic pattern	Mortality (%)	FM (%)[1]
WT	a	100	100
T167A	a	13±10	19±13
E168A	a	0	3.1±3.1
P171A	d	16.7±3.3	40.0±6.7
F229A	d	6.3±6.3	6.3±6.3
E483A	f	3.3±3.3	16.7±3.3
W552A	b	0	0
G689A	e	42±21	48±15
I699A	c	0	0
Y719A	c	26.7±6.7	46.7±6.7
G727A	c	0	0

[1] Percent mortality and functional mortality (FM, defined as dead larvae plus larvae remaining at 1-instar) at 1 μg/cm^2 . Mean ± SD from two replicates of 32 insects each.

3.3 Effect of Residue Substitution on Vip3Af Oligomerization

Residue substitutions may affect the capacity of the Vip3Af protein to form the tetramer (Banyuls et al., 2018b), the form that Vip3 proteins adopt in solution (Zack et al., 2017; Banyuls et al., 2018b; Palma et al., 2017; Sahin et al., 2018; Kunthic et al., 2017). We used gel filtration chromatography to determine the oligomerization state of the Ala-mutants, both as protoxins and after trypsin treatment. First, we tested the wild type Vip3Af (from now on: Vip3Af(WT)) and determined the possible effect of pH on oligomerization. Fig. 2A shows that, at the pH range tested (pH 7, 9, and 11), there was no effect on the tetramerization of the Vip3Af(WT) protoxin. The chromatograms showed a main peak at 24 min, corresponding to a molecular weight of approximately 370 kDa (a tetramer of the 89 kDa protoxin should theoretically be of 356 kDa). The trypsin-treated Vip3Af(WT) also showed just one peak at 24 min (Fig. 2A). An SDS-PAGE analysis of the peak showed the 19 and 65 kDa bands (Fig. 3, lane 1), confirming that trypsin treatment did not induce the separation of the two fragments (Zack et al., 2017; Bel et al., 2017; Banyuls et al., 2018b; Sahin et al., 2018). Mutants T167A and E168A (both giving rise to pattern “a”) showed chromatograms that did not differ from that of the wild type (not shown).

With mutant P171A (which gives rise to pattern “d”), the protoxin eluted at 24 min, revealing a tetrameric form; however, just small fragments (eluting at 34.8 min) were observed after trypsinization (Fig. 2B). SDS-PAGE of the peak at 34.8 min showed a 27 kDa strong band (Fig. 3, lane 2). This chromatography profile was also observed in mutants L209A and M238A (both giving rise to pattern “d”) (not shown). The chromatogram of the F229A protoxin showed big peaks at 18 and 26 min (Fig. 2C), the former coinciding with the exclusion volume of the column and corresponding to protein aggregates. The peak at 26 min indicated a molecular weight of approximately 230 kDa, which would best fit a dimeric form of the protein. This mutant (which renders pattern “d”) also showed only small fragments eluting at 34.8 min after trypsinization. SDS-PAGE of the fraction at 34.8 min revealed a main fragment of 27 kDa (Fig. 3, lane 7).

Neither the protoxin nor the trypsin-treated mutant E483A (which gives pattern “f”) showed any tetramer in solution (Fig. 2D); the main peak of the protoxin eluted at 29.5 min, corresponding to an approximate estimated molecular weight of 122 kDa (best fitting a monomer), whereas the trypsinized protein eluted at around 35 min, corresponding to the 27 kDa fragment and smaller fragment (<10 kDa), as revealed by SDS-PAGE (Fig. 3, lane 4).

Mutant W552A (which gives rise to pattern "b") formed a tetramer both as a protoxin and after trypsin treatment (Fig. 2E); however, upon trypsin treatment, the amount of tetramer was reduced, and a large peak corresponding to small fragments appeared. An analysis of the fraction at 24 min by SDS-PAGE showed the presence of the 38, 35, and 19 kDa fragments (Fig. 3, lane 8). Mutant I699A (which renders pattern "c") also tetramerized as a protoxin and after trypsin treatment (Fig. 2F); however, after trypsinization, it also showed fragments eluting at 35.2 min. This chromatography profile was also observed in the rest of mutants which give rise to pattern "c" (Y719A and G727A) and "e" (G689A). The SDS-PAGE analysis of the fraction at 24 min revealed, among other minor bands, the 53, 35, and 19 kDa fragments (Fig. 3, lane 5), whereas the fraction at 35.2 min revealed a fragment of 17 kDa (Fig. 3, lane 6).

From the chromatographic analysis, we can conclude that the 27 and 17 kDa fragments, once cleaved by trypsin, are released from the structure and no longer form part of the oligomer. However, the tetrameric structure of Vip3Af can still be maintained in the presence of fragments 38, 35, and 19 kDa (such as in mutant W552A, which gives pattern "b") or fragments 53, 35, and 19 kDa (such as mutants rendering pattern "c"). Residues F229 and E483 must have a key role in the oligomerization of the Vip3Af protoxin, since their exchange for alanine prevents tetramer formation even before trypsin treatment.

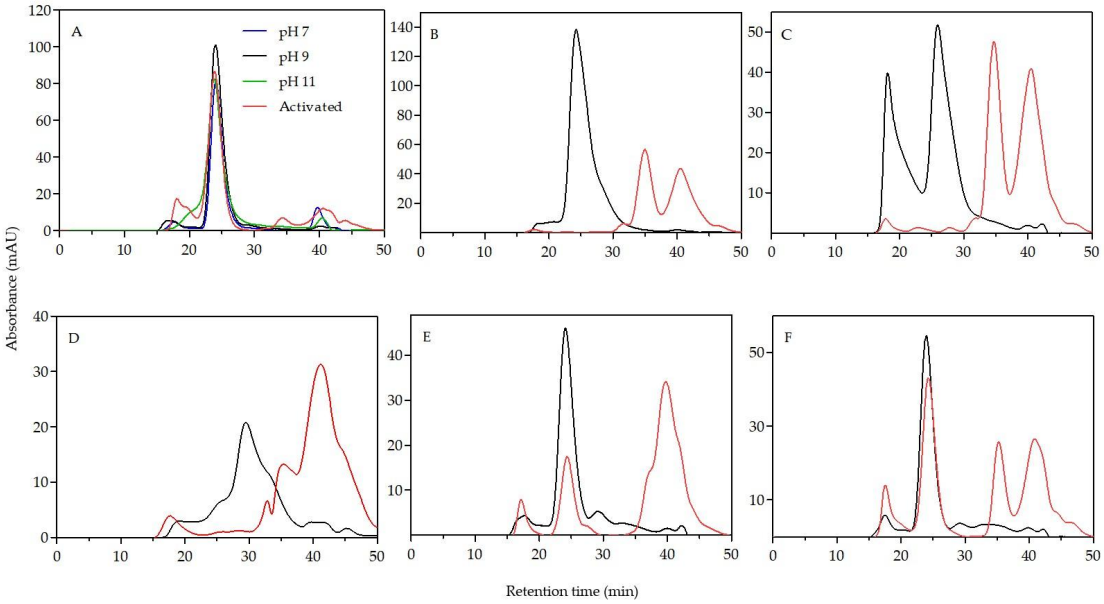


Figure 2. Gel filtration chromatography of Vip3Af(WT) and representative Ala-mutants. A Tris buffer (50 mM Tris, 150 mM NaCl, pH 9.0) was used in all cases (black line: Protoxin; red line: Trypsin-treated). For the Vip3Af(WT), elution was also performed in a phosphate buffer (50 mM phosphate, 150 mM NaCl, pH 7.0) (blue line) and a carbonate buffer (50 mM Na2CO3 , 150 mM NaCl, pH 11.0) (green line). (**A**) Vip3Af1(WT); (**B**) P171A; (**C**) F229A; (**D**) E483A; (**E**) W552A; (**F**) I699A.

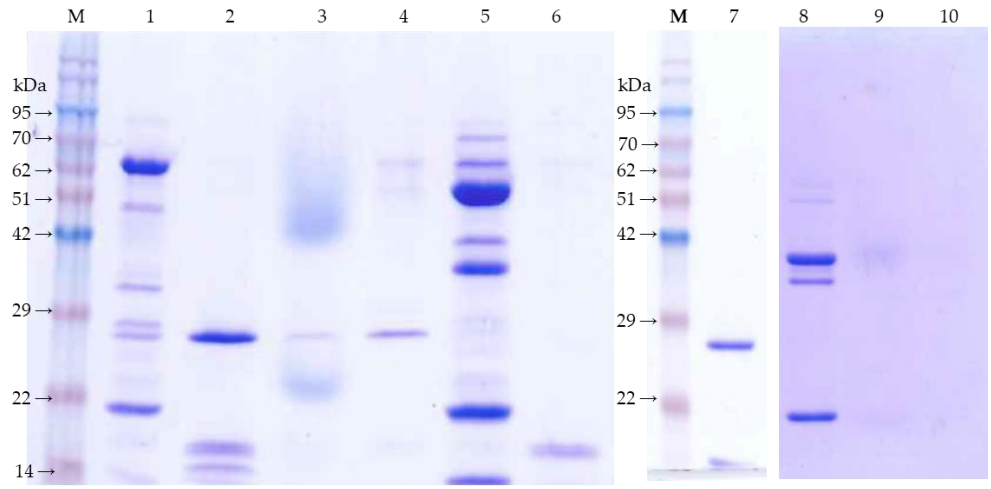


Figure 3. SDS-PAGE analysis of the chromatographic peaks of the trypsin-activated mutants from Figure 2. Lane 1, Vip3Af(WT) peak at 24–26 min; lane 2, P171A peak at 34–36 min; lane 3, P171A peak at 40–42 min; lane 4, E483A peak at 34–36 min; lane 5, I699A peak at 24–26 min; lane 6, I699A peak at 34–36 min; lane 7, F229A peak at 34–36 min; lane 8, W552A peak at 24–26 min; lane 9, W552A peak at 38–40 min; lane 10, W552A peak at 40–42 min.

3.4 Identification of 17, 27, and 38 kDa Tryptic Fragments by Peptide Fingerprinting

The identification of the tryptic fragments was performed after the separation of the fragments by 2D gel electrophoresis and/or size filtration chromatography followed by SDS-PAGE. The results of the peptide fingerprint were matched with those of the tryptic sites in the primary sequence of Vip3Af, and the estimated size of the fragment was taken into account to set the fragment limits. Through 2D gel electrophoresis, we could separate and analyze the 17 and 27 kDa spots from the trypsin-treated F229A mutant. The results of the peptide fingerprint, along with the tryptic sites in the sequence of Vip3Af, indicated that the 27 kDa fragment corresponded to residues from 523/526 to probably the end of the protein. The same type of analysis with the spot of 17 kDa indicated that it corresponded to residues from 523/526 to 661/663. The 17 kDa fragment from the trypsin-treated I699A mutant was analyzed from the chromatographic fraction B15 (peak 34.8 min) of this mutant. The results indicated the same match as the 17 kDa fragment from mutant F229A. The identity of the 38 kDa fragment was determined from the chromatography fraction A11 (24.2 min) from trypsin-treated W552A; the peptide fingerprint indicated that the fragment corresponded to residues from 313/315 to 661/668.

4. Discussion

Banyuls et al. (Banyuls et al., 2018a) defined six proteolytic patterns of mutants with strongly impaired insecticidal activity. With minor modifications in the methodology, we have confirmed and refined such patterns with the aim of revealing the major fragments generated by trypsin and then identifying their position in the primary structure of the protein. The only difference observed with the previous proteolytic patterns is that, using the irreversible trypsin inhibitor to stop the reaction, we obtained a strong band of 35 kDa in patterns “b” and “c”, which was not observed before. We also detected bands smaller than 19 kDa by stopping the electrophoresis before they ran out of gel. Altogether, we ended up with fragments of 53, 38, 35, 27, 19, 17, 15, and <10 kDa, most of them shared by various patterns. We hypothesized that the limits of these fragments may correlate with the structural domains of the wild type protein.

In a previous study, Banyuls et al. (Banyuls et al., 2018a) identified the tryptic fragments of 62 (here referred to as 65), 55 (here referred to as 53), 27, and 20 (here referred to as 19) kDa. Our peptide fingerprint results of fragments of 17, 27, and 38 kDa, taking into account the tryptic sites in the sequence of Vip3Af, allowed us to define their position in the sequence of the protein. Putting all this information together, we propose a map of the tryptic fragments such as the one shown in Figure 4, which defines five domains. In this map, domain I spans the region covered by the 19 kDa fragment (from residues 12 to 198); domain II spans the region from the primary cleavage site to the start of the 38 kDa fragment (from residues 200 to 313/315); domain III spans from the start of the 38 kDa fragment up to the start of fragments of 17 and 27 kDa (from residues E314-E316 to 523/526); domain IV spans the 17 kDa fragment (from residue 524/527 to residue 661/668) and basically consists of the carbohydrate-binding motif common to all Vip3 proteins with the exception of Vip3Ba (Chakroun et al., 2016a); and domain V spans from the end of the 17 kDa fragment (and also the end of the 38 and 53 kDa fragments) to the end of the protein (from residue 662/669 to 788). Compared with the proposed domains by in silico modelling, the domain I proposed by us is in good agreement with domain 1 proposed by Banyuls et al. (Banyuls et al., 2018a) for Vip3Af (from 1 to 188), though there is no further correlation between both models for the rest of domains. However, the boundaries between domain II and III, III and IV, and IV and V in our proposed map have their correspondence with the domains proposed by Sellami et al. (Sellami et al., 2018) for Vip3Aa (at residues 313, 532, and 667, respectively). The agreement between the domain limits proposed by us with some of those defined by in silico modelling supports the predictive value of the tryptic fragments approach to unravel structural domains of the Vip3A proteins.

The results from gel filtration chromatography of the Ala-mutants shed light on the structural role of the proposed domains. Mutants rendering patterns “b”, “c”, and “e” are found forming a tetramer both as protoxins and also after trypsin treatment. Since trypsin digests the 27 kDa fragment (the only one containing domain V), we can conclude that domain V is not necessary to maintain the oligomeric structure. All these mutants, after trypsin treatment, have in common fragments of 19 kDa (domain I) and 35 kDa (domains II and III), plus another larger fragment (either of 38 or 53 kDa) which includes domain IV. Despite the fact that the 17 kDa fragment (which corresponds to domain IV) elutes separately from the tetramer in the chromatography of mutants with patterns “c” and “e”, the tetramer contains domain IV in the structure as part of the 53 kDa fragment. Therefore, according to the results, domains I–III are required to form the tetrameric structure, the need for domain IV is not clear, and domain V is not necessary.

An interesting observation from patterns “b” and “c” is that, in addition to the 19 kDa band, the sum of the remaining main bands gives an apparent molecular weight exceeding that of 65 kDa. In the case of pattern “b”, these bands correspond to fragments of 35 and 38 kDa. In the case of pattern “c”,the strongest bands are those corresponding to fragments of 15, 17, 19, 35, and 53 kDa. Therefore, there must be an alternative splicing of the 65 kDa fragment in mutants rendering these two patterns. The requirement of domain I to form the tetramer, along with domain exchange studies between the 19 kDa fragment and the rest of the protein with Vip3Ab and Vip3Bb (Zack et al., 2017), support the functional role of this domain and rules out the early beliefs that the 19 kDa fragment was non-essential in the insecticidal activity of Vip3 proteins and that only the 65 kDa fragment was the active core. It has been reported that complete deletion of the first 198 N-terminal amino acids in Vip3Aa completely abolishes its toxicity and produces a 62 kDa protein highly sensitive to trypsin degradation (Li et al., 2007). However, some studies have shown that domain I can withstand short N-terminal deletions

without affecting the insecticidal activity (Bhalla et al., 2005; Selvapandiyan et al., 2001). In contradiction to the above results, Gayen et al. (Gayen et al., 2012) reported an active Vip3Aa protein without domain I.

From the distribution of Ala-mutants with decreased insecticidal activity in the primary structure of Vip3Af (Fig. 4), we can observe that they gather into two clusters, except for mutant E483A (the only representative of pattern "f") and mutant W552A (the only representative of pattern "b"). The first cluster contains all mutants with either pattern "a" or "d". Mutations altering the structure and giving pattern "d" are concentrated at the end of domain I and the first part of domain II. This region of the protein, around the primary cleavage site, must have an important role in maintaining the 19 and 65 kDa fragments together, and this might be essential to preserve the overall structure of the tetrameric protein. The second cluster is in domain V and contains all the mutants with either pattern "c" or "e". These mutants destabilize domain V, which is further digested by trypsin with the result of fragment 27 kDa being converted to the 17 kDa fragment.

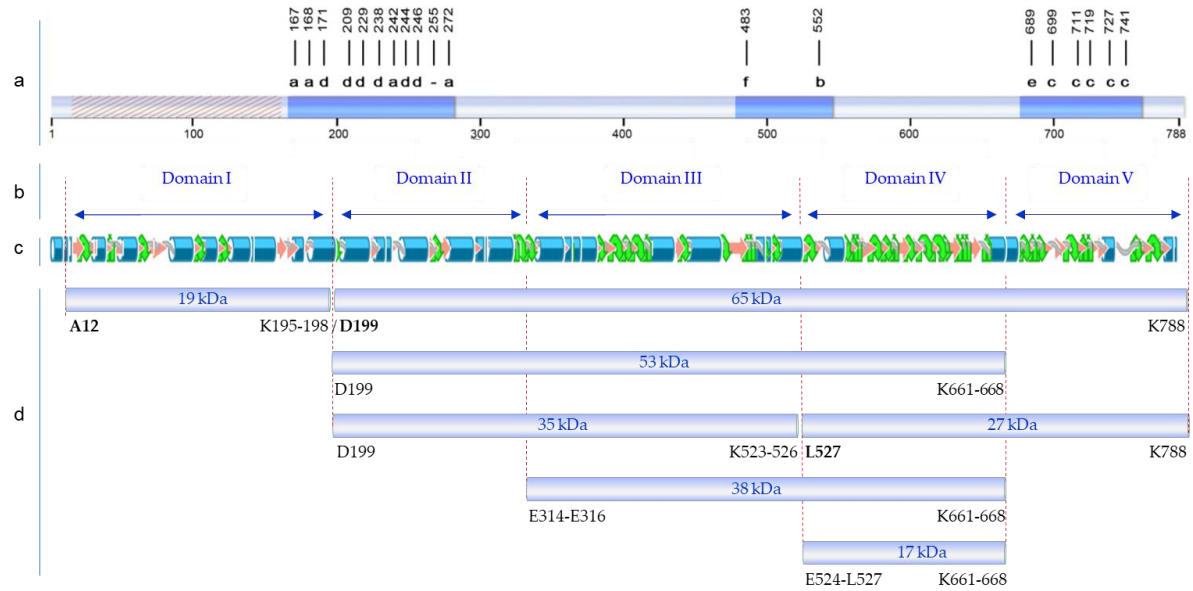


Figure 4. Schematic representation of the Vip3Af protein. (**a**) Distribution of critical residues affecting the insecticidal activity (dashed region: Non-analyzed), with indication of the band pattern after trypsin treatment, following Banyuls et al. (Banyuls et al., 2018a); (**b**) proposed structural domains as defined by the tryptic fragments; (**c**) predicted secondary structure of Vip3Af, following Banyuls et al. (Banyuls et al., 2018a); (**d**) main fragments after trypsin treatment of the Vip3Af protein and their Ala-mutants (amino acid residues identified by Edman's degradation are shown in bold).

5. Conclusions

Using the approach of the trypsin fragmentation of mutants altering the conformation of the Vip3Af protein, we have defined five domains in the structure of Vip3Af which match some of the domains proposed independently by two in silico models. The effect of some the mutations on the ability to form a tetrameric molecule reveals that domains I–III are required for tetramerization, while domain V is not. The involvement of domain IV in the tetramer formation is not clear. The overlapping fragments in the proteolytic patterns suggest a tetramer with a distinct disposition of the monomers, in such a way that the tryptic sites exposed in two molecules are different to those exposed in the other two. Residues around the primary cleavage site are important for maintaining the structure of the

protein, since trypsin processing in mutants of pattern “d” digests most part of the protein and destroys the tetrameric form. Mutants in domain V belonging to pattern “c” destabilize this domain, though they not affect the tetrameric structure after trypsin processing. Because of the high sequence similarity among Vip3 proteins, we think that our domain map proposal may be valid for the Vip3 family of proteins. The information provided here may help to further clarify the 3D structure and its implications in the mode of action of Vip3 proteins.

Chapter 2. Identify the critical amino acid positions of Vip3Af for the insecticidal activity and structure.

2.2 Effect of substitutions of key residues on the stability and the insecticidal activity of Vip3Af from *Bacillus thuringiensis*.

Results are included in:

Banyuls N, Quan Y, González-Martínez RM, Hernández-Martínez P, Ferré J. 2020. Effect of substitutions of key residues on the stability and the insecticidal activity of Vip3Af from *Bacillus thuringiensis*. J Invertebr Pathol 11, 107439. https://doi.org/10.1016/j.jip.2020.107439.

1. Introduction

Worldwide agriculture is continuously facing new challenges to adapt to global change and the human density. According to FAO reports, the rate of human population increase is predicted to reach 9 billion people by 2050 (Alexandratos and Bruinsma, 2012). After the Green Revolution, agriculture has been deeply transformed by means of intensification and technification in order to obtain higher yields and cope with the food market rules. With the consequent increase of the world arable surface accounting up to 1.6 billion ha (Alexandratos and Bruinsma, 2012), the amount of chemicals released to the environments has increased accordingly, summing up more than 166 million tonnes of fertilizers (Alexandratos and Bruinsma, 2012) and up to 3.8 million tonnes of pesticides annually (De et al., 2014; EPA, 2017; Tilman et al., 2001). The overuse of conventional pesticides during the last decades has led to serious environmental and food security concerns and as a consequence, the approval of many active substances has been drawn back. Pesticides such as DDT or HCH are still widely used in developingcountries, whereas in Europe, of the existing 1355 actives substances, only 486 actives remain authorised, 76 out of which are candidates for substitution due to unacceptable hazard (Carvalho, 2006; De et al., 2014; "EU Pesticides database – European Commission," n.d.). In this context, the development of green plant protection products as well as biotechnology-based improved crops (biotech crops) that permit to reduce the employment of chemical pesticides in combination with local agricultural practices is of upmost priority.

Microbial control agents are used as sustainable, health safe and renewable biopesticides, often combined with other chemical pesticides to protect a crop in integrated pest management (IPM) programs. From all known insecticidal microbial control agents, *Bacillus thuringiensis* (Bt) is by far the most used, either as Bt-based bioformulants or as Bt-crops (EPA, 2017; Jouzani et al., 2017; Lacey et al., 2015). Vip3 proteins from *B. thuringiensis* are insecticidal proteins secreted to the environment during the bacterial vegetative growth phase. The mode of action of Vip3 and Cry proteins is similar, differing mainly in the binding to specific receptors. This difference has led to the development of stacked crops co-expressing Cry and Vip3A proteins as to prevent resistance development in susceptible insects. Likewise, other Cry-expressing crops are being pyramided with insect specific RNAi to counteract insect resistance to Cry-expressing crops (Bernardi et al., 2015, 2014; Carriere et al., 2015; Chakroun et al., 2016b; Gayen et al., 2015; Graser et al., 2017; ISAA, n.d.; Kurtz et al., 2007; Ni et al., 2017; Sanchis, 2011). Furthermore, the genetic engineering of Vip3A proteins in other insecticidal microbial agents such as *Beauveria bassiana* is a promising tool to potentiate the pest management strategies in combination with more conventional approaches (Liu et al., 2013; Qin et al., 2010). Additionally, there is room yet to improve the more classic approach of Bt-based formulations either by toxin microencapsulation in inert cells or by nanotechnology (De et al., 2014; Hernandez-Rodríguez et al., 2013; Jouzani et al., 2017; Mahadeva Swamy and Asokan, 2013; Panetta, 1993).

Vip3 proteins are highly active and selective against caterpillar pests, though they do not cover a wide range of target pests. Broadening the spectrum of target pests or the insecticidal potency of the already known proteins by molecular techniques is a shorter and cheaper approach to increase the available resources for the agricultural pest management. Unlike Cry toxins, only few studies exist aiming at improving the insecticidal performance of Vip3 proteins by molecular engineering (Chi et al., 2019; Fang et al., 2007; Gomis-Cebolla et al., 2020; Kahn et al., 2018; Soonsanga et al., 2019; Sopko et al., 2019; Zack et al., 2017). In a previous work, the Vip3Af was analysed by the alanine scanning

technique. As a result, some positions in the primary sequence were identified as being crucial for the insecticidal activity, selectivity or protein stability (Banyuls et al., 2018b). To achieve a deeper insight of the role of the selected key residues on the stability and insecticidal activity of Vip3Af, some of these positions were further investigated by site directed mutagenesis.

2. Materials and methods

2.1. Protein source and site-directed mutagenesis

The *vip3Af(wt)* gene is a His-tagged modification of the *vip3Af1* (GenBank accession No. AJ872070.1) as described in an earlier work (Banyuls et al., 2018a, b). The clone was kindly provided by BASF Belgium Coordination Center – Innovation Center Gent (Ghent, Belgium). A total of 12 single and double mutants of the *vip3Af(wt)* were generated by site directed mutagenesis by the technique of the whole plasmid amplification. Primer design was done according to Zheng et al. (2004). Primer sequences and their characteristics are summarised in Tables 1 and 1S, respectively. Besides the introduction of the desired codon substitution in the two primers of each primer pair, a silent mutation was introduced at least in one of the primers to insert a restriction site for quick screening purposes.

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 6 kb pMAAB 10 plasmid (Beard et al., 2008) containing the *vip3Af(wt*) gene as template. The PCR reaction was carried out with 25–50 ng DNA template, 0.6 µM primer pair, 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 ◦C and 16 cycles of denaturation, annealing and extension phases of 98 ◦C for 20 s, 60 ◦C for 30 s and 72 ◦C for 4 min, respectively. The reaction was finalised with 15 min of final extension at 72 ◦C.

The parental plasmids remaining in the final reaction were digested with FastDigest *Dpn*I (Thermo Scientific, USA) at 37 ◦C for 10 min. The enzymatic digestion of the *Dpn*I was stopped at 80 ◦C for 5 min and the reaction then cooled down on ice. The plasmids carrying the mutant version of *vip3Af* were used to transform *Escherichia coli* DH10ß. Plasmid purification was performed on 2–3 transformed colonies and screened reactions had not led to wrong plasmid constructions due to strand displacement during the elongation step. The restriction product was checked by agarose gel electrophoresis. Plasmids displaying the expected fragment size (Table 2) were further introduced into *E. coli* BL21 for the expression of the mutant Vip3Af protein.

The mutations were confirmed by DNA sequencing. The gene region containing the mutation was amplified in the same way as for the sitedirected mutagenesis, using 5–10 ng template and 30 cycles PCR. The different primer pairs used (Table 2) gave amplification fragments of up to 1000 bp. The PCR product was checked by agarose gel electrophoresis and purified using NucleoSpin Gel and PCR clean-up kit (MN, Germany) and sequenced by the Sanger method.

Table 1 Primers used for the site-directed mutagenesis by the whole plasmid amplification. Primer sequences are grouped in triplets to facilitate the identification of the appropriate mutated codon (highlighted in bold). The nucleotide change with regards to the wild-type is denoted in lower case. Underlined sequences indicate the introduction or removal of a restriction site for screening purposes.

Mutation	Primer	Sequence (5'-3')	Position[1]	Restriction site modification (screening enzyme)
M34L	Fwd	GAC ATT **cTG** AAT ATG ATT TTc AAA ACG GAT ACA GGT GG	94	Deletion (DraI)
	Rev	CGT TTT gAA AAT CAT ATT **CAg** AAT GTC TTT GAT ACC AG	83	
T167S-E168D-P171G	Fwd	C TCT ACA CTT **tCT GAc** ATT ACA **ggT** GCA TAT CAA CGG ATT AAA TAT GTG	489	None (EcoRI)
	Rev	CCG TTG ATA TGC **Acc** TGT AAT **gTC AGa** AAG TGT AGA GTT AAT AAG AAC	478	
K284Q	Fwd	CTA CAA GCA **cAA** GCa TTT CTT ACT TTA ACA ACA TGC	841	Deletion (HindIII)
	Rev	GT AAG AAA tGC **TTg** TGC TTG TAG AGC TGT TAA TAC	822	
E483D	Fwd	GTC ATC AGT **GAt** ACA TTT TTG ACT CCG ATA AAT G	1438	None (EcoRI)
	Rev	CAA AAA TGT **aTC** ACT GAT GAC ACC TAA TGG	1429	
E483Q	Fwd	GTC ATC AGT **cAA** ACA TTT TTG ACT CCG ATA AAT G	1438	None (EcoRI)
	Rev	CAA AAA TGT **TTg** ACT GAT GAC ACC TAA TGG	1429	
E483H	Fwd	GTC ATC AGT **cAc** ACA TTT TTG ACT CCG ATA AAT G	1438	None (EcoRI)
	Rev	CAA AAA TGT **gTg** ACT GAT GAC ACC TAA TGG	1429	
W552H	Fwd	GAC AAT TTA GAG CCG **cAC** AAA GCA AAT AAT AAG AAC GCG	1639	Addition (AciI)
	Rev	GC TTT **GTg** CGG CTC TAA ATT GTC CTC TTC TAT GG	1628	
W552F	Fwd	GAC AAT TTA GAG CCG **Ttc** AAA GCA AAT AAT AAG AAC GCG	1639	None (EcoRI)
	Rev	GC TTT **gaA** CGG CTC TAA ATT GTC CTC TTC TAT GG	1628	
W552Y	Fwd	GAC AAT TTA GAG CCG **Tac** AAA GCA AAT AAT AAG AAC GCG	1639	Addition (RsaI)
	Rev	GC TTT **gtA** CGG CTC TAA ATT GTC CTC TTC TAT GG	1628	
G689S	Fwd	CG ACT CCA **tcG** GCa AGC ATT TCA GGA AAT AAA C	2057	Deletion (NheI)
	Rev	GA AAT GCT tGC **Cga** TGG AGT CGT AAT CCA AG	2048	
G689E	Fwd	CG ACT CCA **GaG** GCa AGC ATT TCA GGA AAT AAA C	2057	Deletion (NheI)
	Rev	GA AAT GCT tGC **CtC** TGG AGT CGT AAT CCA AG	2048	
N682K-G689S	Fwd	CCG **AAc** TCT TGG ATT ACG ACT CCA **tcG** GCT AGC ATT TCA GG	2038	Deletion (EcoRI)
	Rev	GCT AGC **Cga** TGG AGT CGT AAT CCA AGA **cTT** CGG ATT AAT TAA TTC	2032	

1 All primers were non-phosphorylated and synthesised by Sigma-Aldrich.

2 Position at 5′ (forward, "Fwd") and at 3' (reverse, "Rev") primers for each polymerase chain reaction primer pair. Reference gene accession number AJ872070.1.

Table 2 Primers used for testing out the correct change in the mutated proteins and the results of the sequencing (Banyuls et al., 2018a). Underlined primers were used for the sequencing of the amplification products.

Primer	Sequence	Position[1] inthe reference gene	Product size[2](bp)	Vip3Af mutated protein	Codon (aa)(wt / mutation)
Vip3-sc.fw[3]	5' TGCCACTGGTATCA ARGA 3'	78 - 1078	1000	M34L	ATG (M)/ CTG (L)
wt1.rev	5' ACCCAACCAATGCA TGTCCT 3'				
wt1.fw	5' CGATGCGATAAATA CGATGCTTCATA 3'	321 - 1078	757	T167S+E168 D+P171G	ACT (T) + GAA (E) + CCT (P) / TCT (S) + GAC (D) + GGT (G)
wt1.rev	5' ACCCAACCAATGCA TGTCCT 3'			K284Q	AAA (K)/ CAA (Q)
wt2.fw	5' CGGAGGTTATTTAT GGTGATACGG 3'	1166 - 1793	627	E483D	GAA (E)/ GAT (D)
wt2.rev	5' TGGATTACATACTC AGTTTTCGGT 3'			E483Q	GAA (E)/ CAA (Q)
				E483H	GAA (E)/ CAC (H)
				W552H	TGG (W)/ CAC (H)
				W552F	TGG (W)/ TTC (F)
				W552Y	TGG (W)/ TAC (Y)
wt3.fw	5' AAGGACGGAGGATT TTCACAA 3'	1729 - 2281	552	N682K+G689 S	AAT (N) + GGG (G) / AAG (K) + TCG (S)
wt3.rev	5' TCTACATATAATCCGG TATTATTGG 3'			G689S	GGG (G)/ TCG (S)
				G689E	GGG (G)/ GAG (E)

[1] Position at 5' end of the forward and reverse primers for each polymerase chain reaction primer pair. Reference gene accession number AJ872070.1.

[2] Using the reference gene as the template.

[3] in Hernández-Rodríguez *et al.*

2.2. Protein expression and purification

The Vip3Af1 wild type (WT) and the mutated variants harboured in *E. coli* BL21 were expressed as described by Ruiz de Escudero et al. (2014). Briefly, a single *E. coli* colony was grown in LB-ampicillin culture at 37 ◦C with soft shaking (180 rpm). The Vip3Af expression was induced with 1 mM IPTG when the culture reached an OD600 between 1.2 and 1.8. After overnight expression, bacterial cells were pelleted and further treated with 1% lysozyme in PBS (phosphate buffered saline, pH 7.4) followed by two sonication steps. The crude lysate was clarified by centrifugation and the

resulting supernatant was filtered through a 0.2 µm diameter pore membrane before purification. Vip3Af proteins (WT and mutants) were purified from the clarified lysate extracts by isoelectric point precipitation at pH around 5.5 (Chakroun and Ferré, 2014). The pellet was recovered by centrifugation and dissolved in 20 mM Tris-HCl–150 mM NaCl (pH 9.0), aliquoted and stored at − 20 ◦C. Before being used in bioassays, the aliquots were thawed and clarified by centrifugation (16100 × *g*, 4 ◦C, 10 min). Protein purity was checked by SDS-PAGE (Fig. S1) and the protein concentration was calculated densitometrically using the TotalLab 1D v 13.01 software.

2.3. Proteolytic pattern assays

Purified Vip3Af proteins (1.5 mg/ml) were incubated with 5% trypsin (w:w) in a final volume of 500 µl at 37 ◦C for 1 h. The reaction was stopped by taking 10 µl aliquots and adding the AEBSF inhibitor at a final concentration of 1 mM, and let stand at room temperature for 10 min (Bel et al., 2017). Then, SDS-PAGE loading buffer (0.2 M Tris-HCl pH 6.8, 1 M sucrose, 5 mM EDTA, 0.1% bromophenol blue, 2.5% SDS, and 5% β-mercaptoethanol) (2:1, sample:loading buffer) was added followed by 5 min heating at 99 ◦C. Proteolytic fragments were separated in 12% SDS-PAGE. The assay was replicated thrice.

2.4. Insect rearing and insecticidal activity

The bioassays were performed with laboratory populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae), *Spodoptera littoralis* (Lepidoptera: Noctuidae) and *Grapholita molesta* (Lepidoptera: Tortricidae). The populations were maintained on semi-synthetic diet (Greene et al., 1976; Guennelon et al., 1981) in a rearing chamber at 25 ± 2 ◦C, 70 ± 5% RH and 16:8h L:D.

The insecticide potency of Vip3Af (WT) and the modified Vip3Af proteins was estimated from the LC50 values obtained in quantitative surface contamination assays. The surface of 2 cm2 diameter wells filled with semi-synthetic diet was treated with 50 µl of the purified Vip3Af protein. A range of 7 serially diluted concentrations of Vip3Af samples was tested in each bioassay. Proteins were diluted with 20 mM Tris, 150 mM NaCl, pH 9. The same buffer served as a negative control. After the diet surface was dry, one neonate was gently placed into the well and then sealed. Trays were maintained in a climatic chamber at the same conditions as for colony rearing. All bioassays were scored after 7 days for mortality. A total of 256 neonates were used per replicate for each protein tested and the bioassays were replicated twice. The insecticidal potency, regressions lines, and LC50 were calculated with the Polo Plus Probit analysis software (Software, 1987). LC50 values were considered significantly different when fiducial limits did not overlap.

3. Results

To get a better knowledge of the role of some amino acid residues on the stability and insecticidal activity of Vip3Af, selected positions of this protein were modified by site directed mutagenesis. The rationale in choosing each particular substitution was based on (i) multiple sequence alignments of the Vip3 proteins and the information on the insecticidal activity of certain positions available in the literature (Chakroun et al., 2016a), (ii) the critical positions for the structure–function relationship of the Vip3Af identified in a previous work (Banyuls et al., 2018a), (iii) the *in silico* prediction of reactive-structural and exposed-buried residues (Berezin et al., 2004), (iv) the degree of favourable amino acid substitution based on exchange matrices and intrinsic amino acid properties (Betts and

Russell, 2003; Bordo and Argos, 1991; NCBI, n.d.; Russell et al., n.d.) and (v) the positively selected residues described in Wu et al. (2007).

3.1. Protein expression and proteolytic band patterns

Most constructs carrying the mutations were able to express the Vip3Af mutant proteins. Only mutant E483Q and the triple mutant T167S-E168D-P171G did not achieve the expression of enough protein to work with and were dismissed for further analysis. Since the gene sequence and the plasmid were shown to be correct, the failure in expressing these mutants must be due to the nature of the mutation, either by disrupting the structural integrity of the molecule or by rendering such highly unstable protein that is quickly degraded after expression, as described previously (Banyuls et al., 2018a). The remaining 10 mutants were successfully expressed, pre-purified by isoelectric point precipitation and tested for their band pattern after trypsin treatment (Fig. 1).

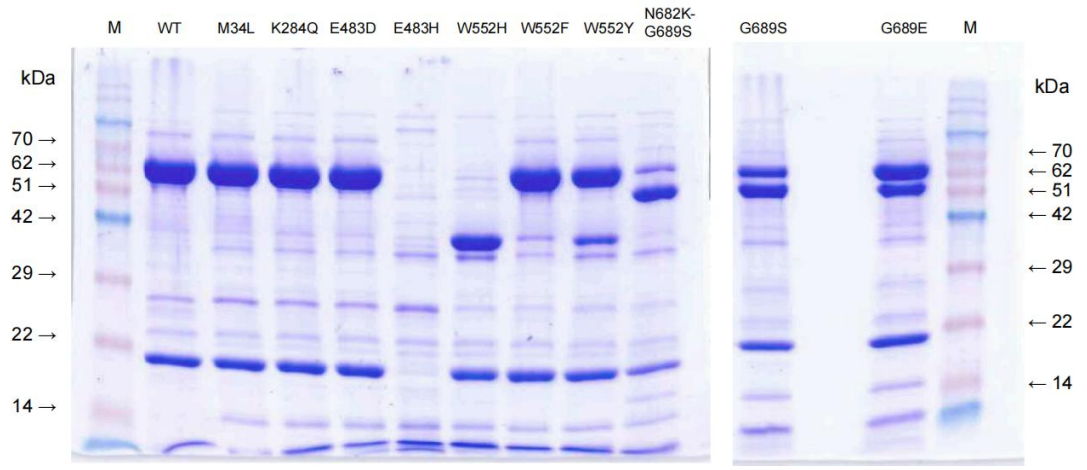


Figure 1. Band patterns after trypsin treatment of the Vip3Af(WT) and its mutant proteins. Protoxins (1.5 mg/ml) were incubated with 5% trypsin at 37 ◦C for 1 h, and then stopped with addition of the AEBSF irreversible trypsin protease inhibitor (1 mM at room temperature for 10 min). M: molecular weight marker.

Vip3Af (WT) rendered two main fragments of approx. 65 kDa and 19 kDa after trypsin treatment (and some minor bands resulting from further processing of the main fragments). This proteolytic band pattern was previously described as pattern “a” (Banyuls et al., 2018a, b). Mutant proteins M34L, K284Q, E483D, and W552F also rendered the expected main fragments of 65 kDa and 19 kDa after trypsin treatment, suggesting that no major change in the structure of the protein had taken place. However, the other mutants showed proteolytic patterns different from that of the wild type protein. The mutant proteins E483H and W552H rendered the proteolytic band patterns “f” (a single band of 27 kDa as the main fragment) and “b” (38 kDa and 19 kDa), respectively, mutant W552Y produced main fragments of 65 kDa, 38 kDa and 19 kDa, which was a mixture of patterns “a” and “b”, and the mutants N682K-G689S, G689S and G689E showed the pattern “e”, consisting on the main bands of 65 kDa, 53 kDa and 19 kDa. These anomalous patterns have been proposed to reflect conformational changes in the structure of the protein exposing tryptic sites otherwise inaccessible and thus, making the structure less stable to midgut proteases (Banyuls et al., 2018b; Quan and Ferr´e, 2019).

3.2. Effect of amino acid residue substitutions on the insecticidal activity

The insecticidal activity of the mutants was compared to that of the WT protein against *S. frugiperda*, *S. littoralis* and *G. molesta*, and the results are summarized in Table 3. The mutant M34L was the only one that increased the insecticidal activity against at least one the insect species tested (3-fold against *S. littoralis*). Although the LC50 value for *S. frugiperda* was 2.5-fold lower than that of the WT, the difference cannot be considered statistically significant according to the overlap in the FL. The rest of the site-directed mutants either did not change the insecticidal activity, decreased it, or abolished it completely. The decreased activity of mutant K284Q did differ significantly from that of the WT protein. For residue 483, the change in mutant E483D did not alter the insecticidal activity whereas E483H completely abolished it for the three insect species. For residue 552, the change in mutant W552H completely abolished the activity against all insects tested, W552Y strongly reduced the activity, and the activity of mutant W552F depended on the insect species tested, ranging from no significant alteration (in *S. frugiperda*) to strong decrease (360-fold in *G. molesta*). For residue 689 the substitutions reduced the activity in all cases.

Table 3 Quantitative parameters from concentration-mortality responses (at 7 days) of Vip3Af1(WT) and SDM-Vip3Af proteins purified by affinity chromatography on *S. frugiperda*, *S. littoralis*, and *G. molesta*.

		Spodoptera frugiperda	*Spodoptera littoralis*	*Grapholita molesta*
Toxins	Pattern	LC50 (95% FL) (ng/cm2)	LC50 (95% FL) (ng/cm2)	LC50 (95% FL) (ng/cm2)
Vip3Af1 (WT)	a	31 (23–40)	7.9 (6.6–9.4)	1.8 (1.1–2.6)
M34L	a	12.6 (3.3–33.6)	2.6 (1.8–3.4)	3.4 (1.7–5.2)
K284Q	a	24 (12–34)	6.0 (4.5–7.7)	3.5 (1.7–8.3)
E483D	a	20.1 (5.3–53.5)	7.3 (4.8–10.5)	3.1 (1.3–7.3)
E483H	f	>10,000	>3000	>3000
W552H	b	>10,000	Non-toxic	Non-toxic
W552F	a	76 (18–157)	14.7 (11.6–18.4)	646 (330–1607)
W552Y	a(+b)	1083 (663–1734)	461 (285–847)	>3000
N682K-G689S	e	99 (41–191)	62 (45–84)	1232 (440–10523)
G689S	e	150 (101–205)	77 (49–117)	534 (218–1266)
G689E	e	422 (262–602)	432 (304–630)	469 (306–673)

Note: The proteins (protoxins) were purified by Ipp (pH = 5.5) and dissolved in Tris buffer (20 mM Tris, 150 mM NaCl, pH = 9.0). The LC50s (at 7 d) were calculated by POLO PLUS software.

4. Discussion

Reverse genetics is one of the most used approach to deepen in the protein knowledge, especially when the protein structure is unknown (Bordo and Argos, 1991). As a logical step forward after the systematic study of the Vip3Af toxin's sequence by alanine scanning (Banyuls et al., 2018a), 12 mutations were build up based on theoretically favourable amino acid changes. Mutations M34L and K284Q were chosen from a protein sequence comparison among different Vip3 proteins (Fig. 2), whereas the rest were chosen based on key residues reported previously whose change significantly affected the insecticidal activity (Banyuls et al., 2018a). Vip3 proteins are found as homotetramers in solution and their activation by proteases consists on the removal of a few amino acids at the

N-terminus and a cleavage at around residue 198 with the result of two fragments of around 19 kDa and 65 kDa which remain together (Banyuls et al., 2018a, b; Chakroun and Ferré, 2014; Zack et al., 2017; Quan and Ferr´e, 2019). At the time when this work was finished, the first 3D structure of a Vip3 protein was released (Zheng et al., 2019). The Vip3 tetramer assembles into a pyramid-shaped structure where the *N*-terminal region forms the tip of the pyramid. Although the released structure was that of the Vip3B protein (deposited with PDB/RCSB under accession code 6V1V), the high homology between this and Vip3A proteins helped explain the results obtained.

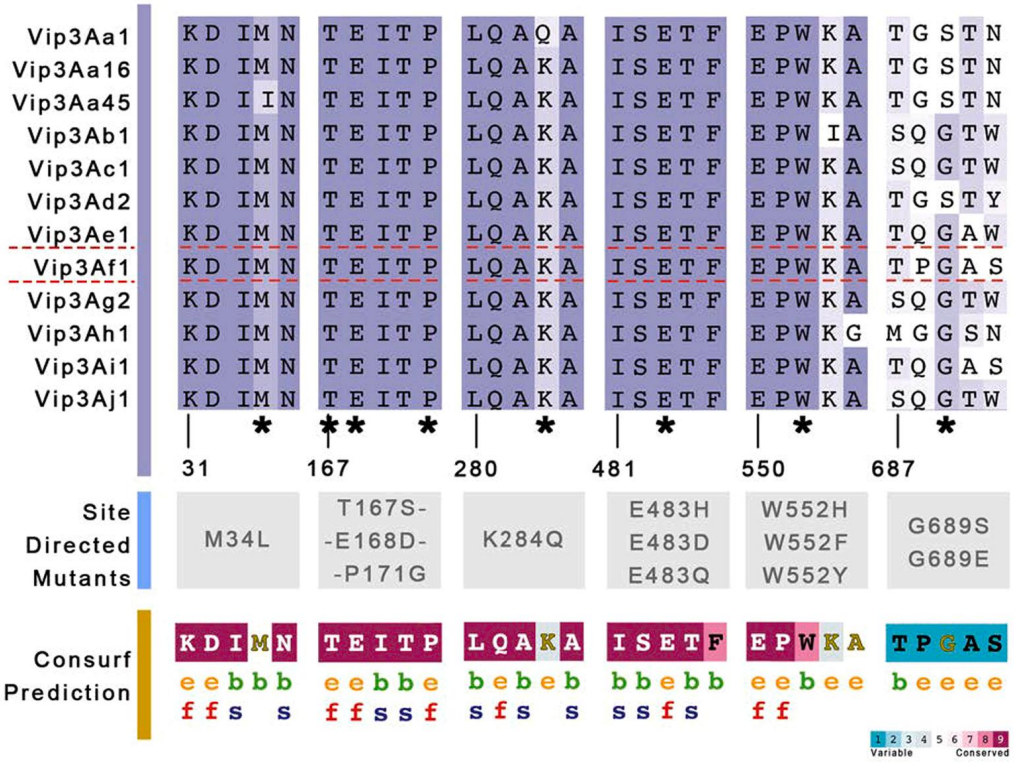


Figure 2. Multiple sequence alignment and consurf prediction of residues buried and exposed for the regions concerning the site-directed mutations of the Vip3Af1 (WT). Conservation of these sites was evaluated by Clustal Omega msa (Sievers et al., 2011) of different Vip3A proteins from *Bacillus thuringiensis*: Vip3Aa1 (GenBank accession number AAC37036), Vip3Aa16 (GenBank accession number AAW65132), Vip3Aa45 (GenBank accession number JF710269), Vip3Ab1 (accession number AAR40284), Vip3Ac1 (named PS49C with Seq. ID 7 in U.S. patent application 20.040.128.716 (Narva and Merlo)), Vip3Ad2 (accession number CAI43276), Vip3Ae1 (accession number CAI43277), Vip3Af1 (accession number CAI43275), Vip3Ag2 (accession number ACL97352), Vip3Ah1 (accession number ABH10614), Vip3Ai1 (accession number KC156693) and Vip3Aj1 (accession number KF826717). The msa (purple panel) is coloured according to BLOSUM62 colour scheme. Amino acid conservation, quality (BLOSUM62 score based on observed substitutions) as visualised by Jalview (Waterhouse et al., 2009). Asterisks (*) highlight the positions that were mutated. The Consurf prediction (Berezin et al., 2004) (yellow panel) represents the predicted functional (f) or structural (s), roles of the residues and the predicted location exposed (e) or buried (b). Residues in yellow indicates low confidence in the prediction due to insufficient data. The conservation-code scale of the consurf prediction is presented at the bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Domain I, at the *N*-end of Vip3A proteins, consists of a bundle of α-helices including the signal peptide, which is not removed after secretion from the bacterial cell (Banyuls et al., 2018a). We found

position Met^{34} the most attractive candidate to seek for a response driven from the *N*-terminus of the protein. On the one hand, this position falls at the end of the signal peptide (SP) according to some authors (Chen et al., 2003; Doss et al., 2002; Estruch et al., 1996; Rang et al., 2005). On the other hand, Met34 falls in a predicted conserved *Tar* multidomain (COG0840, a chemotaxis protein motif) with transmembrane properties (Chakroun et al., 2016a). Met^{34} is a highly-conserved residue among most of the Vip3 proteins (Fig. 2) and is found in a hydrophobic region of helix α1 that docks against the core of the tetramer in a cavity formed by α-helices of the same monomer (Zheng et al., 2019). The change of Met34 to Leu34 did not alter the proteolytic pattern (Fig. 1 and Table 3), indicating that the folding of the protein was not affected. Our results have shown a small though significant increase in the insecticidal activity of M34L against *S. littoralis*, though not against the other two species (Table 3). The leucine amino acid has a higher helical preference than methionine and has a higher hydrophobicity value which contributes to a greater stabilisation of the α-helix, thus, it is likely that this difference is the reason for the slightly higher activity against *S. littoralis*.

A more drastic mutation attempt in the *N*-terminus was intended by creating a triple mutant (T167S-E168D-P171G) based on the three consecutive key residues reported previously (Banyuls et al., 2018a). These residues are in a highly-conserved area (Fig. 2). Furthermore, residues Thr167 and Glu168 play a critical role in maintaining the structure of the protoxin by inter-monomer interactions in the tetrameric structure (Zheng et al., 2019). The key role of (at least) these two residues was confirmed by the fact that the triple mutant protein could not be expressed in enough quantity to be purified or bioassayed.

The residue Lys284 is highly conserved among Vip3A proteins; only in some Vip3Aa sequences, such as the Vip3Aa1, the lysine is substituted to a non-homologous Gln284 (Fig. 2). Residue 284 is located in α-helix 8 in domain II of the closely related Vip3Aa protein (Banyuls et al., 2018a; Zheng et al., 2019). Both lysine and glutamine residues have amphipathic side chains and are frequently found forming part of binding or catalytic active sites, which suggests that the residue in site-284 is exposed to the protein environment. Although the change in the mutant K284Q involves the removal of the positive net charge in the protein local surface in comparison to the WT, the change affected neither the folding (as suggested by the proteolytic pattern) nor the insecticidal activity of the protein (Fig. 1 and Table 3).

Positions Glu483, Trp552 and Gly689 in the Vip3Af1(WT) were previously identified as key residues for the insecticidal activity against *S. frugiperda* and *Agrotis segetum* (Lepidoptera: Noctuidae) (Banyuls et al., 2018a). The substitution of the native residue to an alanine resulted in unique proteolytic patterns that were not observed in any other substitution: E483A gave a single band of 27 kDa (pattern “f”), W552A displayed pattern”b” consisting in two main bands of 40 kDa and the 20 kDa, and G689A gave pattern “e” (62 kDa, 53 kDa and 20 kDa) (Banyuls et al., 2018a). The substitution of glutamic acid, tryptophan or glycine by alanine in this site seems to locally alter the conformation, making trypsin accessible to different cleavage sites other than the native ones. Therefore, we chose these sites for further testing how a change other than alanine could affect the structure and function of the protein.

Residue Glu483 is within a highly conserved region and is crucial in maintaining intra-monomer interactions between domains II and III (Banyuls et al., 2018a; Zheng et al., 2019). The fact that E483Q impairs protein expression reinforces the hypothesis that the side chain of the glutamic acid, negatively charged, contributes to the protein conformation by establishing ionic interactions or salt bridges with other spatially-close residues, especially with those positively-charged. This is further supported by the

non-deleterious effect of mutant E483D, in which the negative charge contribution is ensured by the side chain of the aspartic acid. The lack of a negative charge in E483H and E483A destabilizes the protein, rendering pattern “f” upon exposure to proteases, which must be the cause of the lack of insecticidal activity.

With only one exception known so far (Vip3Ba1), Vip3 proteins share a highly conserved domain identified as a Carbohydrate Binding Domain (CBM type 4, 9) which spans from Ser536 to Gln652 in the Vip3Af1(WT)sequence (Chakroun et al., 2016a). The alanine-scanning in the whole region revealed only one critical residue for the toxicity, the Trp552 (Banyuls et al., 2018a). Aromatic residues Phe, Tyr and Trp are known to be involved in the binding to carbohydrates in CBM by stacking interactions of the aromatic-shape side-chain with the pyranose ring of sugars and by hydrogen bonding (Asensio et al., 2013; Boraston et al., 2004). When Trp552 was changed to alanine or histidine the protein rendered an aberrant proteolytic pattern (pattern “b”) and lost insecticidal activity. In contrast, the substitution by an aromatic amino acid residue, such as in mutants W552F and W552Y, retained the insecticidal activity and did not affect the stability of the protein to proteases (or just did it minimally in W552Y). According to the bioassay and trypsintreatment results, the substitution of Trp552 by phenylalanine is better supported than the change to tyrosine. Based on the Vip3B crystal structure (Zheng et al., 2019), Trp552 (Trp560 in Vip3B) is buried in a hydrophobic core in the protein and it is likely to play an important structural role maintaining the structure of the putative CBM of Vip3Af1 (WT). Thus, the change to a non-aromatic residue might modify the interactions with the surrounding residues causing eventually a local misfolding that accounts for a non-functional activation of the protein by the insect gut proteases.

Finally, position Gly689 was described as being potentially subjected to positive Darwinian selection, though the exchange of the glycine to an alanine was not favorable (Banyuls et al., 2018a; Wu et al., 2007). This position is located in domain V, a domain predominantly formed by ß- sheets. The C-terminal end of the Vip3 proteins is thought to determine the target specificity because of the high sequence divergence among Vip3 proteins (Chakroun et al., 2016a). All Vip3 proteins have a glycine residue at positions 689 or 688 (Fig. 2 and Chakroun et al., 2016a), which may be important for the folding of this domain. The substitution of Gly689 either to a serine or to a glutamic acid decreased the insecticidal activity of the Vip3Af protein (Table 3), similarly to what was observed when the glycine was substituted to an alanine (Banyuls et al., 2018a), confirming the structural role of this small amino acid residue in the folding of domain V.

With the increasing demand of a more sustainable agriculture and the long time and economical costs of development of new active substances, synthetic diversification of the genetic bank of Vip3 proteins might be a shortcut strategy in agricultural biotechnology (Palma and Berry, 2016). Protein engineering is one of such strategies for target diversification. Site-directed mutagenesis is a useful tool to identify sites with critical implications to the function and structure of a protein. The substitutions performed in this study contributed to the understanding of the inter- and intramolecular interactions of key residues in the structure of Vip3Af. Furthermore, one of the residue substitutions (M34L) significantly increased toxicity of the Vip3Af protein against one insect species, though not to the other two, one of the of the same genus. This may be a consequence of the complex mode of action of Vip3 proteins, not totally understood yet. Therefore, genetic engineering of Vip3 proteins with the aim to increase the insecticidal activity should keep into consideration that changes in the amino acid sequence may have different effects depending on the target species, as has already been shown using other approaches (Chen et al., 2003; Selvapandiyan et al., 2001).

Supplementary material

Table 2.2-S1. Characteristics of primers used for the site-directed mutagenesis.

Primer	*T*m (ºC)	Self-annealing *T*m (ºC)	% GC	length/ mutations (bases)	Complementarity of primer pair (bases)
M34L-Fwd	78	70	34	38/ 2	27
M34L-Rev	76	68	29	38/ 2	27
T167S-E168D-P171G-Fwd	83	80	37	49/ 4	40
T167S-E168D-P171G-Rev	82	79	35	48/ 4	40
K284Q-Fwd	78	67	36	36/ 2	23
K284Q-Rev	79	66	33	42/ 2	23
E483D-Fwd	76	64	35	34/ 2	21
E483D-Rev	74	65	37	30/ 1	21
E483Q-Fwd	76	64	35	34/ 1	21
E483Q-Rev	74	65	37	30/ 1	21
E483H-Fwd	77	65	38	34/ 2	21
E483H-Rev	75	66	40	30/ 2	21
W552H-Fwd	81	69	41	39/ 1	23
W552H-Rev	81	71	47	34/ 2	23
W552F-Fwd	80	68	38	39/ 2	23
W552F-Rev	80	70	44	34/ 2	23
W552Y-Fwd	80	68	38	39/ 2	23
W552Y-Rev	80	70	44	34/ 2	23
G689S-Fwd	79	71	45	33/ 2	23
G689S-Rev	79	72	48	31/ 2	23
G689E-Fwd	79	71	45	33/ 2	23
G689E-Rev	79	72	48	31/ 2	23
N682K-G689S-Fwd	86	82	51	41/ 3	33
N682K-G689S-Rev	84	78	42	45/ 3	33

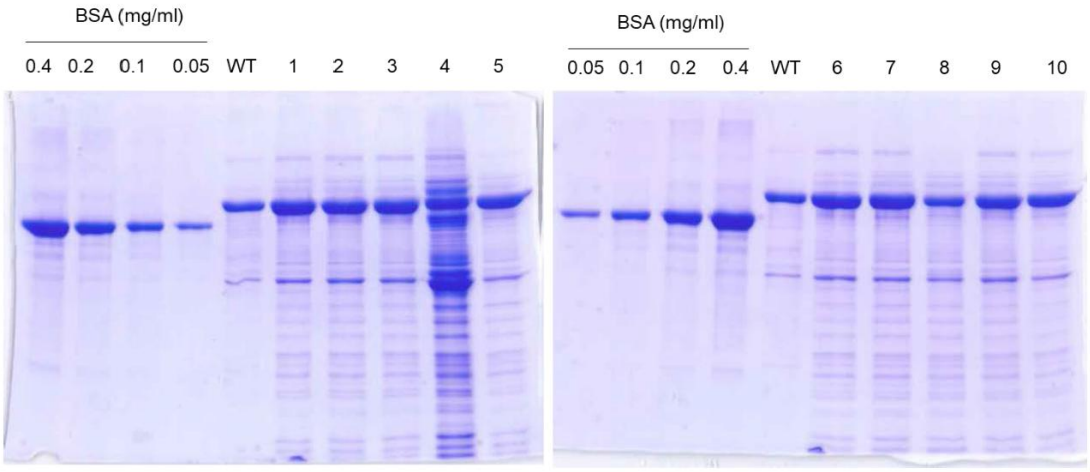


Figure S1. SDS-PAGE of purified Vip3Af protoxins. Vip3Af (WT) and the mutant proteins were purified by isoelectric point precipitation (at pH=5.5) and then dissolved in buffer (20 mM Tris, 150 mM NaCl, pH=9.0). Lanes 1: M34L, 2: K284Q, 3: E483D, 4: E483H, 5: W552H, 6: W552F, 7: W552Y, 8: N682K-G689S, 9: G689S, 10: G689E. WT: Vip3Af(WT). BSA: bovine serum albumin used for measuring concentration.

Chapter 3. Analyze the mode of action of Vip3A, and explore the reason of the resistance

3.1 Artefactual band patterns by SDS-PAGE of the Vip3Af protein in the presence of proteases mask the extremely high stability of this protein.

Results are included in:

Banyuls N, Hernández-Martínez P, Quan Y, Ferré J. 2018. Artefactual band patterns by SDS-PAGE of the Vip3Af protein in the presence of proteases mask the extremely high stability of this protein. Int J Biol Macromol 120, 59–65. https://doi.org/10.1016/j.ijbiomac.2018.08.067.

1. Introduction

Microbial control agents (MCA) have become a widespread resource for agricultural pest management since the 20th century. MCA-based products are environmental safe with no risk to human consumers, allowing a more sustainable production of food. Nowadays there is a large variety of "green" products based on microbial agents, among which *Bacillus thuringiensis* (Bt) based strategies are by far the most widely used (Lacey et al., 2015).

Bacillus thuringiensis Berliner is a Gram-positive sporulating bacterium which produces several entomopathogenic toxins and enzymes of agronomic interest. Among them, δ-endotoxins (Cry and Cyt proteins) are produced during the sporulation growth phase in parasporal inclusions and are highly toxic to a variety of insect pests. A different type of toxins are the vegetative insecticidal proteins (Vip), which are soluble proteins that are secreted during the vegetative growth phase of Bt. Vip3 proteins are a family of Vip proteins active against lepidopteran pests. Cry and Vip proteins differ in their mode of action and, therefore, their combination in the same pest management strategy is becoming a common practice. Nowadays Cry1 and Vip3A proteins are often found co-expressed in some biopesticides and in commercial transgenic plants (Bt-crops) (EFSA et al., 2012; Kurtz et al., 2007; Sanchis, 2011; Carrière et al., 2015; Chakroun et al., 2016a; Moar et al., 2017), constituting an important tool for both conventional and organic farming.

Some details in the mode of action of Vip3A proteins are not yet fully elucidated. It is well accepted that, after ingestion, different gut peptidases process the protein rendering the active toxin, which then crosses the peritrophic membrane and binds to specific receptors in the brush border midgut epithelium (Chakroun et al., 2016a; Lee et al., 2003; Sena et al., 2009; Chakroun and Ferré, 2014; Palma et al., 2014). Soon after the ingestion, the insect larvae stop feeding and moving (Yu et al., 1997). The starvation contributes to the disruption of the peritrophic matrix exposing the ectoperitrophic space to the lumen content (Yu et al., 1997). Following the specific binding event, the Vip3 toxins may either be inserted into the membrane and form pores (Lee et al., 2003; Caccia et al., 2016; Liu et al., 2011), be translocated into the cell cytoplasm bound to the S2 ribosomal protein (Singh et al., 2010), or trigger a signaling cascade that activates an apoptotic process (Jiang et al., 2016; Hernández-Martínez et al., 2017). In all cases, this succession of events leads to the histopathologic alteration in the gut epithelium and the eventual death of the insect (Chakroun and Ferré, 2014; Yu et al., 1997; Abdelkefi-Mesrati et al., 2011; Gomis-Cebolla et al., 2017; Song et al., 2016).

Proteolytic processing is an early step in the insecticidal mode of action of Vip3 proteins. The activation step and the protease sensitivity have been often related to the insecticidal performance (Abdelkefi-Mesrati et al., 2011; Estruch and Yu, 2001; Li et al., 2007; Chakroun et al., 2012; Caccia et al., 2014; Chakroun et al., 2016b; Hernández-Martínez et al., 2013). In an early Vip3Aa report, Estruch and Yu (Chakroun et al., 2012) already proposed that the 89 kDa Vip3A protein acted as a precursor which is promptly cleaved by insect proteases, splitting the protein in two fragments of 20 kDa (corresponding to the 200 first residues of the N-terminus) and of 65 kDa (corresponding to the activated toxin). The identity of these fragments was further confirmed in later studies by Vip3Aa protein sequencing (Bel et al., 2017; Banyuls et al., 2018b), as well as in other Vip3 proteins (Zack et al., 2017). Estruch and Yu also described two additional secondary cleavage sites in the 65 kDa fragment which render two overlapping minor fragments of 45 kDa and 33 kDa. The described proteolytic profile was also found by other authors (Yu et al., 1997; Ben Hamadou-Charfi et al., 2013), whereas the accumulation of the 65 kDa band was not predominant in a study with Vip3Aa12 (Song et

al., 2013). In the case of the Vip3Ca protein, the main activation product is a fragment of approximately 70 kDa (Gomis-Cebolla et al., 2017).

Vip3 proteins do not share any homology with known structures and predictions on their possible 3-D structure have been rarely reported. Structural inferences are mostly reduced to sequence analysis, in silico predictions and observations from protein engineering (Li et al., 2007; Banyuls et al., 2018a; Rang et al., 2005; Wu et al., 2007; Selvapandiyan et al., 2001; Dong et al., 2012; Gayen et al., 2012; Kunthic et al., 2016). It has been recently reported that Vip3A proteins can be found either as a globular protein or as a protein with an elongated shape, and that they are able to aggregate forming homo-tetramers (Palma et al., 2014; Kunthic et al., 2016; Palma et al., 2017; Kunthic et al., 2017).

In a previous work, Bel and coworkers (Bel et al., 2017) showed how an apparent degradation of the Vip3Aa16 protein after activation was in fact a consequence of the interaction of serine-proteases with the Vip3 proteins denatured by the action of sodium dodecyl sulfate (SDS). In this work we extend this study to a Vip3A protein from a different subclass (Vip3Af) to determine whether this phenomenon also applies to other Vip3A proteins. If this was the case, peptidase exposure in the presence of SDS can be a useful tool to infer different folding states among Vip3 proteins that might account for differences in their specificity against target pests.

2. Materials and methods

2.1. Vip3Af expression and purification

The Vip3Af protein was kindly provided Bayer CropScience NV (Ghent, Belgium). The vip3Af1 gene (GenBank accession No. AJ872070. 1) was fused to a His-tag sequence at the N-terminus and cloned in the pMaab10 plasmid (Beard et al., 2008) in E.coli strain BL21. Vip3Af was expressed as reported elsewhere (Ruiz De Escudero et al., 2014).

The protein was purified by metal-chelate affinity chromatography of the crude extract lysate as previously described by Hernández-Martínez et al. (Hernández-Martínez et al., 2013) using HisTrap FF columns (GE Healthcare). Eluted fractions were collected in 2 ml tubes containing EDTA at a final concentration of 5 mM. The first eight fractions were pooled and dialyzed o/n at 4 °C against 20 mM Tris-HCl, 300 mM NaCl, pH 9. The dialyzed protein was maintained in the refrigerator until use.

2.2. Midgut juice and trypsin preparation

Midgut juice was obtained from midguts from 5th instar larvae of Spodoptera frugiperda (Lepidoptera: Noctuidae). The larvae were dissected on ice and the bolus content was extracted by carefully excising the midgut epithelium and the peritrophic membrane. The boluses from eight to ten larvae were mixed and centrifuged at 16,000g at 4 °C for 10 min. The supernatant fraction was immediately snap frozen in liquid nitrogen and stored at −80 °C. Total protein concentration in the midgut juice was quantified with the Bradford reagent using BSA as standard (Bradford, 1976).

A stock solution of 10 mg/ml of commercial trypsin from bovine pancreas (Ref. T8003, Sigma-Aldrich) was prepared with ultrapure water, distributed in small aliquots and stored until use at −20 °C up to one month.

2.3. Proteolytic kinetics assays

The kinetics of the Vip3Af was studied as previously described for the closely related Vip3Aa protein (Bel et al., 2017). Affinity-purified Vip3Af protoxin (50 to 100 μg) was incubated with different concentrations of either trypsin or midgut juice of *S. frugiperda* in 100–280 μl final volume of

20 mM Tris, 300 mM NaCl, pH 9 at 30 °C. At desired times (0.1, 0.5, 1, 2, 6, 24, 48 and 72 h), a volume equivalent to 3 µg of Vip3Af was withdrawn and mixed with SDS-PAGE loading buffer (0.2 M TrisHCl pH 6.8, 1 M sucrose, 5 mM EDTA, 0.1% bromophenol blue, 2.5% SDS, and 5% β-mercaptoethanol) (2:1, sample:loading buffer), followed by a heating step of 5 min at 99 °C. Then, samples were immediately frozen in liquid nitrogen and stored at −20 °C until use. Proteolytic processing fragments were separated in 12% SDS-PAGE.

For a quantitative comparison of the processing rate, the amount of the Vip3Af protoxin (89 kDa) and of the processing products was quantified densitometrically using the TotalLab 1D v 13.01 software. The densitometry values from the 65 kDa, 33 kDa and 20 kDa bands were relativized to the input value in the gel, and the background was corrected. Graphical representation was performed using the software GraphPad Prism v 5.00.

2.4. Size exclusion chromatography

Gel filtration chromatography was carried out with an ÄKTA explorer 100 chromatography system (GE Healthcare) with a Superdex 200 10/300 GL column (GE Healthcare Life sciences, Uppsala, Sweden) equilibrated and eluted with 50 mM phosphate, 300 mM NaCl, pH 7.4, at a flow rate of 0.5 ml/min. To estimate the apparent molecular weight of the chromatographic peaks, the column was calibrated with a protein molecular weight standards kit (HMW calibration kit, GE Healthcare Life Sciences). Samples consisted of Vip3Af protoxin, Vip3Af activated with trypsin (5:100 w:w trypsin:Vip, for 24 h at 30 °C), and Vip3Af incubated with *S. frugiperda* midgut juice (5:100 w:w MJ:Vip for 3 h at 37 °C).

2.5. Biological activity of the digested Vip3Af

The toxicity of the Vip3Af protein activated with midgut juice of *S. frugiperda* at a ratio of 5:100 (MJ:Vip, w:w) at 30 °C for 30 min was tested against *S. frugiperda* neonates. The *S. frugiperda* colony was reared on a semi-synthetic diet (Greene et al., 1976) and maintained under controlled conditions (25 ± 2 °C, 70 ± 5% RH and 16:8 h L:D). Surface contamination bioassays were conducted by applying 50 µl of the Vip3Af in 2 cm2 diameter wells in multiwell plates filled with the semi-synthetic diet. A single protein concentration of 150 ng/cm2 was chosen based on the reported LC50 of the affinity-purified Vip3Af protein (Hernández-Martínez et al., 2013). A single neonate was placed in each well, with 16 neonates per treatment. Bioassays were replicated twice and the mortality was scored at 7 days. During the entire length of the bioassay, trays were maintained under controlled conditions in the insect rearing chamber.

3. Results

3.1. Kinetics of the activation of Vip3Af protoxin with trypsin

The treatment of Vip3Af with 24:100 trypsin (trypsin:Vip, w:w) in Tris-HCl buffer (pH 9) gave the two expected bands of 65 kDa and 20 kDa at all time-points analyzed, plus additional minor bands of approximately 45 kDa, 33 kDa, and 30 kDa immediately after the reaction started (Fig. 1a). As the time course of the reaction progressed, the intermediate bands progressively vanished along with the degradation of the trypsin yet visible in the SDS-PAGE, whereas the intensity of 65 kDa band slightly increased. We also noted the presence of a weak band of 90 kDa from 6 h onwards. When Vip3Af was treated with an even higher concentration of trypsin (120:100 trypsin:Vip, w:w), this effect was even more remarkable (Fig. 1b). At the initial time point of 0.1 h it can be observed a more complete

digestion of the 65 kDa band. As the reaction proceeded, the increase in the 65 kDa band intensity was more notorious, whereas the band of 45 kDa was not conspicuously formed. The band of 20 kDa mostly remained stable and only a slight decrease was observed at the very end of the experiment (Fig. 1b and c).

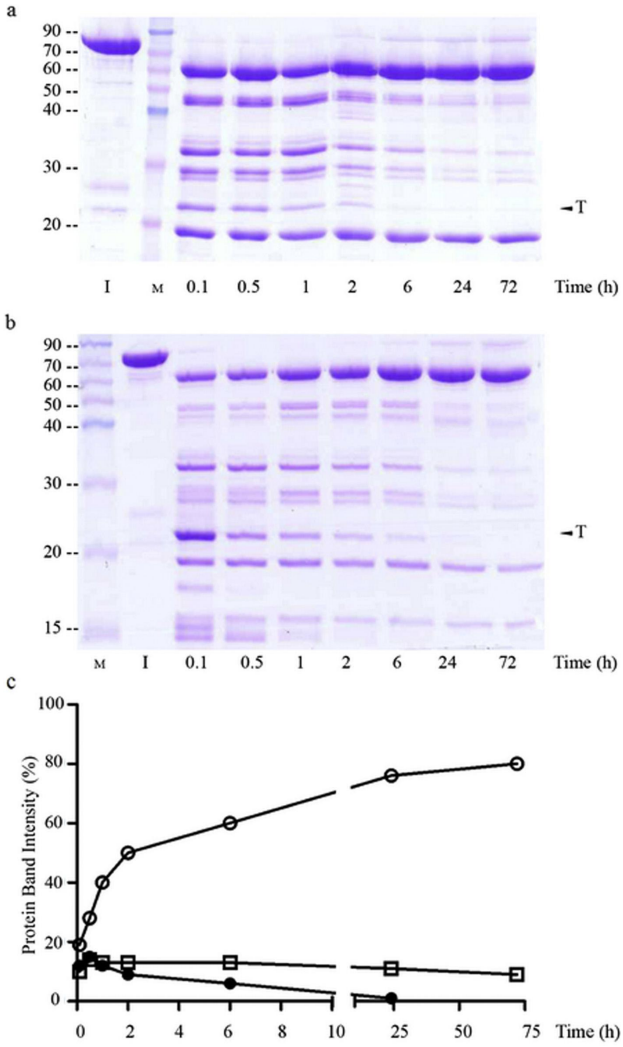


Figure 1. Kinetics of the proteolytic activation of Vip3Af by trypsin. Reactions were performed at 30 °C in Tris-HCl buffer (20 mM Tris, 300 mM NaCl, pH 9) and either (a) 24:100 trypsin (trypsin:Vip, w:w) or (b) 120:100 trypsin (trypsin:Vip, w:w). A sample volume corresponding to 3 µg of Vip protein was withdrawn at different time intervals and subjected to SDSPAGE. (c) Densitometric quantification of bands of 65 kDa (○), 33 kDa (●) and 20 kDa (□) in Fig. 1b. "I": input protoxin. "M": molecular weight markers (kDa). "T": band corresponding to trypsin.

3.2. Kinetics of the activation of Vip3Af protoxin with insect gut proteases

Different SDS-PAGE band patterns were obtained when Vip3Af was treated with different ratios of *S. frugiperda* midgut juice. At a ratio of midgut juice of 0.5:100 (MJ:Vip, w:w), incomplete processing of the 89 kDa protoxin was observed during the first 30 min (Fig. 2a). The main bands of 65 kDa and 20 kDa accumulated over time as the protoxin band (89 kDa) disappeared, accounting for complete digestion of the protoxin form. The increase in intensity of the 65 kDa band was not observed over time, even at higher concentrations of midgut juice (Fig. 2a and b). Interestingly, a band of around 60 kDa was formed from the very beginning of the reaction at the MJ concentration of 0.5:100 (MJ:Vip,

w: w), and even more significantly, at 1.5:100 (MJ:Vip, w:w), (Fig. 2a and b). Other minor bands of about 38 kDa, 33 kDa and 25 kDa appeared to be more intense as the midgut juice concentration was increased (Fig. 2b). Complete digestion of the 65 kDa band was observed in the SDS-PAGE when Vip3Af was treated at the ratio of 5:100 (MJ:Vip, w: w) (Fig. 2c).

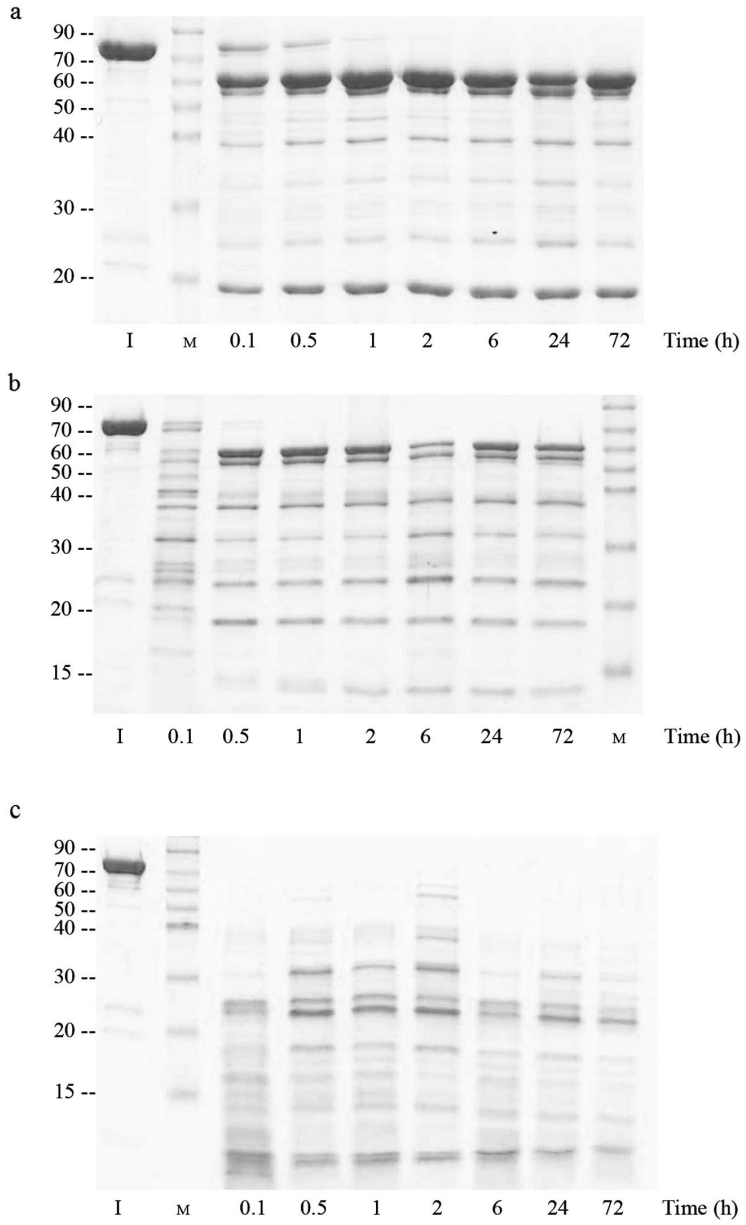


Figure 2. Kinetics of the proteolytic activation of Vip3Af by *S. frugiperda* midgut juice. Reactions were performed at 30 °C in Tris-HCl buffer (20 mM Tris, 300 mM NaCl, pH 9) and either (a) 0.5:100, (b) 1.5:100 or (c) 5:100 midgut juice (MJ) (MJ total protein:Vip, w:w). A sample volume corresponding to 3 µg of Vip protein was withdrawn at different time intervals and subjected to SDS-PAGE. "I": input protoxin. "M": molecular weight markers (kDa).

3.3. Size exclusion chromatography reveal a tetrameric Vip3Af highly stable to further processing

The chromatogram of both the protoxin and the trypsin-activated Vip3Af revealed a single major high molecular weight peak which corresponded to a globular protein of size of approximately 360 kDa

(Fig. 3), which matches the size of a homotetramer of Vip3Af. This result shows that, under native conditions, Vip3Af is found forming a homotetramer and, in the case of the trypsin activated form, the two main fragments (20 kDa and 65 kDa) remain together in the tetramer as they co-elute in the same peak.

To determine whether the proteolytic fragments observed when subjecting the Vip3Af protein to midgut juice are artefactual or not, a sample was subjected to chromatography. Treatment of Vip3Af with a high concentration of midgut juice (40:100, MJ:Vip, w:w, for 3 h) apparently completely degraded the protein (Fig. 4, lane I in inset). Gel filtration of this preparation revealed a single high molecular weight peak which corresponded to the tetrameric form of Vip3Af (Fig. 4). The analysis of this peak by SDS-PAGE showed two main bands of about 65 kDa and 20 kDa (Fig. 4, lanes B1 and B3 in inset), indicating that the midgut juice treatment cleaved the protoxin into these two fragments but did not digest them any further. Other chromatographic peaks were of very low molecular weight and were found to belong to the components of the midgut juice (data not shown).

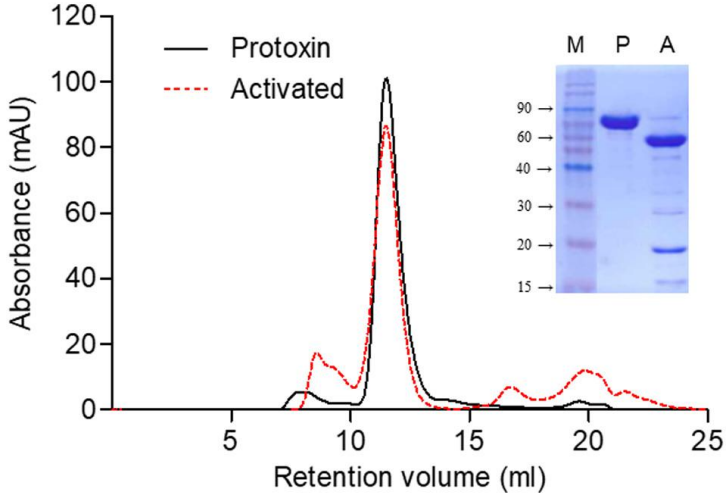


Figure 3. Gel filtration chromatography of the Vip3Af protoxin and the Vip3Af activated toxin after treatment with trypsin. The activation was performed at 30 °C with a trypsin: Vip ratio of 5:100 (w:w) for 24 h. The untreated and trypsin-treated sample were injected into a Superdex 200 10/300 GL column at a flow rate of 0.5 ml/min. The inset shows the protoxin (P) and the trypsin-activated sample (A) as revealed by SDS-PAGE. "M": molecular weight markers (kDa). Absorbance measured at 280 nm.

3.4. Toxicity tests reveal that high concentrations of Midgut juice do not destroy the insecticidal activity of Vip3Af

The single dose bioassays at 150 ng/cm2 (Table 1) gave a mortality and functional mortality higher than 69% in both Vip3Af protoxin and the Vip3Af treated with 5:100 midgut juice (MJ:Vip, w:w) for 72 h at 30 °C, indicating that the profile observed in the SDS-PAGE of an apparently degraded protein (Fig. 2c, last lane) completely retained the insecticidal activity.

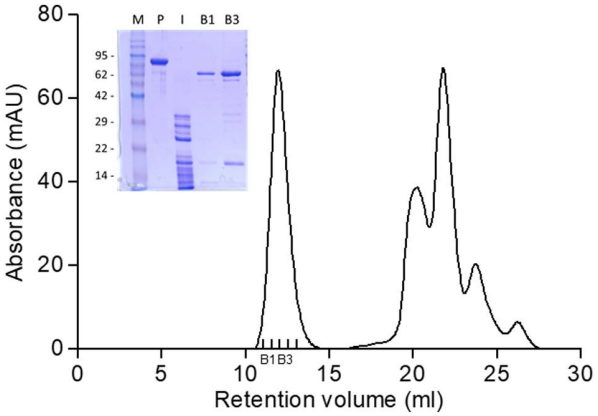


Figure 4. Gel filtration chromatography of Vip3Af treated with *S. frugiperda* midgut juice. The incubation took place at 37 °C, 5:100 midgut juice (MJ total protein:Vip, w:w) for 3 h. The treated sample was injected into a Superdex 200 10/300 GL column at a flow rate of 0.5 ml/min. The inset shows the protoxin (P), the input sample ("I") and the elution fractions B1 and B3 as revealed by SDS-PAGE. "M": molecular weight markers (kDa). Absorbance measured at 280 nm.

Table 1. Susceptibility of *S. frugiperda* to Vip3Af before and after 72 h- treatment with *S. frugiperda* midgut juice (MJ) at a ratio 5:100 (w:w MJ:Vip). Treated and untreated Vip3Af were applied at 150 ng/cm2 in surface contamination bioassays with neonates and the mortality was scored after 7 days. Functional mortality: dead larvae plus stunted larvae (L1 instar). Values represent the mean and standard error of 2 replicates. Mortality in the controls (20 mM Tris, 150 mM NaCl, pH 9) was ≤15%.

Vip3Af treatment	% mortality (mean ± SE)	% functional mortality (mean)
Vip3Af protoxin	69 ± 9	100
Vip3Af treated with midgut juice	88 ± 18	100

4. Discussion

The effect of SDS on exposing secondary cleavage sites in the Vip3Aa protein have been recently described by Bel et al. (Bel et al., 2017). The apparent degradation of Vip3Aa toxins observed by SDS-PAGE was shown to be an in vitro effect caused by the denaturing interaction of the SDS present in the SDS-PAGE loading buffer. While Vip3Aa proteins are progressively unfolded by the SDS, the trypsin and other peptidases present in the insect midgut juice withstand longer the denaturation effect of SDS and heating, retaining the enzymatic activity longer enough to act upon sites that are inaccessible under Vip3Aa-native conditions. This was especially evident at high concentration of proteases.

A similar phenomenon is observed with the Vip3Af protein, suggesting that this might be a common feature of the Vip3A proteins. The interaction of the SDS with proteins during heat denaturation has been commonly assumed to give long unfolded peptides. Nowadays it is known that secondary structures such as α-helices or β-sheets might be formed as a consequence of the protein interaction with the SDS micelles (Parker and Song, 1992). Furthermore, the way in which SDS differently interacts with proteins can be used to define domains and infer in the proteins structure, (e.g. by "gel shifting" of mutated proteins) (Shi et al., 2012). Therefore, the similarities found between Vip3Af and Vip3Aa in this context reflect a similar interaction of both proteins with SDS molecules, which point out to strong similarities in their native structure.

The results obtained herein showed that an apparent degradation also occurs in the Vip3Af protein after being treated with *S. frugiperda* midgut juice (Fig. 2). The progressively emergence of the 65 kDa fragment in the SDS-PAGE over time was not observed at high concentrations of midgut juice (Fig. 2). This difference with the trypsin treatment (Fig. 1) is likely explained by the higher stability of the different proteases in the midgut juice, which must be still present in thereaction mix after 72 h. This apparently degraded sample retained full toxicity against *S. frugiperda* (Table 1). When a Vip3Af sample apparently over-digested by midgut juice was subjected to gel filtration chromatography, the results revealed that Vip3Af had been cleaved into two main fragments of 65 kDa and 20 kDa which co-eluted in a peak corresponding to a high molecular weight species (Fig. 4). This phenomenon accounts for the differential size separation of Vip3Af and the midgut juice proteases, which have lower molecular weights (around 25 kDa and 27 kDa for chymotrypsin and trypsin, respectively). The Vip3Af which eluted from the chromatography column, already free of proteases, is therefore no longer digested when heated in the presence of the loading buffer containing SDS. The Vip3Af eluted in a peak with an apparent molecular weight of around 360 kDa. Assuming a globular conformation of the Vip3A proteins, the monomeric activated form should elute as a peak corresponding to approximately 85 kDa (the sum of the 65 kDa and 20 kDa fragments which co-elute together (Chakroun and Ferré, 2014; Bel et al., 2017; Kunthic et al., 2016)). However, its chromatographic behavior suggests a native conformation of a globular tetrameric form, in agreement to what has been recently shown for other Vip3Aa proteins (Bel et al., 2017; Zack et al., 2017; Kunthic et al., 2016; Palma et al., 2017).

Researchers working with Vip3A proteins commonly activate the protoxins by adding 1:100 to 5:100 trypsin:Vip (w:w) for 1–2 h. After one hour, the protoxin is completely processed at the primary cleavage site into the 65 kDa and the 20 kDa fragments. When Vip3Af protoxin is treated at higher concentrations of trypsin, as in Fig. 1a, several bands other than the 65 kDa and 20 kDa bands are evident even after 1 h incubation. However, these bands essentially disappear at longer incubation times suggesting that they were a product of the high trypsin content in the reaction (which is autodigested with time and thus its effect progressively decreases). We propose that once the protoxin is first treated with trypsin, the excision of the 200 amino acids from the N-terminus probably triggers a compaction of the "core" into a globular form, in agreement to Kunthic et al. (Kunthic et al., 2016). Furthermore, Lee et al. (Lee et al., 2003) showed that the activation step was crucial for the formation of pores in brush border vesicles membranes (BBMV) of susceptible insects, perhaps facilitated by the compacting into a globular shape. It is worth to consider that, in vivo, further activation steps might occur in the brush border membrane of the insect midgut by exopeptidases, such as aminopeptidase and carboxypeptidases, which could account for further folding changes (Song et al., 2016; Caccia et al., 2014; Hernández-Martínez et al., 2013).

Although the X-ray structure of Vip3 proteins is not yet solved, an in silico model of the 3D-structure of Vip3Af (Banyuls et al., 2018a) shows the C-terminal part of the protein predominantly formed by β-sheets, in agreement with the C-terminus model proposed by Wu et al. (Wu et al., 2007). Additionally, there is a Carbohydrate Binding Motif (CBM 4,9; from Ser536 to Gln652 in the Vip3Af protein) predicted within this region, which typically it is mainly composed by β-sheets (Chakroun et al., 2016a; Banyuls et al., 2018a). The secondary structures are differently affected by SDS; while α-helices are quickly unfolded, β-sheets are more robust and resistant to SDS restructuration (Nielsen et al., 2007). Accordingly, Bel and colleagues (Bel et al., 2017) showed that the smaller band of 29 kDa matched the C-terminal end of the Vip3Aa sequence and was not further processed by trypsin in the

presence of SDS, not even at the highest enzyme concentration. Here we show that a 33 kDa band is also relatively stable at high concentrations of trypsin, which is likely to account for the C-terminal end of the Vip3Af protein.

To check whether the apparent degradation previously described was rather anecdotic or, on the contrary, could be generalized to other Vip3A proteins regardless the Vip3 subclass or the insect gut specific proteases, we reproduced the main experiments published by Bel et al. (Bel et al., 2017) with a different Vip3A and a different insect species. The results obtained here supports that this phenomenon seems to be a general feature of Vip3A proteins and confirm the high stability of the Vip3A 65 kDa and 20 kDa fragments.

Chapter 3. Analyze the mode of action of Vip3A, and explore the reason of the resistance

3.2 Critical domains in the specific binding of radiolabelled Vip3Af insecticidal protein to brush border membrane vesicles from *Spodoptera* spp. and cultured insect cells.

Yudong Quan, Maria Lázaro-Berenguer, Patricia Hernández-Martínez, Juan Ferré. 2021. Critical domain in the specific binding of radiolabelled Vip3Af insecticidal protein to brush border membrane vesicles from *Spodoptera* spp. and cultured insect. Appl Environ Microb, under review.

1. Introduction

Globally, an estimated 20–40% of crops is lost due to pests and diseases (FAO, 2009), with insects representing a significant portion of this loss, both by direct damage and indirectly through the transmission of plant diseases (Oerke, 2006). To date the Gram-positive bacterium *Bacillus thuringiensis* (Bt) is known as the most economically successful entomopathogen and it has been estimated to account for the 75-95% of the microbial biopesticide market (Jouzani et al., 2017). Also, Bt insecticidal genes encoding different Cry proteins have been expressed in plants (Bt crops) since 1996 and the area planted to Bt crops represents >53% of the global cultivated area of genetically modified crops in the world (James, 2017; Olson, 2015; Karaborklu et al., 2018). More recently, *vip3* genes have been introduced in elite crop varieties to complement the insecticidal action of the Cry proteins, as well as to help manage the development of insect resistance (Tabashnik et al., 2013; Carrière et al., 2016).

Vip3 proteins contain from 787 to 789 amino acids, with a molecular mass of 89 kDa. Three main groups have been described: Vip3A, Vip3B and Vip3C (Chakroun et al., 2016a). The mode of action of Vip3 proteins has been studied mainly with Vip3Aa protein. It is well accepted that Vip3 proteins are produced as full-length proteins (protoxins) and after ingestion they are activated by midgut proteases which cleave the N-terminal 199 amino acids from the rest of the protein, generating two fragments (19 and 65 kDa) that remain attached to exert their toxic activity. This has been shown by different means (Banyuls, et al., 2018b; Bel et al., 2017; Chakroun and Ferré, 2014) and more recently by the 3D structure of the activated Vip3Aa (Núñez-Ramírez et al., 2020). The activated protein crosses the peritrophic matrix and binds to the apical membrane of brush border epithelial cells, inducing pore formation which eventually leads to the death of the larvae (Lee et al., 2003; Gomis-Cebolla et al., 2017; Liu et al., 2011). Several insect proteins have been identified to serve as putative receptors of Vip3Aa: The ribosomal protein S2 in *Spodoptera litura* (Singh et al., 2010), the scavenger receptor class C protein and fibroblast growth factor receptor-like proteins from cultured Sf9 cells (Jiang et al., 2018a, b) and the 48 kDa tenascin protein in *A. ipsilon* (Osman et al., 2019). In addition to pore formation, induction of apoptosis and internalization of the bound Vip3Aa has been observed (Hernández-Martínez et al., 2017; Hou et al., 2020; Jiang et al., 2016; Nimsanor et al., 2020).

The 3D structure of the Vip3Aa and Vip3B proteins shows that they have a tetrameric organization (Núñez-Ramírez et al., 2020; Zheng et al., 2020), with each monomer containing five structural (and most likely, functional) domains (DI – V) (Núñez-Ramírez et al., 2020; Zheng et al., 2020; Banyuls et al., 2018a; Boonyos et al., 2021; Jiang et al., 2020; Quan and Ferré, 2019). The highly conserved N-terminal domains (DI - III) have been shown to play a crucial role in maintaining the integrity of the tetramer (Quan and Ferré, 2019) whereas the two most C-terminal domains (DIV – DV), which are highly variable, do not seem to be necessary for maintaining the tetrameric structure (Núñez-Ramírez et al., 2020; Quan and Ferré, 2019), though they are critical for the insecticidal activity *in vivo* (Banyuls et al., 2018a, Quan and Ferré, 2019; Gayen et al., 2012; Li et al., 2007; Selvapandiyan et al., 2001). Domains IV and V are carbohydrate-binding modules (CBM) and it is very likely that their function is to interact with carbohydrates of the membrane (Chakroun et al., 2016a; Núñez-Ramírez et al., 2020; Boonyos et al., 2021).

In the present work we have optimized the binding assay, which has allowed us to show ^{125}I-Vip3Af specific binding to *Spodoptera exigua* and *Spodoptera frugiperda* midgut brush border membrane vesicles (BBMV) and to test whether the membrane binding sites are shared or not with other Vip3 and Cry proteins. In addition, we have identified the Vip3Af domains involved in the

specific binding by using truncated versions of the Vip3Af protein. The role of the domains involved in the specific binding has also been confirmed *ex vivo* with Sf21 cells.

2. Materials and methods

2.1 Source of proteins and expression

The insecticidal proteins used in the present work were obtained from different sources. The Vip3Af1 protein (NCBI accession No. CAI43275, from now on Vip3Af), three Vip3Af ala-mutant proteins (Vip3Af F229A, Vip3Af W552A, and Vip3Af I699A), and the Vip3Ca2 protein (NCBI accession No. AEE98106, from now on Vip3Ca) were overexpressed in *Escherichia coli* WK6 carrying the expression vector pMaab 10 (kindly supplied by Bayer CropScience N.V., Ghent, Belgium, (currently BASF Belgium Coordination Center–Innovation Center Gent)). Vip3Aa16 (from now on Vip3Aa) was prepared from recombinant *E. coli* BL21 expressing the *vip3Aa16* gene (Accession No. AY739665) (Mesrati et al., 2005). Protein expression and lysis of Vip3Af, its mutants, and Vip3Ca was carried out following the conditions described previously (Gomis-Cebolla et al., 2017), whereas expression of Vip3Aa was performed as described elsewhere (Mesrati et al., 2005). Cry1Ac and Cry1Fa were obtained from recombinant *B. thuringiensis* strains EG11070 and EG11069, respectively (from Ecogen Inc., Langhorn, PA). Crystal purification and solubilization was performed as described before (Estela et al., 2004).

2.2. Purification of Vip3 and Cry1 proteins

To be used for radiolabeling, the Vip3Af protein was subjected to isoelectric point precipitation (Ipp) and to anion-exchange chromatography. After cell lysis, the pH of the Vip3Af lysate was lowered with 0.1 M acetic acid to pH 5.6. After 10 min centrifugation at 16100x*g* at 4ºC, a portion of the pellet was dissolved with buffer Tris/NaCl (20 mM Tris, 150 mM NaCl, pH 9.0) and dialyzed against 20 mM Tris, pH 9.0 overnight. Afterwards, the protein was filtered prior to anion-exchange purification in a HiTrap Q HP column using an ÄKTA explorer 100 chromatography system (GE Healthcare, United Kingdom). Proteins were eluted with a 100-ml linear gradient (0 to 80%) of 1 M NaCl. The collected 1 ml fractions were subjected to SDS-PAGE. Fractions containing Vip3Af were stored, and the protein concentration quantified by Bradford. The fraction with the maximum concentration of Vip3Af was used for radiolabeling.

The Vip3 proteins to be used as competitors in binding assays were purified from crude extracts (lysed cells) by metal-chelate affinity chromatography using 1 ml HisTrap FF columns (GE Healthcare) (Hernández-Martínez et al., 2013). The fractions containing the Vip3 protein were pooled and dialyzed overnight against 20 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8.6, and stored at −80 ˚C. The purified Vip3 proteins were trypsin-treated (5% w/w trypsin from bovine pancreas, SIGMA T8003, Sigma-Aldrich, St. Louis, MO, USA) at 37 ˚C for 1 h, centrifuged at 16100x*g* for 10 min at 4˚C, and the supernatant quantified by Bradford (Bradford, 1976) (Fig. S1).

Trypsin-activated Cry1Ac and Cry1F proteins were further dialyzed in 20 mM Tris-HCl (pH 9) and filtered prior to be purified by anion-exchange chromatography in a HiTrap Q HP column as described elsewhere (Estela et al., 2004) (Fig. S1).

For cell toxicity assays, Vip3Af was subjected to Ipp as described above. Then, a portion of the pellet was dissolved with 20 mM Tris, 150 mM NaCl, pH 9.0, and dialyzed against the same buffer overnight. The concentration of the Vip3Af protoxin was determined, before trypsin activation, by densitometry after SDS-PAGE using bovine serum albumin (BSA) as standard and the TotalLab 1D

v13.01 software. The Ipp purified protein was flash frozen in liquid nitrogen and stored at ─80 ˚C until used.

2.3. Generation and purification of truncated Vip3Af molecules

According to our previous results, the Vip3Af ala-mutants F229A, W552A and I699A destabilize the protein making some sites more accessible to trypsin and generating fragments containing different combinations of the structural domains (Banyuls et al., 2018a; Quan and Ferré, 2019). We have used the trypsin treatment approach to generate Vip3Af truncated molecules with a different domain composition which, in principle, would maintain the original 3D structure as the full length protein since they would be derived from the original folded protoxin.

The main fragments resulting from treatment of F229A with trypsin were the 17 kDa and 27 kDa fragments (Quan and Ferré, 2019). The 17 kDa fragment corresponds to domain IV and the 27 kDa fragment to domains IV and V (Banyuls et al., 2018a; Quan and Ferré, 2019). To obtain pure preparations of these fragments, the F229A protoxin was first purified by HisTrap FF column and dialyzed in Tris buffer (20 mM Tris-HCl, pH 8.6) overnight. The protoxin was then incubated with trypsin (5% w/w, 37ºC, 1 h) and the fragments separated by anion-exchange chromatography in a HiTrap Q HP column (5-ml bed volume), equilibrated in the same dialysis buffer, in an ÄKTA explorer 100 chromatography system, eluted with a 100-ml linear gradient (0 to 80%) of 1 M NaCl. Individual peaks containing the 17 kDa fragment (DIV) and the 27 kDa fragment (DIV-V) were collected, subjected to SDS-PAGE, quantified by Bradford (Bradford, 1976), frozen in liquid nitrogen and stored at ─80˚C (Fig. S2). Fractions A5 and A9 were used for competition analyses and for cell toxicity assays.

The incubation of W552A and I699A with trypsin generates, in addition to the N-terminal 19 kDa fragment (from amino acid 12 to 198, corresponding to domain I) (Banyuls et al., 2018a; Quan and Ferré, 2019), a main fragment of around 37 kDa (for W552A) or one of around 53 kDa (for I699A) which, along with the 19 kDa fragment maintain the tetrameric structure of the protein (Quan and Ferré, 2019). The 37 kDa fragment consisted mostly of on domains II and III (Quan and Ferré, 2019). The 53 kDa fragment was previously identified as containing domains II, III and IV (Banyuls et al., 2018a).

To obtain pure preparations of the 19+37 kDa (DI-III) and 19+53 kDa (DI-IV) truncated molecules, the W552A and I699A mutants were first purified by Ipp (pH 5.6), subjected to trypsin treatment (5% w/w, 37ºC, 1 h) and finally purified by gel filtration chromatography in a Superdex 200 column (GE Healthcare Life sciences, Superdex 200 10/300 GL, Uppsala, Sweden) equilibrated and eluted with Tris/NaCl buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.6). Under these conditions, the tetrameric molecules can be separated from small fragments that do not form oligomers (Fig. S3). Individual peaks containing the pure fragments were collected, subjected to SDS-PAGE, quantified by Bradford (Bradford, 1976), frozen in liquid nitrogen and stored at ─80˚C. Fractions A11 and A12 were used for competition analyses and for cell toxicity assays. The Vip3Af protein was also subjected to the same protocol to serve as a control in the assays with the truncated molecules (referred to in these experiments as DI-V). Fig. S3 shows the chromatograms and SDS-PAGE of the collected fractions. Fractions A11 and A12 were used for competition analyses and for cell toxicity assays.

2.4. BBMV preparation

Midguts from fifth instar larvae of *S. exigua* and *S. frugiperda* were used to prepare BBMV by the differential magnesium precipitation method (Wolfersberger et al., 1987). BBMV were immediately

frozen in liquid nitrogen and stored at −80°C until used (less than one month). The protein concentration of BBMV preparations was determined by Bradford (Bradford, 1976) using BSA as a standard.

2.5. Radiolabeling of Vip3Af and the DI-III molecule

Chromatographically purified Vip3Af protoxin (25 µg) or the DI-III molecule (25 µg) were labeled with 0.3 mCi of [^{125}I]-NaI by the Chloramine T method (Chakroun and Ferré, 2014; Van Rie et al., 1989). The mixture was passed through a PD10 desalting column (GE Healthcare Life Sciences, United Kingdom) to separate the labeled protein from the excess of free radioactive iodine. The protein peak fractions were collected and stored at 4ºC. For optimal results, binding assays were performed within 10 days of protein labeling. The specific activity of labeled proteins was 3.81 mCi/mg for Vip3Af protoxin and 0.75 mCi/mg for the DI-III molecule, respectively.

2.6. Binding assays with 125I-labeled Vip3Af and the 125I-DI-III molecule to BBMV

Prior to be used in binding assays, radiolabeled Vip3Af protoxin was trypsin treated (5% w/w, 37ºC, 1 h) and stored at 4ºC until used. BBMV, which had been stored at −80ºC, were thawed on ice, centrifuged for 10 min at 16,000x*g* at 4ºC, then the supernatant removed and the pellet re-suspended in binding buffer (20 mM Tris, 1 mM $MnCl_2$, 0.1% BSA, pH 7.4). The binding assay consisted of incubating the ^{125}I-Vip3Af (0.37 nM) or ^{125}I-DI-III (0.40 nM) with 0.1 mg/ml BBMV (except when different concentrations of BBMV were tested) for 1 h at room temperature (RT) in a 0.1 ml final volume of binding buffer. The reaction was stopped by centrifuging the tubes at 16,100x*g* for 10 min at 4°C, the supernatant removed and the pellet washed twice (centrifuged at 16,100x*g* for 5 min at 4°C) with 500 µl of cold binding buffer. The radioactivity in the final pellet was measured in a model 2480 WIZARD2 gamma counter. An excess of unlabeled protein (370 nM of trypsin-treated Vip3Af or 400 nM of the DI-III molecule) was used to estimate the nonspecific binding.

To search for the optimal conditions for Vip3Af specific binding, a fixed amount of trypsin-treated ^{125}I-Vip3Af (0.37 nM) was used with 0.1 mg/ml BBMV to test the effect of NaCl concentration, $MnCl_2$ and the type of blocking agent: BSA or membrane blocking agent (MBA, GE Healthcare UK).

Autoradiography of ^{125}I-Vip3Af or ^{125}I-DI-III bound to BBMV was performed by re-suspending the binding assay pellets in 10 µl Milli-Q water and mixing them (2:1 v:v) with loading buffer (0.2 M Tris-HCl, pH 6.8, 1 M sucrose, 5 mM EDTA, 0.1% bromophenol blue, 2.5% SDS, and 5% β-mercaptoethanol). After heating at 99ºC for 5 min, the mixture was subjected to 12% SDS-PAGE. When finished, the gel was dried (at 50ºC for 1 h) and exposed to an X-ray film.

2.7. Cell culture maintenance

Spodoptera frugiperda derived Sf21 cells were cultured as a monolayer at 25°C 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 into fresh medium once a week.

2.8. Toxicity assays with Vip3Af and truncated Vip3Af molecules

For cell toxicity assays, Sf21 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 cell suspension (6×10^5 cells/ml) was added per well and the plates were let stand at 25°C for at least 45

min. Cell viability assays were performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Stockert et al., 2012) after exposure of cells to Vip3Af proteins. At least five concentrations of the Ipp purified Vip3Af protein (10 µl), either as a protoxin or as a trypsin-activated, were tested. The 20 mM Tris, 150 mM NaCl, pH 9.0 buffer was used as a negative control and 2% Triton X-100 as positive control. After 24 h or 48 h incubation at 25°C, 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, Maennedorf, Switzerland). The percent viability was calculated in relation to the absorbance of the cells treated with buffer (defined as 100% viable) and that of the cells treated with Triton X-100 (defined as 0% viable) (Martínez-Solís et al., 2018). Duplicates of each condition were performed in each assay and assays were replicated at least three times.

Vip3Af truncated molecules (DI-III, DI-IV, DIV-V, DV), purified by Ipp or by chromatography, were tested for cell toxicity as described above, though only at a single concentration (100 µg/ml). As a control, Vip3Af was purified after trypsin activation following the same methodology used for the truncated molecules and tested in parallel (referred to in these experiments as DI-V). Duplicates of each condition were performed in each assay and assays were replicated at least twice.

2.9. Binding of ^{125}I-DI-III to Sf21 cells

Specific binding of the labelled DI-III was analyzed on Sf21 cells. Prior to binding assays, Sf21 cells were detached and recovered by centrifugation at 500×*g* for 5 min at RT, and then washed twice with binding buffer. The pellet of cells was gently suspended in binding buffer to a concentration of approximately 4×10^7 cells/ml, calculated by Countess Automated Cell counter (Invitrogen). Total binding was determined by incubating different amounts of cells with ^{125}I-DI-III (0.4 nM) in a final volume of 0.1 ml in binding buffer. The same experiment was done adding an excess of unlabeled DI-III (400 nM) to each tube to calculate the nonspecific binding. After 1 h at RT, the reaction was stopped by centrifuging at 500×*g* for 10 min at RT, and the pellets were washed with 500 µl of binding buffer twice. Radioactivity was measured in a model 2480 WIZARD2 gamma counter. Two replicates were performed.

3. Results

3.1 Specific binding of ^{125}I-Vip3Af to S. exigua and S. frugiperda BBMV

Only marginal specific binding of ^{125}I-Vip3Af could be obtained using conditions described with radiolabeled Vip3Aa or Vip3Ca (20 mM Tris, 150 mM NaCl, 1 mM $MnCl_2$, 0.1% BSA, pH 7.4) (Chakroun and Ferré, 2014; Kahn et al., 2018). Therefore, new conditions were explored by testing the influence of NaCl and the blocking agent (none, BSA or MBA) on *S. frugiperda* BBMV (Table 1). The results revealed that NaCl had a strong negative effect on the specific binding. Leaving out this salt in the assay allowed to observe specific binding of ^{125}I-Vip3Af, with the best result using BSA as the blocking agent. All subsequent binding assays were performed in the absence of NaCl.

Table 1 Effect of NaCl, $MnCl_2$, and type of blocker on the total and specific binding of trypsin-treatment ^{125}I-Vip3Af to *S. frugiperda* BBMV[1].

Binding conditions					^{125}I -Vip3Af binding (% of input)		Percentage of specific binding over total (%)
Buffer	pH	NaCl (mM)	Divalent cation (1 mM)	Blocker (0.1%)	Specific[2]	Total	
20 mM Tris-HCl	7.4	150	-	-	0	9.8	0
			$MnCl_2$	-	0	4.8	0
			$MnCl_2$	BSA	0.1	6.9	1.4
			$MnCl_2$	MBA	0.4	3.1	12.9
		-	-	-	15.5	45.2	34.3
		-	$MnCl_2$	-	20.2	50.1	40.3
		-	$MnCl_2$	BSA	18.6	28.6	65.1
		-	$MnCl_2$	MBA	9.1	23.8	38.4

[1] Assays were performed with trypsin treatment 0.37 nM labeled ^{125}I-Vip3Af, and 0.1 mg/ml of BBMV proteins.

[2] Specific binding was determined adding 370 nM excess of unlabeled Vip3Af (affinity purified by HisTrap FF column).

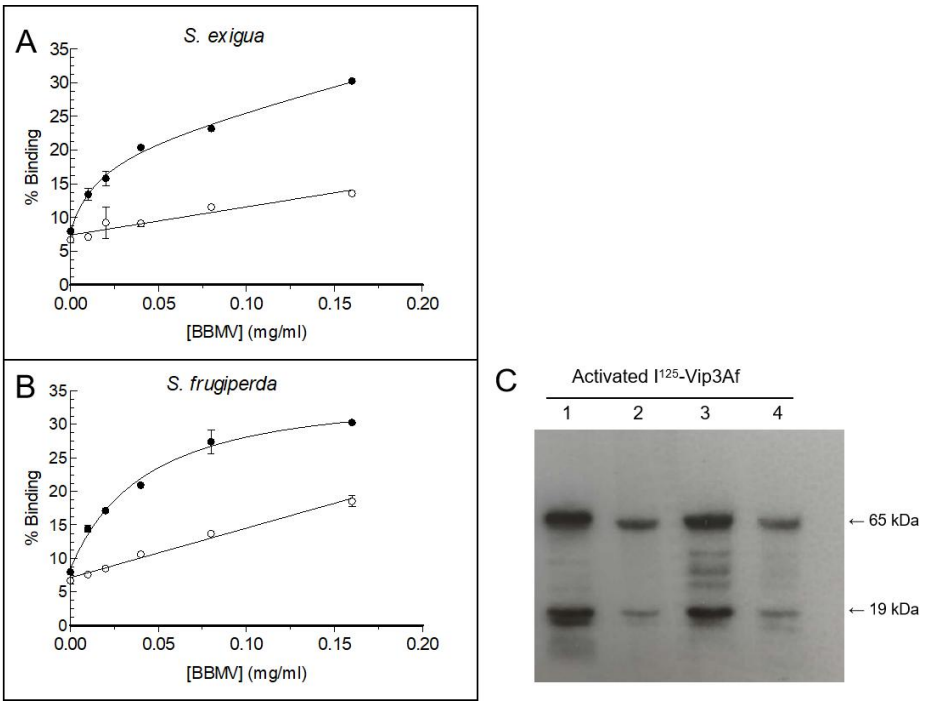


Figure 1. Specific binding of ^{125}I-Vip3Af to *S. exigua* (A) and *S. frugiperda* (B). Binding of ^{125}I-Vip3Af at increasing concentrations of BBMV; specific binding is the difference between total and nonspecific binding. The nonspecific binding was calculated using an excess (370 nM) of unlabeled Vip3Af toxin (purified by HisTrap FFolumn). , Total binding; ○, Nonspecific binding. (C) Autoradiography of ^{125}I-Vip3Af in different reaction samples. Pellets obtained after centrifugation of the reaction mixture were subjected to SDS-PAGE and exposed to the X-ray film. Lane 1: labeled toxin used in the assay (input); 2: sample without BBMV (precipitated protein or protein bound to the tube walls); 3: pellet of 0.1 mg/ml *S. frugiperda* BBMV (total binding); 4: pellet of 0.1 mg/ml *S. frugiperda* BBMV in the presence of 885 nM unlabeled Vip3Af (nonspecific binding).

3.2 Competition binding assays with ^{125}I-Vip3Af and Vip3 and Cry1 proteins

Trypsin-treated Vip3 and Cry1 proteins were used at increasing concentrations to compete with the binding of ^{125}I-Vip3Af (Fig. 2). The analysis of the homologous curve (using Vip3Af as competitor) yielded an equilibrium dissociation constant (K_d) of 71±7 and 89±2 nM for *S. exigua* and *S. frugiperda,* respectively, with a concentration of binding sites (R_t) of 354±32 and 350±21 pmol/mg BBMV protein for *S. exigua* and *S. frugiperda,* respectively.

Binding assays of ^{125}I-Vip3Af in the presence of unlabeled heterologous competitors were carried out to evaluate whether Vip3Af shares binding sites with other Vip3 or Cry1 proteins. No competition was observed when Cry1Ac or Cry1Fa were used as competitors (Fig. 2). In contrast, Vip3Aa and Vip3Ca were able to compete for the ^{125}I-Vip3Af binding. In the case of Vip3Aa, the competition curve completely overlapped with that of Vip3Af, indicating that both proteins bound to the same sites and with the same affinity to BBMV of the two *Spodoptera* species. However, Vip3Ca could not displace completely the ^{125}I-Vip3Af binding, which means that Vip3Ca only recognizes part of specific sites of Vip3Af.

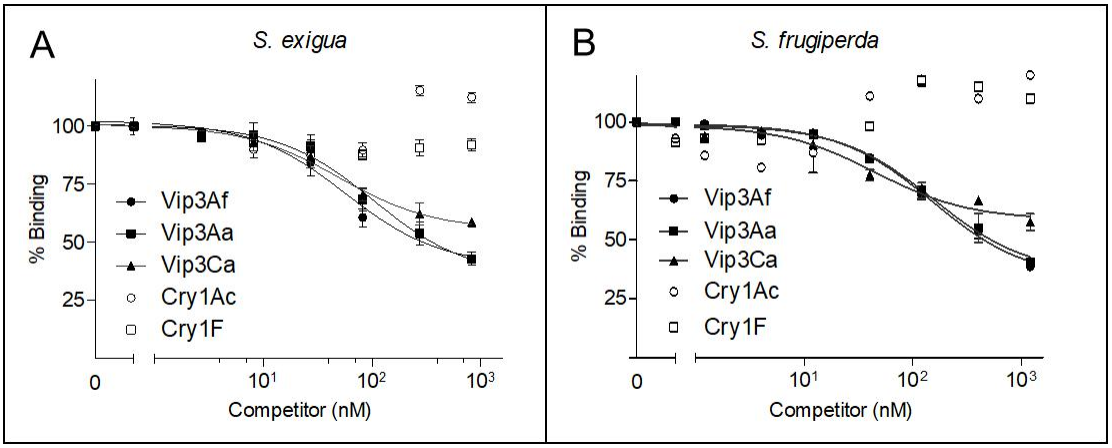


Figure 2. Heterologous competition of Vip3 and Cry1 proteins with ^{125}I-Vip3Af. Curves represent binding of ^{125}I-Vip3Af at increasing concentrations of unlabeled competitor, using BBMV from *S. exigua* (A) or *S. frugiperda* (B). Data points represent the mean and standard error from three to nine replicated experiments.

3.3 Competition binding assays with ^{125}I-Vip3Af and truncated molecules

To determine the involvement of the different Vip3Af domains in the specific binding, Vip3Af truncated molecules were tested in competition assays with ^{125}I-Vip3Af. The results were similar with BBMV from both *S. exigua* and *S. frugiperda* (Fig. 3). The truncated molecules DI-III and DI-IV, competed similarly to the trypsin-activated Vip3Af protein (DI-V) for the ^{125}I-Vip3Af binding sites (Fig. 3A and B). All three molecules have in common that they conserve domains I to III and that they hold the tetrameric conformation. In contrast, the truncated molecules DIV-V or DIV did not displace ^{125}I-Vip3Af within biologically relevant concentrations (Fig. 3C and D). Therefore, the results suggest that the minimum portion of Vip3Af required for specific binding is the one containing domains I to III.

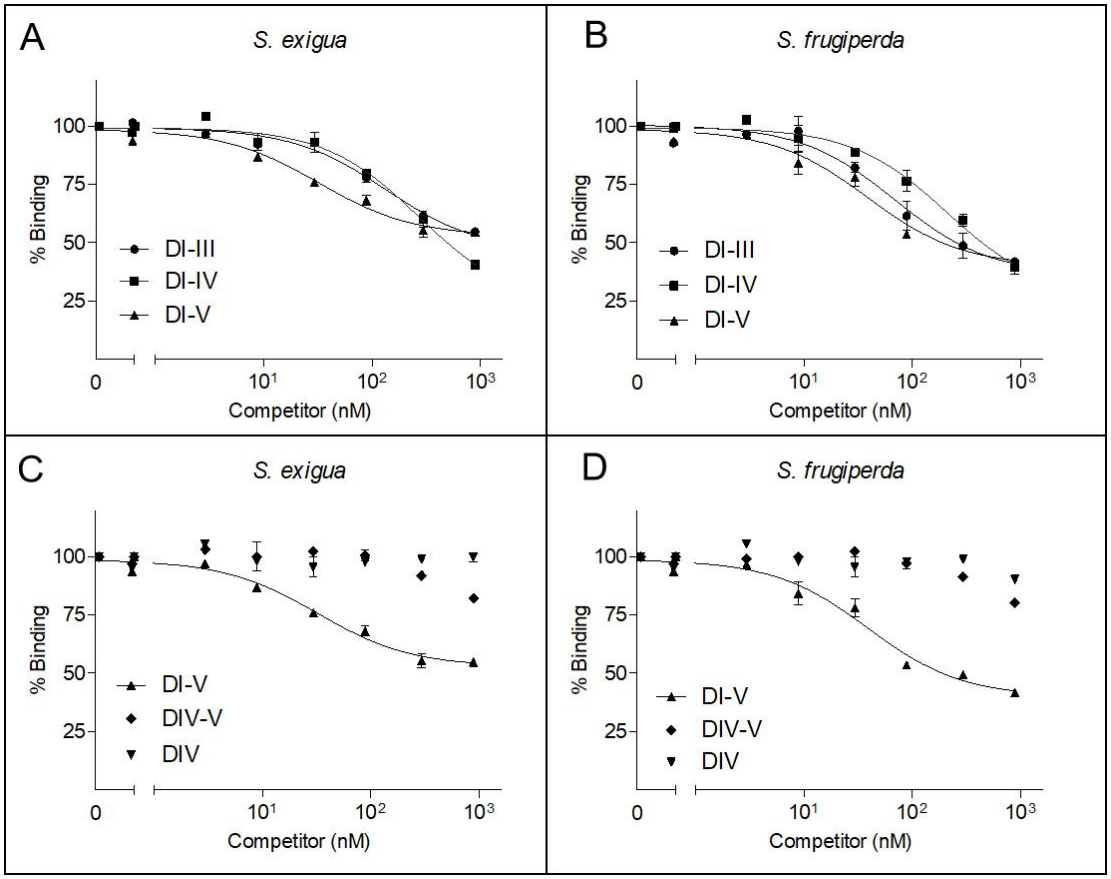


Figure 3. Heterologous competition of truncated Vip3Af molecules with ^{125}I-Vip3Af. Curves represent binding of ^{125}I-Vip3Af at increasing concentrations of unlabeled competitor, using BBMV from *S. exigua* (A and C) or *S. frugiperda* (B and D). As a control for the competitors, unlabeled trypsin-treated Vip3Af was prepared using the same protocol as the truncated molecules and is represented as DI-V in the figures. Data points represent the mean and standard error from three to nine replicated experiments.

3.4 Specific binding of the ^{125}I-DI-III molecule to *S. frugiperda* and *S. exigua* BBMV

To further support the role of Vip3Af domains I to III in the specific binding, the DI-III molecule was radiolabeled and used in binding assays. Specific binding was confirmed by incubating ^{125}I-DI-III (0.4 nM) with increasing concentrations of *S. exigua* and *S. frugiperda* BBMV in the presence or absence of an excess of unlabeled DI-III (400 nM) (Fig. 4). At 0.1 mg/ml BBMV, the total binding of ^{125}I-DI-III was 30%, of which approximately 60% was specific. Autoradiography after SDS-PAGE separation of the proteins in the *S. exigua* BBMV pellet showed that both the 37 kDa fragment (which corresponded to domains II to III of the protein) and the 19 kDa fragment bound to the BBMV (Fig. 4B). These two bands were much less intense when the assay contained an excess of unlabeled DI-III.

Competition binding experiments were conducted with ^{125}I-DI-III in the presence of increasing concentrations of unlabeled competitors using the truncated molecules (DI-III, DI-IV, DIV-V, and DV) and the trypsin activated Vip3 proteins (Vip3Af, Vip3Aa, Vip3Ca) (Figs. 5 and 6). The results confirmed that domains I to III are critical for the specific binding and that domains IV and V did not act as competitors (Fig. 5A and B). Competition of ^{125}I-DI-III with Vip3 trypsin activated proteins rendered similar results as when using ^{125}I-Vip3Af, with Vip3Af and Vip3Aa completely displacing the binding of with ^{125}I-DI-III and with Vip3Ca competing only partially (Fig. 6A and B). These results

confirm that the specific binding observed *in vitro* is mainly due to the three most N-terminal domains and that more than one population of binding sites exists for Vip3Af since Vip3Ca can displace just a fraction of the binding.

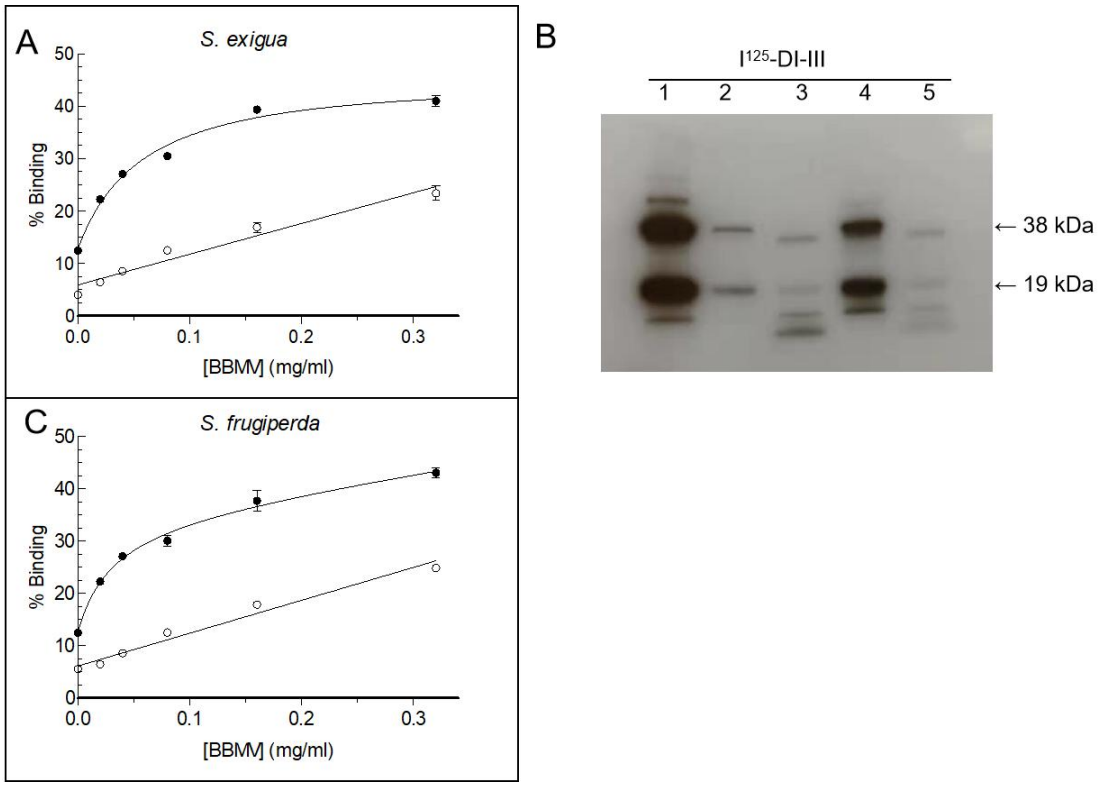


Figure 4. Specific binding of ^{125}I-DI-III at increasing concentrations of BBMV from *S. exigua* (A) and *S. frugiperda* (C). , Total binding; , Nonspecific binding. (B) Autoradiography of ^{125}I-DI-III in different reaction samples. Lane 1: labeled ^{125}I-DI-III (input); 2: sample without BBMV (precipitated protein or protein bound to the tube walls); 4: pellet of 0.1 mg/ml *S. exigua* BBMV (total binding); 3 and 5: pellet of 0.1 mg/ml *S. exigua* BBMV in the presence of 400 or 1200 nM unlabeled DI-III, respectively (nonspecific binding).

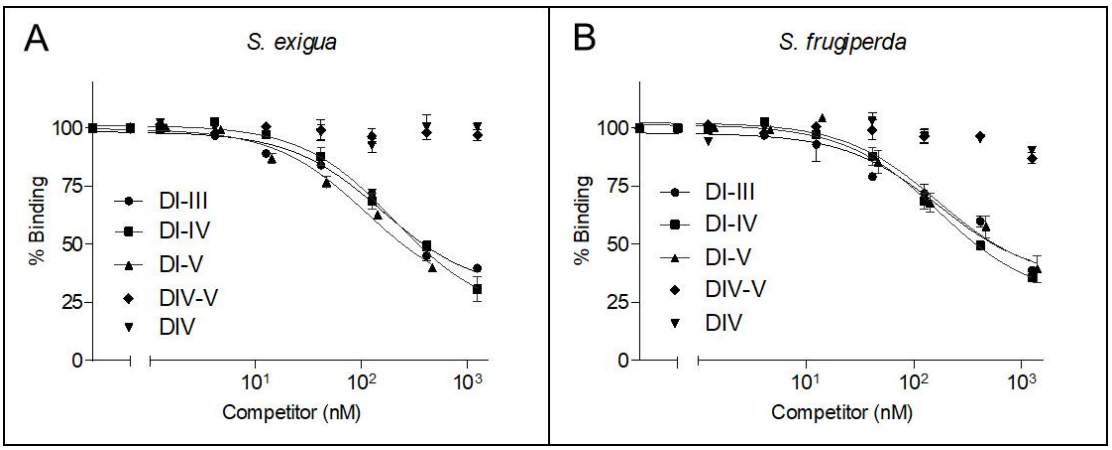


Figure 5. Heterologous competition of truncated proteins with ^{125}I-DI-III to BBMV from *S. exigua* (A) and *S. frugiperda* (B). Data points represent the mean and standard error from three to nine replicated experiments. Note that DI-V refers to the trypsin-activated Vip3Af purified in the same way as the truncated fragments.

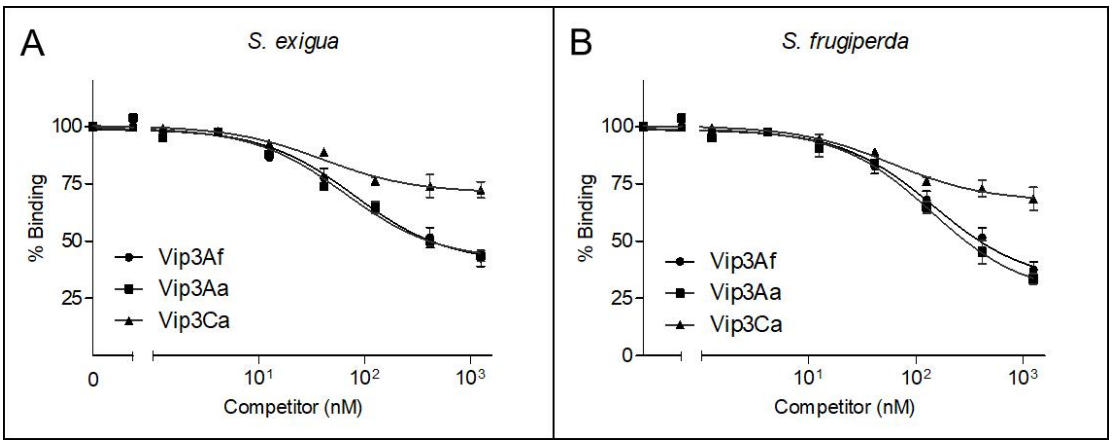


Figure 6. Heterologous competition of Vip3 proteins with ^{125}I-DI-III to *S. exigua* **(A)** and *S. frugiperda* **(B)**. Data points represent the mean and standard error from three to nine replicated experiments.

Table 2 K_d and R_t of Vip3A and truncated domains proteins with BBMV from *S. exigua* and *S. frugiperda.*

Proteins	*Spodoptera exigua*		*Spodoptera frugiperda*	
	K_d (nM)	R_t (pmol/mg)	K_d (nM)	R_t (pmol/mg)
Vip3Af	71 ± 7	354 ± 32	89 ± 2	350 ± 21
DI-IV	72 ± 1	291 ± 7	94 ± 1	426 ± 14

[a]Results represent the mean ± standard error (SEM) of the results from four to six replicates.

3.5. Toxicity of Vip3Af and its truncated molecules to Sf21 cells

To determine the appropriate conditions for the assays, the Ipp purified Vip3Af protein was first tested both as protoxin and as trypsin activated form, at different concentrations. The results showed that the protoxin had no effect on the viability of Sf21 cells, even at the highest concentration tested (Fig. 7A and B), whereas the trypsin-activated Vip3Af was toxic in a dose-dependent manner. Moreover, the loss of cell viability was slightly higher when Sf21 cells were exposed to the activated protein for 48 h than when cells were exposed for 24 h.

Based on the above results, the truncated molecules were tested at 100 μg/ml both as Ipp purified and as chromatography purified. As a control, the trypsin-activated Vip3Af protein (DI-V) was purified in the same way as the truncated molecules and tested in parallel. The results showed that the molecule DIV-V had basically no effect on the viability of Sf21 cells (Fig. 7C), even when the concentration was increased to 300 μg/ml (data not shown), whereas the DI-III and DI-IV molecules were as active as the trypsin-activated Vip3Af protein (DI-V). These results indicate that the fragment containing DI-III is the minimum Vip3Af fragment required for toxicity to Sf21 cells.

3.6. Specific binding assays of 125I-DI-III to Sf21 cells

Since the DI-III truncated molecule was toxic to Sf21 cells, we set out to demonstrate specific binding of ^{125}I-DI-III to these cells using the same approach as with BBMV. Figure 7D shows that ^{125}I-DI-III binds specifically and in a concentration-dependent manner to Sf21 cells. Therefore, the DI-III fragment is also the minimum Vip3Af fragment required for binding to Sf21 cells.

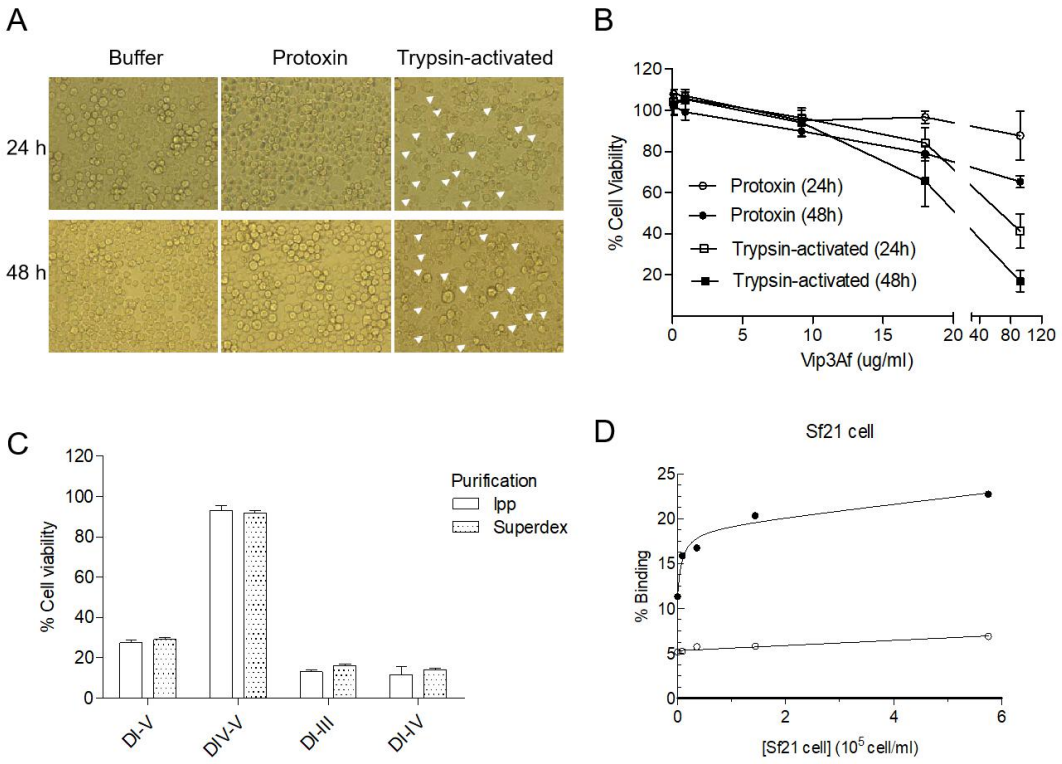


Figure 7. Morphology (A) and cell viability (B, C) of Sf21 cells exposed to Vip3Af and its truncated molecules for 48 h. Cell viability assays were repeated more than three times, each time including at least three replicates. The bars represent the mean and standard error of the mean. (D) Specific binding of ^{125}I-DI-III to Sf21 cells. ●, Total binding; ○, Nonspecific binding.

4. Discussion

The first steps towards understanding the mode of action of Vip3 proteins were given at the time of their discovery from Bt in 1996 (Estruch et al., 1996). To date, although more than 101 Vip3 proteins have been reported (Crickmore et al., 2016), their mode of action remains somewhat elusive (Chakroun et al., 2016a; Syed et al., 2020; Chakrabarty et al., 2020), though it is well accepted that their activity requires binding to membrane receptors in the midgut of target insects (Chakroun and Ferré, 2014; Lee et al., 2003; Abdelkefi-Mesrati et al., 2011; Lee et al., 2006). Several insect proteins have been proposed as functional receptors for Vip3Aa (Singh et al., 2010; Jiang et al., 2018a, b; Osman et al., 2019). However, very little is known regarding how Vip3 proteins interact with the membrane receptors and which Vip3 domains are involved.

So far, evidence that Vip3 proteins bind specifically to the brush border membrane of the midgut epithelial cells has been shown, almost exclusively, in studies working with the Vip3Aa protein. Specific binding of Vip3Aa has been shown in *Spodoptera littoralis, S. frugiperda*, *Agrotis ipsilon*, *Heliothis virescens, Helicoverpa zea,* and *Helicoverpa armigera* (Chakroun and Ferré, 2014; Liu et al., 2011; Abdelkefi-Mesrati et al., 2011; Lee et al., 2006; Chakroun et al., 2016b; Pinos et al., 2020; Yu et al., 1997). Only a few studies have shown binding of other Vip3 proteins, Vip3Af and Vip3Ca among them, and except for the latter (Kahn et al., 2018), always indirectly as heterologous competitors of Vip3Aa or in qualitative or semiquantitative assays (Chakroun and Ferré, 2014; Gomis-Cebolla et al., 2017; Sena et al., 2009). However, direct binding of radioactively labeled Vip3Af had never been reported before. By excluding NaCl from the binding assay buffer we have been able to show specific

binding of Vip3Af to BBMV from *S. exigua* and *S. frugiperda* larvae and perform competition binding assays to determine whether Vip3Af binding sites are shared with other Bt toxins. Autoradiography of the BBMV pellet after the binding assay showed that both the 19 kDa and 65 kDa fragments bind specifically (because their binding is competed by an excess of unlabeled protein) to the BBMV (Fig. 1), in agreement with previous reports that showed that these two fragments remain strongly associated after trypsin activation (Chakroun and Ferré, 2014; Núñez-Ramírez et al., 2020).

Competition binding studies are a potent tool that provides information on the potential for cross-resistance among Bt toxins since alteration of binding sites is the main mechanism of resistance to Cry proteins (Ferré and Van Rie, 2002; Ferré et al., 2008; Jurat-Fuentes et al., 2021). All studies performed with insects resistant to either Cry or Vip3A proteins have shown lack of significant cross-resistance between these two families of proteins (Gomis-Cebolla et al., 2018; Pickett et al., 2017; Anilkumar et al., 2019; Jackson et al., 2007; Mahon et al., 2012; Fang et al., 2007; Tabashnik and Carrière, 2020; Quan et al., 2021), which is supported by the fact that they do not share binding sites (Chakroun et al., 2016a; Chakroun and Ferré, 2014; Kahn et al., 2018; Sena et al., 2009; Gouffon et al., 2011). Our results with *S. exigua* and *S. frugiperda* support the lack of shared binding sites between Cry1 and Vip3 proteins, and the occurrence of common sites for Vip3 proteins (Fig. 2). Our results also expand the binding site model of Vip3 proteins (Gomis-Cebolla et al., 2017; Kahn et al., 2018) in that they show that Vip3Ca is not able to compete for all Vip3Af binding sites, suggesting that Vip3Af has some binding sites to which Vip3Ca does not bind (Fig. 8).

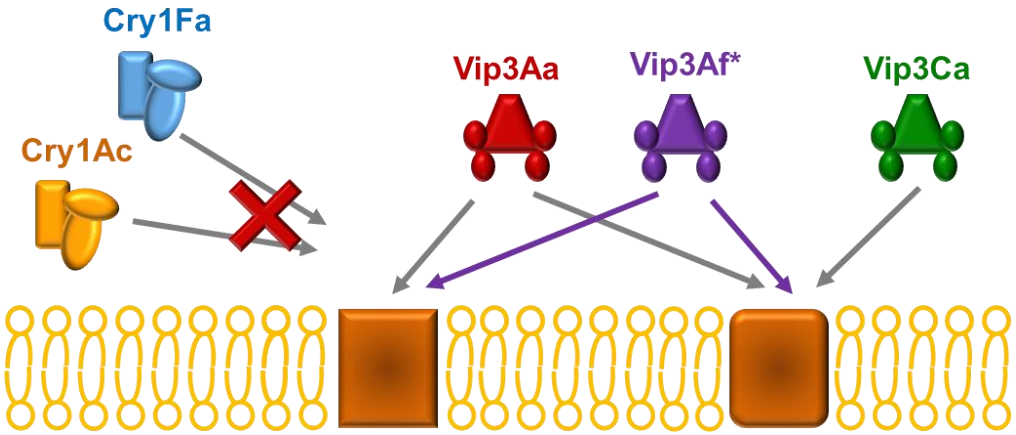


Figure 8. Proposed binding site model for Vip3 and Cry1 toxins in *Spodoptera* spp.

Three independent groups have recently reported the 3D structure of Vip3Aa and Vip3B proteins (Núñez-Ramírez et al., 2020; Zheng et al., 2020; Jiang et al., 2020) which has firmly established 5 structural domains that had been previously proposed (Quan and Ferré, 2019). The structure of the trypsin-activated Vip3Aa has shed light on the long-time puzzling observation that the 19 kDa and 65 kDa fragments remain strongly associated after trypsin action (Núñez-Ramírez et al., 2020). The 3D structures confirmed that Vip3 protoxins spontaneously form homotetramers in solution, which adopted a "pyramid-shaped" structure. Upon protease activation, and probably triggered by binding to a receptor, the tetrameric molecule suffers a drastic change conferring it a new conformation with a "syringe-like" structure, with the "needle" being a four-helix coiled coil involving domain I from the

four monomers (Núñez-Ramírez et al., 2020).

By the use of Vip3Af ala-mutants unstable to trypsin, we have been able to generate and purify Vip3Af truncated molecules with a different domain composition. Instead of using cloned fragments, we preferred to use the alternative approach with the aim of maintaining the original 3D structure of the remaining part of the truncated molecule, especially the tetrameric structure. From three Vip3Af ala-mutants (W552A, I699A, and F229A) we produced the DI-III and DI-IV molecules, which maintain the tetrameric structure (Quan and Ferré, 2019), and the DIV and DIV/V molecules, which contain the carbohydrate binding modules (Núñez-Ramírez et al., 2020; Zheng et al., 2020; Jiang et al., 2020) and cannot form tetrameric structures in the absence of the rest of the protein (Quan and Ferré, 2019). In competition binding assays with BBMV we could demonstrate that the truncated molecules containing domains I to III were able to compete for binding with ^{125}I-Vip3Af, whereas domains IV or IV/V were not (Fig. 3). These results were confirmed by labeling the DI-III truncated molecule, which showed specific binding to BBMV (Fig. 4). Only those molecules retaining domains I to III were able to compete with the ^{125}I-DI-III molecule for BBMV binding (Fig. 5). As with ^{125}I-Vip3Af, Vip3Af and Vip3Aa completely displaced binding of the DI-III truncated molecule, whereas Vip3Ca competed only partially (Fig. 6).

Specific binding of the ^{125}I-DI-III molecule to Sf21 cells was also observed by performing binding assays in the presence and absence of unlabeled competitor. Our results are in agreement with those reported with cloned Vip3Aa fragments using fluorescence-based cell binding assays with Sf9 cells (Jiang et al., 2020). The cloned DI-III and DII-III fragments displayed similar binding to the cells as the full-length Vip3Aa (protoxin), whereas the binding of the cloned DIII or DI-II fragments was less efficient, and no binding was observed for the DIV-V fragment. We have also tested the functional role of the truncated molecules by measuring their toxicity to Sf21 cells. In agreement with the binding data, only the trypsin-activated Vip3Af protein (DI-V) and the truncated molecules maintaining domains I to III were toxic (Fig. 7C), indicating that the binding observed to the cells was functional. The results from the *in vitro* binding to BBMV and the *ex vivo* binding to Sf21 cells, along with the functional analysis (toxicity to Sf21 cells) and results from other authors (Jiang et al., 2020) strongly indicate that domains I to III are critical in maintaining the functional core of the Vip3 proteins. Domain III contains three antiparallel β-sheets that form a β-prism fold strikingly similar to that found in the Cry insecticidal δ-endotoxins (Núñez-Ramírez et al., 2020; Li et al., 1991). Therefore, it is a strong candidate to be the domain interacting with the membrane receptor, either alone or in combination with domain II or domains I+II. Although the C-terminal variable domains IV and V do not seem to play a role in either the *in vitro* and *ex vivo* binding or in the toxicity to Sf21 culture cells, it has been widely demonstrated that mutations in those domains drastically decrease the Vip3A insecticidal activity and that their presence is required for *in vivo* toxicity (Banyuls et al., 2018a; Quan and Ferré, 2019; Gayen et al., 2012; Li et al., 2007; Selvapandiyan et al., 2001; Chi et al., 2017; Soonsanga et al., 2019). Our results point out that these domains are more critical for *in vivo* activity (insecticidal activity) than for *ex vivo* activity (cell toxicity to Sf21 cells). Therefore, further research is needed to determine the role of these domains *in vivo*.

In conclusion, the results in this study provide evidence of the critical role of the N-terminal domains (domains I to III) in the mode of action of Vip3Af and probably all Vip3 proteins. The role of the highly variable C-terminal domains (domains IV and V) remains elusive, though they are required for full toxicity *in vivo*. It is likely that their role *in vivo* is to increase the stability of the tetrameric structure and to bind to glycosylated molecules in the apical membrane to help the Vip3 bind to the

specific functional receptors.

Supplementary material

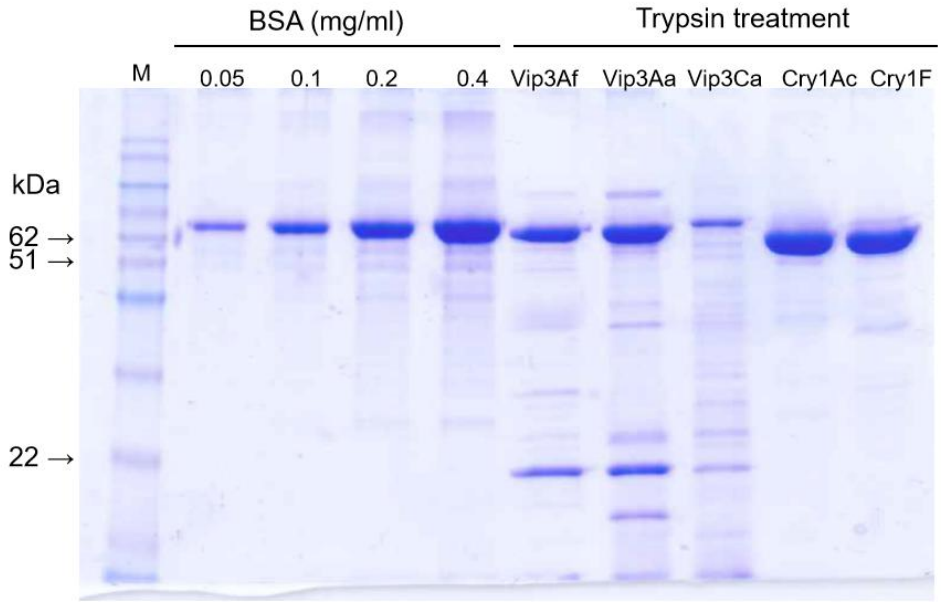


Figure S1. SDS-PAGE analysis of activated Vip3 and Cry proteins used as competitors in binding assays.

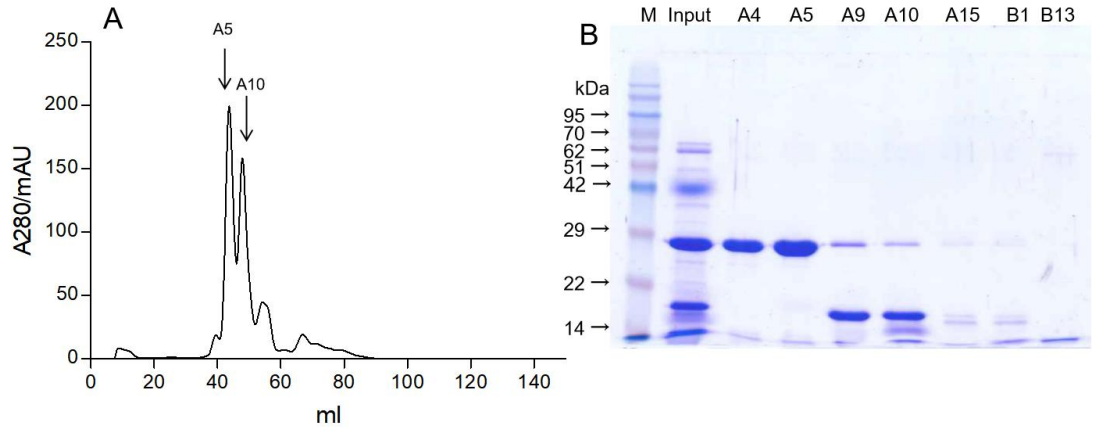


Figure S2. Purification of truncated molecules DIV-V and DIV by anion exchange chromatography in a HiTrap Q HP column (A). SDS-PAGE of the collected peaks from the chromatogram (B).

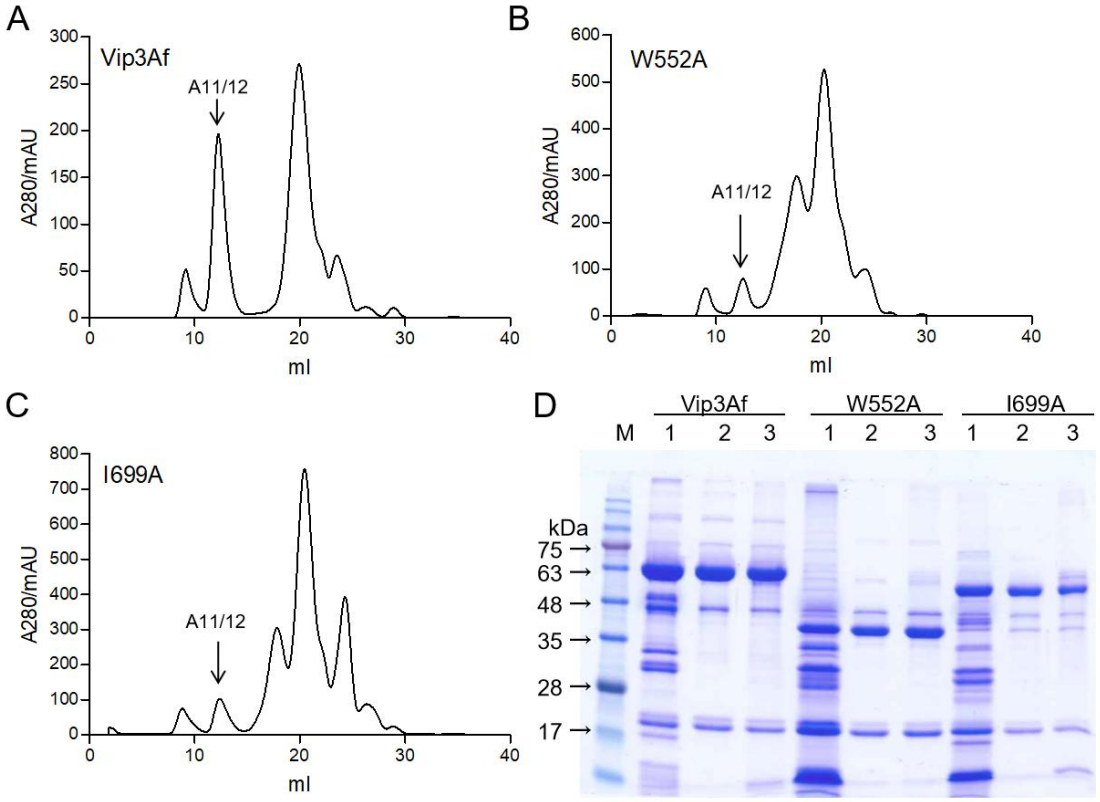


Figure S3. Purification of Vip3Af (A) and truncated molecules DI-III (B) and DI-IV (C) by gel filtration chromatography in a Superdex 200 column. SDS-PAGE of the collected peaks from the chromatograms (D). 1, Input; 2, fraction A11; 3, fraction A12.

Chapter 3. Analyze the mode of action of Vip3A, and explore the reason of the resistance

3.3 The rapid evolution of resistance to Vip3Aa insecticidal protein in *Mythimna separata* (Walker) is not related to alter binding to midgut receptors.

Results are included in:

Quan Y, Yang J, Wang Y, Hernández-Martínez P, Ferré J, He K. 2021. The rapid evolution of resistance to Vip3Aa insecticidal protein in *Mythimna separata* (Walker) is not related to altered binding to midgut receptors. Toxins 13, 364. https://doi.org/10.3390/toxins13050364.

1. Introduction

Oriental armyworm, *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) (Fig. S1) is a polyphagous pest that can feed on more than 300 species, including some important ones, as a staple food, such as corn, rice, and wheat (Jiang et al., 2011; Rashid et al., 2013). It is a major migratory agricultural insect pest in East Asia. Outbreaks of *M. separata* have caused devastating damage to grain production and economic losses (Zeng et al., 2013). For control of this pest, various chemical pesticides, mainly organophosphates and pyrethroids, have been heavily employed, which consequently lead to resistance in field populations, as was reported in Shaanxi and Shanxi Provinces of China (Zhang et al., 2018). *Bacillus thuringiensis* (Bt) insecticidal proteins are a good alternative to synthetic insecticides and their use is being considered in the suppression of this pest (Su et al., 2020). Bt-based insecticides account for 75–95% of the microbial biopesticide market, and Bt crops (genetically modified crops expressing Bt insecticidal proteins) are being commercialized globally (James, 2017). However, as with any other insecticide, field populations of target pests have already developed resistance to Bt insecticides and Bt crops due to their extensive and long-term use (Ferré and Van Rie, 2002; Jurat-Fuentes et al., 2021; Tabashnik et al., 2013).

Thus far, all cases of reported field resistance to Bt insecticides or Bt crops were due to resistance to the Cry proteins (Ferré and Van Rie, 2002; Jurat-Fuentes et al., 2021; Tabashnik et al., 2013). Vip3 proteins are a different class of Bt insecticidal proteins produced during the vegetative growth phase, which do not share sequence homology and binding sites with Cry proteins (Chakroun et al., 2016a). Vip3A proteins have high activity and specificity against lepidopteran pests, and Vip3Aa19 and Vip3Aa20 have been first expressed as single insecticidal proteins in cotton and corn (Chakroun et al., 2016a; Chakrabarty et al., 2020). More recently, Vip3A proteins are being used in the pyramided strategy of combining them with Cry proteins in the same crop to increase activity and delay insect resistance (Carrière et al., 2015). Although practical field resistance to Vip3 proteins has never been reported, a relatively high frequency of Vip3Aa resistant alleles has been detected in Australian populations of *Helicoverpa armigera* and *Helicoverpa punctigera* (Mahon et al., 2012) and in US populations of *Helicoverpa zea* (Yang et al., 2020). Laboratory selection has also rendered Vip3Aa resistance in other insect species such as *Heliothis virescens* (Pickett et al., 2017), *Spodoptera litura* (Barkhade et al., 2010), and *Spodoptera frugiperda* (Bernardi et al., 2016). However, no mechanistic data on field- or laboratory-evolved resistance to Vip3 proteins have ever been reported. Understanding the genetic and biochemical basis of resistance is crucial in the development of strategies to delay or prevent the evolution of resistance.

In this study, we aimed to determine the potential of an important Asian agricultural pest species, *M. separata*, to evolve resistance against three Bt proteins expressed in Bt crops: Vip3Aa, Cry1Ab, and Cry1Fa. Since resistance was rapidly developed only against Vip3Aa, cross resistance against the Cry proteins and Vip3Aa binding to brush border membrane vesicles (BBMV) from larvae midguts were analyzed.

2. Materials and Methods

2.1. Insect Colonies

The laboratory colony of *M. separata* was established with 2500 eggs collected by putting an egg-laying substrate in a cornfield in Gongzhuling (Jilin Province, China) in June 2016. Insects were

reared on an artificial diet at 24 ± 1 ◦C, photoperiod of 14:10 h (L:D), 70–80% RH. After two generations at the insectary (on an artificial diet), abundant eggs hatched (about 3000–5000 larvae) almost on the same day, and neonates were used to start bioassays and selection.

2.2. Source of Toxins

For bioassays and selection, the Vip3Aa19 protein was used, and it was provided by the Beijing DBN Biotech Center (DBNBC, Beijing, China). Trypsin-activated Cry1Ab and Cry1F (98% pure proteins) were produced and shipped by Marianne P. Carey, Case Western Reserve University, USA. The Vip3Aa protein used in the binding assays was Vip3Aa16 (NCBI Accession No. AAW65132) and the *Escherichia coli* clone containing the *vip3Aa16* gene was kindly provided by the Laboratory of Biopesticides, Centre of Biotechnology of Sfax (Sfax, Tunisia).

2.3. Bioassays and Selection Process

Bioassays were performed with an artificial diet (Yang et al., 2018) by the surface contamination method and conducted in 24-well (1.9 cm2) trays that contained approximately 1 mL of diet. Once solidified (about 10–15 min at room temperature), the diet was overlaid with an aqueous solution of Vip3Aa or Cry1 proteins dissolved in phosphate buffer saline (PBS) and allowed to air dry again. About six to nine different concentrations (48 larvae per concentration at generation 1 to 5, and 24 larvae per concentration from generation 6) of the tested proteins were used, which were chosen to produce between 20–90% mortality. One neonate larva (hatched during the previous 12 h) was added per well, covered with a perforated plastic membrane. Trays were maintained at the insectaria for seven days. The bioassay negative control consisted of just PBS; only bioassays in which the mortality in the control insects was less than 16% were considered. The mortality was recorded and judged dead if the larva did not move after repeatedly poked by a brush. Each bioassay was biologically duplicated twice on different dates.

Before starting the selection process, the toxicities of Vip3Aa, Cry1Ab, and Cry1F on *M. separata* (susceptible) were estimated (Table S1). The selection pressure was thus adjusted to 60 µg/g for Vip3Aa19 ($LC_{95–99}$), 60 µg/g for Cry1Ab ($LC_{75–80}$) and 100 µg/g for Cry1F ($LC_{65–72}$), and larvae (3000–5000 per generation and selection line) were maintained for 7–10 days. Larvae that survived and had at least molted to the second instar were gently picked out and transferred to a fresh diet without insecticidal protein until pupation. For each generation, neonates were monitored for susceptibility and subjected to selection, as described above. A sample of the population was maintained without selection to serve as a control, and the insects were reared in the same conditions as those subjected to selection.

2.4. Protein Purification for Binding Analysis

Conditions for bacterial culture and expression of the Vip3Aa16 were described previously (Chakroun and Ferré, 2014). The Vip3Aa16 from the supernatant of an *E. coli* cell lysate was purified using a HisTrap FF affinity purification column (GE Healthcare, UK), as described elsewhere (Hernández-Martínez et al., 2013). Fractions (1 mL) were collected and those containing Vip3Aa were dialyzed against Tris-NaCl buffer (20 mM Tris, 150 mM NaCl, pH 9) overnight and then stored at −80 ◦C until used. The purity was checked by SDS–PAGE, and the concentration of protein was quantified by Bradford (Bradford, 1976) before use.

2.5. Vip3Aa Radiolabeling

HisTrap FF purified Vip3Aa (25 μg) was labeled using 0.3 mCi of 125INa using the chloramine-T method, as previously described (Chakroun and Ferré, 2014; Van Rie et al., 1989). Then, the labeled protein was purified and separated from free 125I by a PD10 desalting column (GE Healthcare, UK). The purity of collected fractions was checked by subjecting an equal radioactivity signal (20,000 cpm) from each fraction to SDS-PAGE with further exposure of the dried gel (52 ◦C, 1 h) to an X-ray film at −20 ◦C. The purest125I-Vip3Aa fraction was used for all the binding assays and stored at 4 ◦C. The estimated specific activity of the labeled protein was 3.9 mCi/mg.

2.6. BBMV Preparation

Fifth instar larvae of *M. separata* from both the susceptible (Ms-S) and the resistant (Ms-R) lines of the eighth and ninth selection generation (>3061-fold resistant) were dissected and the midguts (without the bolus content) in MET buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris, pH 7.5) were immediately frozen in liquid nitrogen and preserved at −80 ◦C until required. BBMV were prepared by the differential magnesium precipitation method (Wolfersberger et al., 1987), frozen in liquid nitrogen, and stored at −80 ◦C. The concentration of BBMV preparations was determined by Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The enrichment of apical membranes in the BBMV preparation was determined by measuring the activities of the apical membrane enzyme leucine aminopeptidase N, which provided an approximately fivefold enrichment.

2.7. Binding Assays with ^{125}I-labeled Vip3Aa

Prior to being used in the binding assays, the Vip3Aa protoxin (both the 125I labeled and the unlabeled sample) was subjected to trypsin activation (trypsin from bovine pancreas, SIGMA T8003, Sigma-Aldrich, St. Louis, MO, USA) at 5% trypsin for 1 h at 37 ◦C. To determine the specific binding, the activated 125I-Vip3Aa (0.39 nM) was incubated for 1 h with different concentrations of *M. separata* BBMV (resuspended in binding buffer) at room temperature in a 100 uL final volume of binding buffer (20 mM Tris, 1 mM MnCl2, 0.1% BSA, pH 7.4). The reaction was stopped by centrifugation at 16,000× *g* for 10 min at 4 ◦C, and the pellet was washed twice with 500 μL of cold binding buffer (with centrifugation of 5 min after each wash). An excess of unlabeled Vip3Aa toxin (390 nM) was added to some samples to calculate the nonspecific binding. The radioactivity retained in the pellet was measured in a model 2480 WIZARD2 gamma counter (PerkinElmer, Downers Grove, IL, USA). The assay was repeated twice.

Homologous competition assays were performed as described above but using a fixed amount of BBMV (0.1 mg/mL) and increasing amounts of unlabeled activated Vip3Aa. The assay was repeated twice. The dissociation constant (*K*d) and the concentration of binding sites (*R*t) were calculated using the LIGAND program (Munson and Rodbard, 1980).

2.8. Statistical Analysis

Response (mortality)-dose data from bioassays were subjected to Probit analysis by the PoLoPlus V 1.0 program (LeOra Software Company, Petaluma, CA, USA) to generate the LC_{50} and LC_{95} values with 95% fiducial limits (FL), chi-square (χ^2) and slope with standard errors (slope ± SE). LC_{50} values were considered significantly different if their 95% FL did not overlap. The level of resistance was expressed as the resistance ratio (RR_{50}) and was calculated by the formula: RR_{50} = (LC_{50} of resistance line)/(LC_{50} of susceptible line).

3. Results

3.1. Response to Selection with Vip3Aa and Cry1 Toxins in *M. separata*

Bioassays with Vip3Aa, Cry1Ab, and Cry1F proteins with a recently established *M. separata* population rendered LC_{50} values of 0.86, 0.14, and 1.45 µg/cm2 for Vip3Aa, Cry1Ab, and Cry1F, respectively (Table S1). Three different lines were selected with Vip3Aa, Cry1Ab, and Cry1F separately. Immediately upon start of the selection regimes, the sensitivity of *M. separata* to Vip3Aa decreased significantly (Table S2). As early as in one or two generations of selection, the LC_{50} value of Vip3Aa was 26.8 and 238 µg/cm2, which was about 31- and 278-fold higher, compared with the susceptible control insects. At the eighth generation of continuous selection, the LC_{50} value was >1684 µg/cm2 , with an at least 3061-fold resistance ratio (RR_{50}) relative to the unselected population (Table 1). On the contrary, in the other two selection lines, no significant change in the susceptibility to the Cry1 proteins (Cry1Ab or Cry1F) was observed during the eight or ten generations of selection (Table 1, Tables S3 and S4). Considering that the laboratory population started with 2500 eggs collected from the field, and at the very least one allele for Vip3Aa resistance had to be present in that sample, we can make an estimate of the frequency of Vip3Aa resistance alleles of 0.0002 (1 in 5000 gene copies). However, because of the rapid increase in resistance in the very early generations of selection, the initial allele frequency must have been higher than this value.

Table 1. Response of *M. separata* to selection with Vip3Aa, Cry1Ab, and Cry1F.

Generation[a]	Protein	LC_{50} (95% FL) µg/cm^2	LC_{95} (95% FL) µg/cm^2	RR_{50} [b]	Slope ± SE	χ^2
Ms-S	Vip3Aa	0.55 (0.26, 1.14)	213 (66, 850)	/[c]	0.71 ± 0.07	7.8
F9	Vip3Aa	>1684	–[d]	>3061	/	/
Ms-S	Cry1Ab	0.14 (0.08, 0.23)	6.65 (3.04, 20.8)	/	0.99 ± 0.11	5.9
F8	Cry1Ab	0.39 (0.20, 0.60)	49 (15, 139)	2.7	0.76 ± 0.08	6.9
Ms-S	Cry1F	1.73 (0.70, 4.25)	271 (55, 8540)	/	0.75 ± 0.10	13.9
F9	Cry1F	2.60 (1.77, 3.69)	60 (35, 125)	1.5	1.29 ± 0.12	5.1

[a] The values of the control population (Ms-S) are referred to as the last generation of selection. [b] RR_{50} = Ms-R LC_{50}/Ms-S LC_{50}. [c] / = Not applicable. [d] – = Impossible to obtain due to the high resistance level.

3.2. Cross-Resistance Evaluation of Vip3Aa-Resistant *M. separata* Strain to Cry1 Proteins

To determine whether the subpopulation that responded to the Vip3Aa selection could have also developed cross resistance to the Cry1 toxins, the Vip3Aa selected insects were tested with Cry1Ab and Cry1F in the ninth generation. The results showed that LC_{50} of Cry1Ab toxin was significantly increased to 0.39 µg/cm2 in the ninth Vip3Aa-selected strain (Ms-R) (95% FL overlap test). However, we did not detect any difference of Cry1F in the Ms-S and Ms-R strain, and the LC_{50} value was about 1.15 µg/cm2 (Table 2).

Table 2. Evaluation of cross resistance to Cry1 proteins in the Vip3Aa-selected population of *M. separata* (ninth generation).

Strains	Proteins	LC_{50} (95% FL) μg/cm^2	RR_{50} [a]	Slope ± SE	χ^2
Ms-S	Cry1Ab	0.14 (0.08, 0.23)	/	0.99 ± 0.11	5.9
	Cry1F	1.95 (1.25, 3.08)	/	0.97 ± 0.11	7.8
Ms-R	Cry1Ab	0.39 (0.24, 0.62)	3	0.93 ± 0.12	4.1
	Cry1F	1.15 (0.93, 2.26)	/	1.15 ± 0.12	10.5

[a] RR_{50} = Ms-R LC_{50}/Ms-S LC_{50}.

3.3. ^{125}I-Vip3Aa Binding to BBMV of Susceptible and Resistant *M. separata*

Specific binding of Vip3Aa to *M. separata* BBMV was shown by incubating a fixed amount of labeled 125I-Vip3Aa with increasing concentrations of BBMV, in the presence or absence of an excess of unlabeled Vip3Aa (Fig. 1a). Around 20% of 125I-Vip3Aa (total binding) used in the assay bound to the BBMV, of which approximately 70% was specific. BBMV from the susceptible and resistant insects showed similar profiles and percent of specific binding, indicating that the resistance of Vip3Aa was not due to an absence of binding to the epithelial membrane. This result was further confirmed by performing competition binding assays (Fig. 1b). Again, very similar curves were obtained giving estimated equilibrium dissociation constants (*K*d) and concentration of binding sites (*R*t) very similar and non-significantly different (Table 3).

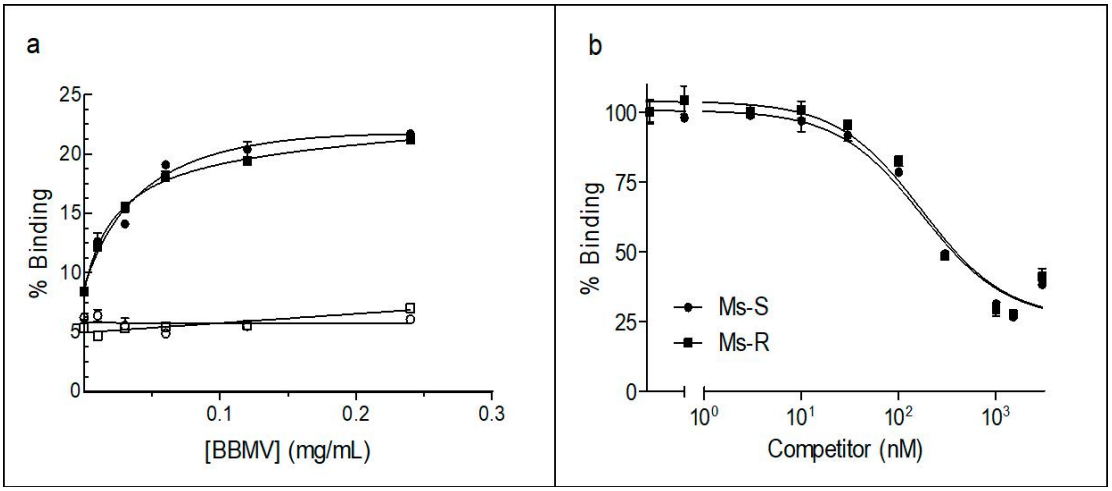


Figure 1. Binding of ^{125}I-Vip3Aa (0.39 nM) to the susceptible Ms-S (circles) and the resistant Ms-R (squares) *M. separata* BBMVs: (**a**) total (full symbols) and nonspecific binding (open symbols) at increasing concentrations of BBMVs. The nonspecific binding was estimated in the presence of an excess of unlabeled Vip3Aa (390 nM); (**b**) binding of ^{125}I-Vip3Aa at increasing concentrations of unlabeled Vip3Aa with 0.1 mg/mL BBMVs.

Table 3. Equilibrium dissociation constant (K_d) and concentration of binding sites (R_t) of Vip3Aa with BBMVs from susceptible and resistant *M. separata*.

Insects	K_d (nM)	R_t (pmol/mg)
Ms-S	40 ± 6	75 ± 22
Ms-R	41 ± 6	79 ± 17

4. Discussion

Monitoring resistance is important for the widespread and long-term use of commercial adoption of Bt crops since it provides information on how likely is that insect pest populations can evolve resistance to the insecticidal proteins. Resistance to Vip3Aa has been reported in several species (Mahon et al., 2012; Yang et al., 2020; Pickett et al., 2017; Barkhade et al., 2010; Bernardi et al., 2016), in all cases, after laboratory selection of field populations. In the present study, we subjected three subpopulations of *M. separata* to laboratory selection with Vip3Aa, Cry1Ab, or Cry1F proteins, all of them already expressed in Bt corn and highly effective against corn pests (Carrière et al., 2015; Chang et al., 2007; He et al., 2003; Buschman et al., 2001; Siebert et al., 2008). Only Vip3Aa showed a rapid response to selection, whereas the Cry1 proteins were unable to elicit such a response. A similar rapid response to Vip3Aa selection was also found in *H. virescens*, reaching a >2300-fold level of resistance at the tenth generation (Pickett et al., 2015). This rapid evolution of selection under laboratory conditions is in contrast with results obtained with Cry1 proteins, such as in this work and others. A Cry1Ab-resistant population of *Ostrinia furnacalis* (Guenée) acquired around 100-fold resistance level only after 35 generations of selection (Xu et al., 2010) and an *Ostrinia nubilalis* population developed more than 3000-fold resistance to Cry1F after 35 generations of selection (Pereira et al., 2011). This difference in response to selection, in addition to reflecting a much higher frequency of resistance alleles for Vip3Aa, may suggest differences in the mechanisms of resistance to Vip3Aa and Cry1 proteins. Our results also detected no significant cross resistance to the Cry1 proteins in the Vip3Aa-selected line of *M. separata*.

Many studies have reported that the alteration of membrane receptors is a common evolutionary mechanism conferring high levels of resistance to Cry proteins, such as mutations in the aminopeptidases N, cadherin, or ABC transporters that serve as putative receptors for Cry proteins (Ferré and Van Rie, 2002; Jurat-Fuentes et al., 2021). However, Vip3 proteins do not share binding sites with Cry proteins (Chakroun et al., 2016a; Chakroun and Ferré, 2014; Kahn et al., 2018; Sena et al., 2009; Gouffon et al., 2011). Although some proteins (from Sf9 or Sf21 cells) have been identified to bind the Vip3Aa protein and thus to serve as receptors (Singh et al., 2010; Jiang et al., 2018a, b), their relationship with Vip3A resistance has never been established. Nevertheless, specific binding of Vip3 proteins to lepidopteran BBMV has been shown and it is generally accepted that binding to specific receptors is the basis for the specificity of Vip3 proteins (Kahn et al., 2015; Lee et al., 2003; Lee et al., 2006). Here, we have tested the binding of 125I-Vip3Aa to BBMV from susceptible and resistant *M. separata* insects to see whether we could find differences that could explain resistance. The lack of qualitative or quantitative binding differences between susceptible and resistant insects is in line with previous results with other Vip3Aa-resistant strains from other insect species for which Vip3Aa binding differences were not found (Chakroun et al., 2016b; Pinos et al., 2021). Slower activation of Vip3Aa by midgut juice of *H. armigera* larvae has been shown in Vip3Aa-resistant insects, though because of the small differences with the susceptible insects, it did not seem to be the main reason for resistance (Chakroun et al., 2016b). Additionally, Vip3Aa-resistant *H. virescens* larvae showed dramatically reduced levels of membrane-bound alkaline phosphatase, but its involvement in resistance could not be demonstrated (Pinos et al., 2020). Therefore, unlike for Cry proteins, altered binding to membrane receptors seems not to be the main mechanism of resistance to Vip3Aa proteins, and other mechanisms should be explored (Pinos et al., 2021). Other steps in the mode of action of Vip3Aa, either prior (such as protease activation or peritrophic matrix sequestration) or after (pore forming,

signal transduction, apoptosis, mitochondria disruption, etc.) binding to the membrane should be responsible for blocking the toxic action of the protein.

In conclusion, the biochemical basis of resistance to Vip3A proteins is still unknown and deserves further study. Additionally, alleles for Vip3Aa resistance seem to be relatively common, and thus, the use of Vip3A proteins alone, without combining them with Cry proteins, is not an appropriate strategy for the long-term implementation of this technology in pest control. The reported synergistic action of some combinations of Vip3Aa and Cry1 proteins (Yang et al., 2018; Lemes et al., 2014) also favors the combined use of these two types of insecticidal proteins for better and most long-term use of the Bt-crops technology.

Supplementary Materials

Table 3.3-S1. The susceptibility of collected *M. separarta* against Vip3A and Cry1 toxins.

Toxins	Methods[1]	LC_{50} (95%FL)	LC_{95} (95%FL)	Slope±SE	χ^2
Vip3Aa	μg/g	1.64 (0.55, 3.53)	35 (17, 148)	1.24 ± 0.13	10.0
	μg/cm^2	0.86 (0.29, 1.86)	185 (90, 779)	1.24 ± 0.15	11.1
Cry1Ab	μg/g	6.54 (2.44, 12.00)	289 (104, 3002)	0.96 ± 0.12	6.6
	μg/cm^2	0.16 (0.06, 0.29)	6.89 (2.49, 71.5)	0.60 ± 0.14	6.6
Cry1F	μg/g	26.1 (17.6, 37.5)	4700 (2043, 15403)	0.20 ± 0.03	12.5
	μg/cm^2	1.71 (1.16, 2.46)	308 (134, 1010)	0.73 ± 0.07	10.0

Note: [1] method: "μg/g" (toxin/diet) was performed by directly mixed the toxin and diet described previously [36]; "μg/cm^2"was the quantitative surface contamination assays.

Table 3.3-S2. Response of *M. separata* to selection with Vip3Aa.

Generation	LC_{50} (95%FL) μg/cm^2	LC_{95} (95%FL) μg/cm^2	RR_{50} [a]	Slope±SE	χ^2
Ms-S	0.86 (0.29, 1.86)	185 (90, 779)	/	1.24 ± 0.15	11.1
F1	26.8 (5.5, 84.7)	>4736	31	0.73 ± 0.16	4.4
F2	239 (106, 977)	>100000	278	0.69 ± 0.11	17.9
Ms-S	0.81 (0.45, 1.42)	206 (76, 880)	/	0.68 ± 0.07	7.8
F5	224.9 (86.4, 816.8)	>500000	277	0.48 ± 0.10	3.7
F6	>320	/	>400	/	/
F7	>1052	/	>1200	/	/
Ms-S	0.55 (0.26, 1.14)	213 (66, 850)	/	0.71 ± 0.07	7.8
F9	>1684	/	>3061	/	/

Table 3.3-S3. Response of *M. separata* to selection with Cry1Ab.

Generation	LC_{50} (95%FL) μg/cm^2	LC_{95} (95%FL) μg/cm^2	RR_{50} [a]	Slope±SE	χ^2
Ms-S	0.16 (0.06, 0.29)	6.89 (2.49, 71.5)	/	0.60 ± 0.14	6.6
F1	0.38 (0.17, 0.73)	91 (19, 2954)	2.5	0.69 ± 0.11	6.0
F2	0.19 (0.11, 0.30)	18.2 (7.9, 68.8)	1.2	0.83 ± 0.07	28.7

Ms-S	0.21 (0.15, 0.29)	3.25 (1.87, 7.78)	/	1.38 ± 0.60	4.8
F6	0.18 (0.12, 0.26)	4.69 (2.49, 12.04)	0.9	1.16 ± 0.13	0.6
Ms-S	0.11 (0.03, 0.25)	14.2 (4.04, 178)	/	0.78 ± 0.12	13.0
F7	0.28 (0.18, 0.42)	16.6 (7.43, 54.0)	2.5	0.93 ± 0.09	7.4
Ms-S	0.14 (0.08, 0.23)	6.65 (3.04, 20.8)	/	0.99 ± 0.11	5.9
F8	0.39 (0.20, 0.60)	49 (15, 139)	2.7	0.76 ± 0.08	6.9

Table 3.3-S4. Response of *M. separata* to selection with Cry1F.

Generation	**LC_{50} (95%FL) μg/cm²**	**LC_{95} (95%FL) μg/cm²**	**RR_{50} [a]**	**Slope±SE**	χ^2
Ms-S	1.71 (1.16, 2.46)	308 (134, 1010)	/	0.73 ± 0.07	10.0
F2	2.06 (0.66, 4.64)	236 (58, 6240)	1.2	0.80 ± 0.11	9.9
F4	5.63 (3.13, 12.8)	937 (174, 38381)	3.3	0.74 ± 0.14	6.8
Ms-S	1.45 (0.32, 3.67)	62 (158, 9022)	/	0.63 ± 0.12	9.1
F7	10.6 (4.4, 18.3)	435 (160, 4375)	7.3	1.02 ± 0.22	0.6
F8	2.99 (1.82, 4.77)	134 (61, 440)	2.1	0.99 ± 0.10	10.6
Ms-S	1.73 (0.70, 4.25)	271 (55, 8540)	/	0.75 ± 0.10	13.9
F9	2.60 (1.77, 3.69)	60 (35, 125)	1.5	1.29 ± 0.12	5.1

[a] RR_{50} = Ms-R LC_{50}/Ms-S LC_{50}

Figure S1. *Mythimna separata* larvae and adult.

General discussion

Bacillus thuringiensis (*Bt*) is the most economically successful entomopathogen to date. The use of its insecticidal Cry proteins has been popular and efficient to control pests in agriculture in the past decade. One of the most important biotechnology to pest control is the expression of *Bt* genes in transgenic crops as plant-incorporated protectants. Benefits driving adoption of Bt crops include high efficacy, improved health safety due to lower contamination with mycotoxins (in maize), and greater gross margins with higher yields. With long-term commercial use of Cry proteins, target pests would respond this pressure by evolving resistance. To delay this resistance, the second generation GM crops expres two or more pyramided *Bt* genes to provide superior resistance management options and increase the potential life expectancy of the biotech plants (Bates et al., 2005). The vegetative insecticidal proteins (Vip), particularly Vip3 proteins, share no sequence homology with Cry proteins and no cross-resistance has been found among them since they have different modes of action. Vip3A proteins have been regarded as interesting candidates to complement Cry proteins in Bt crops to broaden the insecticidal spectrum. To understand the insecticidal Vip3 proteins mechanism, our lab has identified several critical amino acids positions affecting toxicity by using the alanine scanning methodology (Banyuls et al., 2018a). In this thesis I have investigated interaction between Vip3 and Cry proteins, analyzed the effect of these critical amino acids (of Vip3Af) in the structure and function, and identified the Vip3A domains involved in the specific binding to the membrane receptors.

Interactions of the combinations of Vip3 and Cry proteins.

In this chapter, we mainly investigated the interaction between Vip3 and Cry proteins in toxicity. Bioassays in *Mythimna separata* showed the LC_{50} values of Vip3Aa16, Vip3Aa19 and Vip3Ca proteins did not show any significant difference. In previous studies, the Vip3Ca protein revealed low efficacy against *S. frugiperda*, *S. littoralis*, *S.exigua* and *O. nubilalis* compared with Vip3Aa proteins (Palma et al., 2012; Hernández-Martínez et al., 2013). According to both the LC_{50} and LC_{95} values, Vip3Ca could be recommended in controlling *M. separata.* The toxicities of Cry proteins showed Cry1Ab/Cry1Ac/Cry2Aa > Cry1Fa/Cry1Ah/Cry2Ab > Cry1Ie, against this pest. The interaction between Vip3 and Cry proteins was detected by the synergism observed between Vip3 and Cry proteins (from 3-9 fold). Synergism was found in the group Cry1Fa+Vip3Aa16, Cry1Ie+Vip3Aa16, Cry1Ah+Vip3Aa16; no antagonism was observed for any group. A number of investigations have confirmed that synergism and antagonism may occur between Vip3 and Cry proteins (Lemes et al., 2014; Bergamasco et al., 2013; Figueiredo et al., 2019). This suggests that combination of these two types of proteins would confer, to Bt crops, a better and maybe broader target species control, favoring the pest resistance management.

In a later study we tested the susceptibility to Vip3Aa and Vip3Ca in Cry1A-, Cry2A-, Dipel- and Vip3-resistant insect colonies from different species to determine whether resistance to other insecticidal proteins confers cross-resistance to Vip3 proteins. The results showed that there were no cross-resistance to Vip3Aa and Vip3Ca in Cry1Ac-resistant *Trichoplusia ni*, Cry1Ab-resistant *Ostrinia furnacalis*, Cry2Ab-resistant colonies of *T. ni* and *H. armigera*, and Dipel-resistant *Plodia interpunctella,* strains. Despite the slight effects on the LC50 value or growth inhibition, by both Vip3Aa and Vip3Ca, observed in the resistant strains (either Cry1A or Cry2Ab resistant) compared to the susceptible insects, the fiducial limits (95%) were overlapping. These results are in agreement to

those of previous studies showing the lack of cross-resistance between Vip3 and Cry proteins in different species (Fang et al., 2007; Jackson et al., 2007; Mahon et al., 2007, 2012; Chakroun et al., 2016b; Pickett et al., 2017). In chapter 3.3, we also selected and obtained the Vip3Aa resistance (>3601 fold) strain in *M. separata* only after 8 generations of selection, and found no significant cross-resistance to Cry1Ab or Cry1F. Similar results were reported in the Vip3Aa resistance *H. virescens* that showed no cross-resistance to Cry1Ab and Cry1Ac (Pickett et al., 2017). In contrast, there was strong cross-resistance between Vip3Ca and Vip3Aa in *H. armigera* (Vip3Aa-resistant strain).

In addition, unlike to other species (Palma et al., 2012; Hernández-Martínez et al., 2013), *O. furnacalis* (both susceptible and Cry1Ab-resistant strains), showed no susceptibility to Vip3Aa, and only about 50% growth inhibition was observed at 100 μg/g. However, Vip3Ca was highly active to *O. furnacalis* larvae from both strains, susceptible and Cry1Ab-resistant. The LC_{50} value of Vip3Ca for the susceptible strain was comparable to that of Cry1Ab, one of the most toxic Cry proteins against this pest. A slightly significant increase of LC_{50} of Vip3Ca in Cry1Ab-resistant *O. furnacalis* was detected compared with the susceptible strain, but it was only around 3-fold.

Several studies demonstrated that the binding sites play critical roles in the Cry resistance mechanism, but Vip3Aa does not share binding sites with Cry1 or Cry2 proteins (Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014). Our results about the lack of cross-resistance between Vip3 and Cry proteins are in agreement with the differences in the mode of action of them. The Vip3Aa and Vip3Ca share binding sites in *Mamestra brassicae* (Gomis-Cebolla et al., 2017) and *S. frugiperda* (Kahn et al., 2018) which is probably the reason of the cross-resistance between Vip3 proteins.

In conclusion, our results supported the lack of cross-resistance between Vip3 and Cry proteins and the occurrence of cross-resistance between Vip3Ca and Vip3Aa. In addition, the synergism effect of the combined use of the Cry1Ab/Vip3Aa16 (or Cry1F/Vip3Aa16) pair in *M. separata*, and the rapid evolution of Vip3Aa resistance *M. separata* (though not to Cry1Ab or Cry1F) suggested that the strategy of application Vip3 proteins (or genes) was better in combination with Cry proteins in the modern agriculture.

Analysis of critical amino acid positions in Vip3Af for the structure and insecticidal activity

Banyuls defined six proteolytic patterns (pattern “a” to “f”) of ala-mutants with strongly impaired insecticidal activity located at three clusters (Banyuls et al., 2018a). In this chapter, we started with these critical ala-mutants to figure out the how these critical amino acids affected the structure of the protein. We identified the SDS-PAGE bands from the ala-mutants, giving characteristic patterns generated after trypsin activation, and identified the position of the fragments in the primary structure of the Vip3Af protein. Our peptide fingerprint results of fragments of 17, 27, and 38 (or 35) kDa, taking into account the tryptic sites in the sequence of Vip3Af, allowed us to define their position in the sequence of the protein. This information, along with that of Banyuls et al. (Banyuls et al., 2018a), allowed us to propose a map of the Vip3Af protein with five domains. Domain I ranges amino acids (aa) 12–198, domain II aa199–313, domain III aa314–526, domain IV aa527–668, and domain V aa669–788. The domain limits proposed were further confirmed by the 3D structure of Vip3Aa and Vip3B (Núñez-Ramírez et al., 2020; Zheng et al., 2020).

The results from gel filtration chromatography of the ala-mutants revealed that mutants rendering patterns “b”, “c”, and “e” are found forming a tetramer, both as protoxins and also after trypsin

treatment. It was further confirmed that these trypsin-treated patterns ("b", "c", and "e") were either missing domain V or domain IV and V. Therefore, just domains I-III were necessary to form the tetrameric structure. Moreover, in the trypsin-digested patterns "d" or "f" no tetramer was observed since they lack domains I-III.

The critical ala-mutants of Vip3Af affected not only the protein structure (chapter 3.1) but also the insecticidal activity against *S. frugiperda* and *Agrotis segetum* (Banyuls et al., 2018a). In this chapter, we focus on these critical residues again, and further seek the functional group of these critical amino acids in the structure and insecticidal activity. Twelve mutations were build up based on theoretically favorable amino acid changes, but only 10 mutations were successfully cloned and stably expressed. The toxicities revealed that merely a significant increase in the insecticidal activity of M34L (Met^{34} to Leu^{34}) against *Spodoptera littoralis* was observed from all the 10 stable mutations, though not against *Spodoptera frugiperda* and *Grapholita molesta.*

In this chapter, we showed that domains I-III of Vip3Af are necessary to maintain the integrity of the tetramer, that the side chain of the aspartic acid at Glu^{483} (at domain III) and the aromatic residue Trp^{552} (at domain IV) are pivotal to insecticidal activity.

Analysis of the shared binding sites and identification of binding domains of Vip3 proteins, and explore their possible involvement in the Vip3A resistance.

Vip3 protoxin has been demonstrated susceptible to proteolytic cleavage by digestive enzymes present in the host digestive fluids once ingested (Abdelkefi-Mesrati et al., 2011; Caccia et al., 2014; Sellami et al., 2015). A trypsin-like primary cleavage site (aa 198/199) of Vip3Aa was identified and yielded two bands of 65 and 20 kDa in SDS-PAGE after incubation with midgut juice (or trypsin) (Bel et al., 2017). The results obtained herein showed that an apparent degradation in the SDS-PAGE also occurs in the Vip3Af protein after being treated with *S. frugiperda* midgut juice, and progressively the emergence of the 65 kDa fragment in the SDS-PAGE over time was not observed at high concentrations. This apparently degraded sample retained full toxicity against *S. frugiperda*, something that suggested that this overdegradation observed by SDS-PAGE might be not reflecting what happened *in vivo*. As explained by Bel et al., the over-digestion probably was an artefact from the preparation of the sample before SDS-PAGE, and that the protein structure was broken by heat and then further digested by proteases acting on previously non accessible sites (Bel et al., 2017). This assumption was confirmed by a Vip3Af sample apparently over-digested by midgut juice was subjected to gel filtration chromatography, the results revealed that Vip3Af had been only cleaved into two main fragments of 65 kDa and 20 kDa which co-eluted in a peak corresponding to a high molecular weight species. This phenomenon accounts for the differential size separation of Vip3Af and the midgut juice proteases, which have lower molecular weights (around 25 kDa and 27 kDa for chymotrypsin and trypsin, respectively). In addition, both the protoxin and the activated Vip3Af elutes in size exclusion chromatography as a tetramer, which rendered two bands (65 and 20 kDa) by SDS-PAGE. This result confirmed that the primary cleavage by trypsin or *S. frugiperda* midgut juice does not affect the tetrameric conformation and that the 65 and 20 kDa fragments are maintained strongly associated. All these results were further confirmed by the publication of the 3D structure of Vip3Aa, which demonstrated that this proteolytic cleavage is necessary to induce the change of the Vip3Aa protoxin into the activation conformation (Núñez-Ramírez et al., 2020).

Recently, although it is still not clear how Vip3 proteins reach at the membrane of insect midgut cell (Lepidoptera), binding of Vip3A proteins to this membrane has been observed *in vivo* and *in vitro*

(Lee et al., 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Gouffon et al., 2011; Chakroun and Ferré, 2014). In addition, according to the 3D structure of Vip3Aa and Vip3Bc, a rational mode of action of Vip3 proteins was proposed that the protease digestion of Vip3 proteins (or activation) is required for conformational changes (or remodeling), possibly in conjunction with the factors (mostly the binding to receptors), that lead to the formation of a four-helix coiled coil among the N-terminal domain of the four monomers, permeate the lipid bilayer (Núñez-Ramírez et al., 2020; Byrne et al., 2021). A new specific binding condition of Vip3Af was developed by removing the sodium chloride from the binding buffer previously published for Vip3Aa binding (Chakroun and Ferré, 2014). Specific binding of Vip3Af to *S. exigua* and *S. frugiperda* larvae BBMV suggested both the 19 kDa and 65 kDa fragments bind specifically. Competition experiments indicated that Vip3Af do not have shared binding sites with Cry1, Vip3C is not able to compete for all Vip3Af binding sites, and Vip3Aa competed for all Vip3Af binding sites. The results are in agreement with previous reports that showed the lack of shared binding sites between Cry1 and Vip3Aa, and shared binding sites among Vip3 proteins (Chakroun and Ferré, 2014; Gomis-Cebolla et al., 2017; Kahn et al., 2018).

Several different truncated molecules with a different domain composition were generated from the critical Vip3Af ala-mutants that had been shown to affect oligomerization and the insecticidal activity in *S. frugiperda* (Chapter 3.1). The competition binding assays with BBMV revealed that the truncated molecules containing domains I to III were able to compete for binding with ^{125}I-Vip3Af, whereas domains IV or IV/V were not. These results were confirmed by labeling the DI-III truncated molecule (^{125}I-DI-III), and only those molecules retaining domains I to III were able to compete with the ^{125}I-DI-III molecule for BBMV binding. As with ^{125}I-Vip3Af, Vip3Af and Vip3Aa completely displaced binding of the ^{125}I-DI-III truncated molecule, whereas Vip3Ca competed only partially.

In addition, specific binding of the ^{125}I-DI-III molecule to Sf21 cells was also observed, and those molecules retaining domains I to III showed toxicity against Sf21 cells. In a previous study, the cloned DI-III and DII-III of Vip3Aa fragments displayed similar binding to the Sf9cells than the full-length Vip3Aa (protoxin), and no binding was observed for the DIV-V fragment (Jiang et al., 2020).

Domain III contains three antiparallel β-sheets (from 3D structure) that form a β-prism fold (domain II of Cry toxins) strikingly similar to that found in the Cry insecticidal δ-endotoxins (domain II of CryA toxins) (Núñez-Ramírez et al., 2020; Li et al., 1991). Domain IV (of Vip3Bc) (or CBM) shares a twisted β-sheet "jelly roll" topology with Cry1A toxins (domain III of Cry1A) (Byrne et al., 2021). These two domains are strong candidates to be interacting with the membrane receptor. Our results indicated that domain III of Vip3, either alone or in combination with domain II or domains I+II, also play a critical role in interacting with the membrane receptor, but the C-terminal (domains IV and V) do not seem to play a role in either the *in vitro* and *ex vivo* binding. However, it has been widely demonstrated that domains IV and V presence is required for *in vivo* toxicity (Banyuls et al., 2018a; Quan and Ferré, 2019; Chi et al., 2017; Soonsanga et al., 2019). A previous study that Tyr^{776} (domain V) of Vip3Aa64 was important to retain the activity against *S. exigua* during high temperature storage (Soonsanga et al., 2019). Therefore, in addition to binding to the membrane, there are other processes relevant to domains IV/V (such as the stability), in the mode of action of Vip3 proteins *in vivo*.

The alteration of membrane receptors is a common evolutionary mechanism conferring high levels of resistance to Cry proteins (Ferré and Van Rie, 2002; Jurat-Fuentes et al., 2021). Also, specific binding of Vip3 proteins to lepidopteran BBMV has been shown and it is generally accepted that binding to specific receptors is the basis for the specificity of Vip3 proteins (Kahn et al., 2018; Lee et al., 2003; Lee et al., 2006). However, no qualitative or quantitative binding differences of Vip3Aa

between susceptible and Vip3Aa-resistant *M. separata* (> 3061 fold) were detected. The analysis of binding difference of Vip3Aa-resistant strains of *H. armigera* and *H. virescens* also revealed the altered binding to membrane receptors seems not to be the main mechanism of resistance to Vip3Aa proteins (Chakroun et al., 2016b; Pinos et al., 2020). Taken together, unlike for Cry proteins, altered binding to membrane receptors seems not to be the main mechanism of resistance to Vip3Aa proteins. So the Vip3A-resistant mechanism must reside in other step of the mode of action (such as proteolysis, peritrophic matrix sequestration, post-binding events, etc.).

In conclusion, the results of this thesis are important and essential to establish the strategy to delay the growing number of cases of Cry resistance and to guide the pyramiding strategy in *Bt*-crops (expressing *vip3* and *cry* genes). The identification of binding domains (DI-III of Vip3Af) contributes to define the role of the structural domains of Vip3 proteins.

Conclusion

1. Significant synergism interactions were detected combining Vip3Aa16 with Cry1 toxins (Cry1Ab/Vip3Aa16; Cry1Fa/Vip3Aa16; Cry1Ie/Vip3Aa16; Cry1Ah/Vip3Aa16) (with factors ranging from 2 to 9) .

2. No cross-resistance between Cry and Vip3 proteins were observed, in contrast, the strong cross-resistance to Vip3Ca was detected in the Vip3 resistant strains.

3. Five domains of Vip3Af were mapped: domain I ranges amino acids (aa) 12–198, domain II aa199–313, domain III aa314–526, domain IV aa527–668, and domain V aa669–788.

4. A tetrameric conformation of Vip3Af was detected in protoxin or activated form, Moreover, the tetramer was also observed for that proteins lacking domain IV and V. In contrast, no tretrameric form was observed when lacking domain I, II, or III.

5. Residue change of Met^{34} to Lys^{34} (M34L, M to L) significantly increased the toxicity *S. littoralis*, and critical position 483 required an acidic residue, and position 552 an aromatic residue plays an important role in the Vip3Af structure and toxicity.

6. An established proteolytic process of Vip3A was digested to two strongly associated fragments of approximately 20 kDa and 65 kDa (as tetramer) and that are no further processed even at high peptidase concentrations.

7. In *Spodoptera* spp. Vip3Af protein shares all the binding sites with Vip3Aa, partial sites with Vip3Ca, it does not share sites with Cry1 proteins.

8. The truncated proteins, domain I-III of Vip3Af is sufficient to compete all the ^{125}I-Vip3Af to *Spodoptera* spp. BBMVs *in vitro*, whereas domain IV/V is not.

9. Domain I-III (or domain I-IV) as toxic as trypsin-activated Vip3Af (domain I-V) in Sf21 cells, but non-toxic *in vivo* (*S. frugiperda* or *S. exigua*), suggesting the potential discrepancy of the mode of action of Vip3 proteins *in vivo* and *ex vivo*.

10. A rapid Vip3Aa resistance strain was selected in *M. separata*, only after 8 generation, which not observed in Cry1Ab or Cry1F. And this Vip3Aa resistance mechanism was not related to the binding to the receptors.

References

- Abdelkefi-Mesrati L, Boukedi H, Chakroun M, Kamoun F, Azzouz H, Tounsi S, Rouis S, Jaoua S. 2011. Investigation of the steps involved in the difference of susceptibility of *Ephestia kuehniella* and *Spodoptera littoralis* to the *Bacillus thuringiensis* Vip3Aa16 toxin. J Invertebr Pathol 107, 198–201.
- Abdelkefi-Mesrati L, Boukedi H, Dammak-Karray M, SellamiBoudawara T, Jaoua S, Tounsi S. 2011. Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*. J Invertebr Pathol 106, 250–254.
- Abdelkefi-Mesrati L, Rouis S, Sellami S, Jaoua S. 2009. *Prays oleae* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3LB differs from that of Cry1Ac toxin. Mol Biotechnol 43, 15–19.
- Abulreesh HH, Osman GEH, Assaeedi ASA. 2012. Characterization of insecticidal genes of *Bacillus thuringiensis* strains isolated from arid environments. Indian J Microbiol 52, 500 –503.
- Adamczyk JJ, Mahaffey JS. 2008. Efficacy of Vip3a and Cry1Ab transgenic traits in cotton against various lepidopteran pests. Fla Entomol 91, 570–575.
- Adang M, Crickmore N, Jurat-Fuentes JL. 2014. Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In: Dhadialla TS, Gill S (Eds.), Advances in Insect Physiology, Insect Midgut and Insecticidal Proteins, vol. 47. Academic Press, San Diego, CA, pp. 39–87.
- Alexandratos N, Bruinsma J. 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working Paper 12-03.
- Andrews RE, Bibilos MM, Bulla LA. 1985. Protease activation of the entomocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki.* Appl Environ Microbiol 50, 737–742.
- Anilkumar KJ, Rodrigo-Simón A, Ferré J, Pusztai-Carey M, Sivasupramaniam S, Moar WJ. 2008. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). Appl Environ Microbiol 74, 462–469.
- Arthurs S, Dara SK. 2019. Microbial biopesticides for invertebrate pests and their markets in the United States. J Invertebr Pathol 165, 13–21.
- Asensio JL, Ardá A, Ca˜nada FJ, Jiménez-Barbero J. 2013. Carbohydrate-aromatic interactions. Acc Chem Res 46, 946–954.
- Asokan R, Swamy HM, Arora DK. 2012. Screening, diversity and partial sequence comparison of vegetative insecticidal protein (*vip3A*) genes in the local isolates of *Bacillus thuringiensis* Berliner. Curr Microbiol 64, 365–370.
- Atsumi S, Miyamoto K, Yamamoto K, Narukawa J, Kawai S, Sezutsu H, Kobayashi I, Uchino K, Tamura T, Mita K, Kadono-Okuda K, Wada S, Kanda K, Goldsmith MR, Noda H. 2012. Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, *Bombyx mori*. PNAS 109, E1591–98.
- Attathom T. 2002. Biotechnology for insect pest control, Chap. 2, Biotechnology for sustainable bioproduction. Proc Sat Forum Sustainable agricultural system in Asia, Nagoya.
- Azevedo JL, Maccheroni W Jr, Pereira JO, Araújo WL. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron J Biotechnol 3, 40–65.

- Banyuls N, Hernández-Martínez P, Quan Y, Ferré J. 2018b. Artefactual band patterns by SDS-PAGE of the Vip3Af protein in the presence of proteases mask the extremely high stability of this protein. Int J Biol Macromol 120, 59–65.
- Banyuls N, Hernández-Rodríguez CS, Van Rie J, Ferré J. 2018a. Critical amino acids for the insecticidal activity of Vip3Af from *Bacillus thuringiensis*: Inference on structural aspects. Sci Rep 8, 7539.
- Banyuls N, Quan Y, González-Martínez RM, Hernández-Martínez P, Ferré J. 2020. Effect of substitutions of key residues on the stability and the insecticidal activity of Vip3Af from *Bacillus thuringiensis*. J Invertebr Pathol 11, 107439.
- Baranek J, Kaznowski A, Konecka E, Naimov S. 2015. Activity of vegetative insecticidal proteins Vip3Aa58 and Vip3Aa59 of *Bacillus thuringiensis* against lepidopteran pests. J Invertebr Pathol 130, 72–81.
- Barkhade UP, Thakare AS. 2010. Protease mediated resistance mechanism to Cry1C and Vip3A in *Spodoptera litura*. Egypt Acad J Biol Sci 3, 43–50.
- Bates SL, Zhao J, Roush RT, Shelton AM. 2005. Insect resistance management in GM crops: past, present and future. Nat Biotechnol 23, 57–62.
- Baxter SW, Badenes-Pérez FR, Morrison A, Vogel H, Crickmore N, Kain W, Wang P, Heckel DG, Jiggins CD. 2011. Parallel evolution of *Bacillus thuringiensis* toxin resistance in Lepidoptera. Genetics 189, 675–679.
- Baxter SW, Zhao JZ, Gahan LJ, Shelton AM, Tabashnik BE, Heckel DG. 2005. Novel genetic basis of field-evolved resistance to Bt toxins in *Plutella xylostella*. Insect Mol Biol 14, 327–34.
- Beard CE, Court L, Boets A, Mourant R, Van Rie J, Akhurst RJ. 2008. Unusually high frequency of genes encoding vegetative insecticidal proteins in an Australian *Bacillus thuringiensis* collection. Curr Microbiol 57, 195–199.
- Bel Y, Banyuls N, Chakroun M, Escriche B, Ferré J. 2017. Insights into the structure of the Vip3Aa insecticidal protein by protease digestion analysis. Toxins 9, 131.
- Bel Y, Siqueira HA, Siegfried BD, Ferré J, Escriche B. 2009. Variability in the *cadherin* gene in an *Ostrinia nubilalis* strain selected for Cry1Ab resistance. Insect Biochem Mol Biol 39, 218–223.
- Bell RA, Owens CD, Shapiro M, Tardif JR. 1981. Development of mass-rearing technology. In: Doane CC, McManus, ML (Eds.), The gypsy moth: research toward integrated pest management. U.S. Dep. Agric., Washington, DC. pp. 599–633.
- Ben Hamadou-Charfi D, Boukedi H, Abdelkefi-Mesrati L, Tounsi S, Jaoua S. 2013. *Agrotis segetum* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 differs from that of Cry1Ac toxin. J Invertebr Pathol 114, 139–143.
- Berezin C, Glaser F, Rosenberg J, Paz I, Pupko T, Fariselli P, Casadio R, Ben Tal N. 2004. ConSeq: the identification of functionally and structurally important residues in protein sequences. Bioinformatics 20, 1322–1324.
- Bergamasco VB, Mendes DR, Fernandes OA, Desidério JA, Lemos MV. 2013. *Bacillus thuringiensis* Cry1Ia10 and Vip3Aa protein interactions and their toxicity in *Spodoptera* spp. (Lepidoptera). J Invertebr Pathol 112, 152–158.
- Bernardi O, Amado D, Sousa RS, Segatti F, Fatoretto J, Burd AD, Omoto C. 2014. Baseline Susceptibility and Monitoring of Brazilian Populations of *Spodoptera frugiperda* (Lepidoptera:

Noctuidae) and *Diatraea saccharalis* (Lepidoptera: Crambidae) to Vip3Aa20 Insecticidal Protein. J Econ Entomol 107, 781–790.

- Bernardi O, Bernardi D, Amado D, Sousa RS, Fatoretto J, Medeiros FCL, Conville J, Burd T, Omoto C. 2015. Resistance risk assessment of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and *Diatraea saccharalis* (Lepidoptera: Crambidae) to Vip3Aa20 insecticidal protein expressed in corn. J Econ Entomol 108, 2711–19.
- Bernardi O, Bernardi D, Horikoshi RJ, Okuma DM, Miraldo LL, Fatoretto J, Medeiros FCL, Burd T, Omoto C. 2018. Selection and characterization of resistance to the Vip3Aa20 protein from *Bacillus thuringiensis* in *Spodoptera frugiperda*. Pest Manag Sci 72, 1794–1802.
- Bernardi O, Bernardi D, Ribeiro RS, Okuma DM, Salmeron E, Fatoretto J, Medeiros FCL, Burd T, Omoto C. 2015. Frequency of resistance to Vip3Aa20 toxin from *Bacillus thuringiensis* in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) populations in Brazil. Crop Prot 76, 7–14.
- Berry C, Crickmore N. 2016. Structural classification of insecticidal proteins – towards an *in silico* characterisation of novel toxins. J Invertebr Pathol 142, 16-22.
- Betts MJ, Russell RB. 2003. In: Bioinformatics for Geneticists. John Wiley & Sons, Ltd, Chichester, UK, pp. 289–316.
- Bhalla R, Dalal M, Panguluri SK, Jagadish B, Mandaokar AD, Singh AK, Kumar PA. 2005. Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis*. FEMS Microbiol Lett 243, 467–472.
- Boaventura D, Ulrich J, Lueke B, Bolzan A, Okuma D, Gutbrod O, Grbei S, Zeng Q, Dourado PM, Martinelli S, Flagel L, Head G, Nauen R. 2020. Molecular characterization of Cry1F resistance in fall armyworm, *Spodoptera frugiperda* from Brazil. Insect Biochem Mol Biol 116, 103280.
- Boonserm P, Davis P, Ellar DJ, Li J. 2005. Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J Mol Biol 348, 363–382.
- Boonserm P, Mo M, Angsuthanasombat C, Lescar J. 2006. Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. J Bacteriol 188, 3391–3401.
- Boonyos P, Trakulnalueamsai C, Rungrod A, Chongthammakun S, Promdonkoy B. 2020. Antagonistic effect of truncated fragments of *Bacillus thuringiensis* Vip3Aa on the larvicidal activity of its full-length protein. Protein Peptide Left 32586243.
- Boraston AB, Bolam DN, Gilbert HJ, Davies GJ. 2004. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382, 769–781.
- Bordo D, Argos P. 1991. Suggestions for “safe” residue substitutions in site-directed mutagenesis. J Mol Biol 217, 721–729.
- Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of dye-binding. Anal Biochem 72, 248-254.
- Bravo A, Gill, SS, Soberón M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49, 423–435.
- Bravo A, Gómez I, Conde J, Munoz-Garay C, Sanchez J, Miranda R, Zhuang M, Gill SS, Soberón M. 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim Biophys Acta 1667, 38–46.

- Bulla LA Jr, Bechtel DB, Kramer KJ, Shethna YI. 1980. Ultrastructure, physiology, and biochemistry of *Bacillus thuringiensis*. CRC Crit Rev Microbiol 8, 147–203.
- Buntin GD, Lee RD, Wilson DM, Mcpherson RM. 2001. Evaluation of yieldgard transgenic resistance for control of fall armyworm and corn earworm (Lepidoptera: Noctuidae) on corn. Fla Entomol 81, 37–42.
- Burgess EPJ. 1987. Population dynamics of *Mythimna separata* and its parasitoid, *Cotesia ruficrus*, on maize in New Zealand. N Z J Agric Res 30, 203–208.
- Burkness EC, Dively G, Patton T, Morey AC, Hutchison WD. 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions: implications for resistance management. GM Crops 1, 337–343.
- Buschman L, Sloderbeck P, Witt M. 2001. Efficacy of Cry1F corn for the control of southwestern corn borer and corn earworm, 2000. Arthropod Manage. Tests 26, M2.
- Byrne MJ, Iadanza MG, Perez MA, Maskell DP, George1 RM, Hesketh EL, Beales PA, Zack MD, Berry C, Thompson RF. 2021. Cryo-EM structures of an insecticidal Bt toxin reveal its mechanism of action on the membrane. Nat Commun 12, 2791.
- Caccia S, Chakroun M, Vinokurov K, Ferré J. 2014. Proteolytic processing of *Bacillus thuringiensis* Vip3A proteins by two *Spodoptera* species. J Insect Physiol 67, 76–84.
- Caccia S, Di Lelio I, La Storia A, Marinelli A, Varricchio P, Franzetti E, Banyuls N, Tettamanti G, Casartelli M, Giordana B, Ferré J, Gigliotti S, Ercolini D, Pennacchio F. 2016. Midgut microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism. Proc Natl Acad Sci 113, 201521741.
- Caccia S, Hernández-Rodríguez CS, Mahon RJ, Downes S, James W, Bautsoens N, Van Rie J, Ferré J. 2010. Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2A insecticidal proteins in two Helicoverpa species. PLoS One 5, e9975.
- Cannon RJC. 1995. *Bacillus thuringiensis* in pest control. In Hokkanen HMT, Lynch JM (Eds). Biological Control: Benefits and Risks. Cambridge University Press, UK, pp. 190–197.
- Carrière Y, Crickmore N, Tabashnik BE. 2015. Optimizing pyramided transgenic Bt crops for sustainable pest management. Nat Biotechnol 33, 161–168.
- Carrière Y, Fabrick JA, Tabashnik BE. 2016. Can pyramids and seed mixtures delay resistance to Bt Crops? Trends Biotechnol 34, 291-302.
- Carvalho FP. 2006. Agriculture, pesticides, food security and food safety. Environ Sci Policy 9, 685–692.
- Cerda R, Avelino J, Gary C, Tixier P, Lechevallier E, Allinne C. 2017. Primary and secondary yield losses caused by pests and diseases: assessment and modeling in coffee. PloS One 12, e0169133.
- Chakrabarty S, Jin M, Wu C, Chakraborty P, Xiao Y. 2020. *Bacillus thuringiensis* vegetative insecticidal protein family Vip3A and mode of action against pest Lepidoptera. Pest Manag Sci, 76, 1612–1617.
- Chakroun M, Banyuls N, Bel Y, Escriche B, Ferré J. 2016a. Bacterial vegetative insecticidal proteins (Vip) from entomopathogenic bacteria. Microbiol Mol Biol Rev 80, 329–350.
- Chakroun M, Banyuls N, Walsh T, Downes S, James B and Ferré J. 2016b. Characterization of the resistance to Vip3Aa in *Helicoverpa armigera* from Australia and the role of midgut processing and receptor binding. Sci Rep 6, 24311.

- Chakroun M, Bel Y, Caccia S, Abdelkefi-Mesrati L, Escriche B, Ferré J. 2012. Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein. J Invertebr Pathol 110, 334–339.
- Chakroun M, Ferré J. 2014. *In vivo* and *in vitro* binding of Vip3Aa to *Spodoptera frugiperda* midgut and characterization of binding sites by ^{125}I radiolabeling. Appl Environ Microbiol 80, 6258–6265.
- Chandler D, Davidson G, Grant WP, Greaves J, Tatchell GM. 2008. Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability. Trends Food Sci Technol 19, 275–283.
- Chang X, Chang XY, He KL, Wang ZY, Bai SX. 2007. Resistance evaluation of transgenic Bt maize to oriental armyworm. Acta Phytophyl Sin 34, 225–228.
- Chang XQ, Shu Z, Liang L. 2015. Insight into the ultrastructure of antennal sensilla of *Mythimna separata* (Lepidoptera: Noctuidae). J Insect Sci 15, 124.
- Chen J, Yu J, Tang L, Tang M, Shi Y, Pang Y. 2003. Comparison of the expression of *Bacillus thuringiensis* full-length and N-terminally truncated vip3A gene in *Escherichia coli*. J Appl Microbiol 95, 310–316.
- Chen WB, Lu GQ, Cheng HM, Liu CX, Xiao YX, Xu C, Shen ZC, Wu KM. 2017. Transgenic cotton coexpressing Vip3A and Cry1Ac has a broad insecticidal spectrum against lepidopteran pests. J Invertebr Pathol 149, 59–65.
- Chi B, Li H, Zhang J, Wei P, Gao J, Liu R. 2019. Correction to: In silico structurebased identification and validation of key residues of Vip3Aa involving in lepidopteran brush border receptor binding. Appl Biochem Biotechnol 187, 1581.
- Chi B, Luo G, Zhang J, Sha J, Liu R, Liu T, Gao J. 2017. Effect of C-terminus site-directed mutations on the toxicity and sensitivity of *Bacillus thuringiensis* Vip3Aa11 protein against three lepidopteran pests. Biocontrol Sci Techn 27: 1363-1372.
- Coates BS, Siegfried BD. 2015. Linkage of an ABCC transporter to a single QTL that controls *Ostrinia nubilalis*, larval resistance to the *Bacillus thuringiensis*, Cry1Fa toxin. Insect Biochem Mol Biol 63, 86–96.
- Coates BS, Sumerford DV, Siegfried BD, Hellmich RL, Abel CA. 2013. Unlinked genetic loci control the reduced transcription of aminopeptidase N 1 and 3 in the European corn borer and determine tolerance to *Bacillus thuringiensis* Cry1Ab toxin. Insect Biochem Mol Biol 43, 1152–1160.
- Crickmore N, Baum J, Bravo A, Lereclus D, Narva K, Sampson K, Schnepf E, Sun M. Zeigler D.R. 2016. *Bacillus thuringiensis* toxin nomenclature. http://www.btnomenclature.info/.
- Crickmore N, Berry C, Panneerselvam S, Mishra R, Connor TR, Bonning BC. 2020. A structure-based nomenclature for *Bacillus thuringiensis* and other bacteriaderived pesticidal proteins. J Invertebr Pathol. https://doi.org/10.1016/j.jip.2020.107438.
- Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62, 807–813.
- Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A., Dean, D.H., 2013. *Bacillus thuringiensis* toxin nomenclature. http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/.

- De A, Bose R, Kumar A, Mozumdar S. 2014. Targeted delivery of pesticides using biodegradable polymeric nanoparticles. Fundam Appl Control Release Drug Deliv. 629–639.
- De Barjac H, Bonnefoi A. 1968. A classification of strains of *Bacillus thuringiensis* Berliner with a key to their differentiation. J Invertebr Pathol 11, 335–347.
- de Maagd RA, Bakker P, Staykov N, Dukiandjiev S, Stiekema W, Bosch D. 1999a. Identification of *Bacillus thuringiensis* delta-endotoxin Cry1C domain III amino acid residues involved in insect specificity. Appl Environ Microbiol 65, 4369–4374.
- de Maagd RA, Bakker PL, Masson L, Adang MJ, Sangadala S, Stiekema W, Bosch D. 1999b. Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol Microbiol 31, 463–471.
- de Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu Rev Genet 37, 409–433.
- Debost-Legrand A, Warembourg C, Massart C, Chevrier C, Bonvallot N, Monfort C, Rouget F, Bonnet F, Cordier S. 2016. Prenatal exposure to persistent organic pollutants and organophosphate pesticides, and markers of glucose metabolism at birth. Environ Res 146, 207–217.
- Derbyshire DJ, Ellar DJ, Li J. 2001. Crystallization of the *Bacillus thuringiensis* toxin Cry1Ac and its complex with the receptor ligand Nacetyl-D-galactosamine. Acta Crystallogr Sect D 57, 1938–1944.
- Deutsch CA, Tewksbury JJ, Tigchelaar M, Battisti DS, Merrill SC, Huey RB, Naylor RL. 2018. Increase in crop losses to insect pests in a warming climate. Science 361, 916–919.
- Devine GJ, Furlong MJ. 2007. Insecticide use: contexts and ecological consequences. Agric Human Values 24, 281–306.
- Dong F, Shi RP, Zhang SS, Zhan T, Wu GB, Shen J, Li Z. 2012. Fusing the vegetative insecticidal protein Vip3Aa7 and the N terminus of Cry9Ca improves toxicity against *Plutella xylostella* larvae. Appl Microbiol Biot 96, 921-929.
- Dong F, Zhang S, R. Shi, S. Yi, F. Xu, Z. Liu. 2012. Ser-substituted mutations of Cys residues in Bacillus thuringiensis Vip3Aa7 exert a negative effect on its insecticidal activity. Curr Microbiol 65, 583–588.
- Donovan WP, Engleman JT, Donovan JC, Baum JA, Bunkers GJ, Chi DJ, Clinton WP, English L, Heck GR, Ilagan OM, Krasomil-Osterfeld KC, Pitkin JW, Roberts JK, Walters MR. 2006. Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. Appl Microbiol Biotechnol 72, 713–719.
- Doss VA, Kumar KA, Jayakumar R, Sekar V. 2002. Cloning and expression of the vegetative insecticidal protein (*vip3V*) gene of *Bacillus thuringiensis* in *Escherichia coli*. Protein Expr Purif 26, 82–88.
- Dow JA. 1984. Extremely high pH in biological systems: a model for carbonate transport. Am J Phys 246, R633–636.
- Drake V, Gatehouse A. (Eds.) 1995. Insect migration in relation to weather and climates. In *Insect Migration: Tracking Resources through Space and Time*; Cambridge University Press: London, UK, pp. 93–150.
- EFSA. 2012. Panel on Genetically Modified Organisms, Scientific opinion on application (EFSA-GMO-DE-2010-82) for the placing on the market of insect-resistant genetically modified

maize MIR162 for food and feed uses, import and processing under regulation (EC) no 1829/2003 from syngenta, EFSA J. 10, 2756. https://doi.org/10.2903/j.efsa.2012.2756.

- Endo H, Azuma M, Adegawa S, Kikuta S, Sato R. 2017. Water influx via aquaporin directly determines necrotic cell death induced by the *Bacillus thuringiensis* Cry toxin. FEBS Lett 591, 56–64.
- English L, Readdy TL. 1989. Delta endotoxin inhibits a phosphatase in midgut epithelial membranes of *Heliothis virescens*. Insect Biochem 19, 145–152.
- EPA (Environmental Protection Agency), 2017. Biopesticides, https://www.epa.gov/pesticides/biopesticides.
- EPA, 2017. Pesticides Industry Sales and Usage 2008 – 2012 Market estimates.
- EPPO Global Database. Available online: https://gd.eppo.int/taxon/PSEDSE/distribution (accessed on 20 August 2018).
- Esker P, Savary S, McRoberts N. 2012. Crop loss analysis and global food supply: focusing now on required harvests. CAB Rev 7, 1–14.
- Estela A, Escriche E, Ferré J. 2004. Interaction of *Bacillus thuringiensis* toxins with larval midgut binding sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae). Appl Environ Microbiol 70,1378–1384.
- Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. P Natl Acad Sci USA 93, 5389–5394.
- Estruch JJ, Yu CG. September 2001. Plant pest control. US patent 6,291,156 B1. 58.
- EU Pesticides database – European Commission [WWW Document], n.d. URL http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN (accessed 3.18.17).
- Fabrick JA, Mathew LG, Tabashnik BE, Li X. 2011. Insertion of an intact CR1 retrotransposon in a cadherin gene linked with Bt resistance in the pink bollworm, *Pectinophora gossypiella*. Insect Mol Biol 20, 651–665.
- Fabrick JA, Ponnuraj J, Singh A, Tanwar RK, Unnithan GC, Yelich AJ, Li X, Carrie`re Y, Tabashnik BE. 2014. Alternative splicing and highly variable cadherin transcripts associated with field-evolved resistance of pink bollworm to Bt cotton in India. PLoS ONE 9, e97900.
- Fang J, Xu X, Wang P, Zhao JZ, Shelton AM, Cheng J, Feng MG, Shen Z. 2007. Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl Environ Microbiol 73, 956 –996.
- FAO (UN Food Agric. Organ.). 2009. Global agriculture towards 2050. High Level Expert Forum, FAO, Rome, Italy.
- Federici BA, Park HW, Sakano Y. 2006. Insecticidal protein crystals of *Bacillus thuringiensis*. In part Microbiol Monogr, pp. 195–236.
- Ferré J, Van Rie J, MacIntosh SC. 2008. Insecticidal genetically modified crops and insect resistance management (IRM), p41-86. In Romeis J, Shelton AM, Kennedy GG (ed), Integration of insect-resistant genetically modified crops within IPM programs. Springer Science Business Media BV, Dordrecht, Netherlands.
- Ferré J, Van Rie J. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 47, 501–533.

- Figueiredo CS, Lemes ARN, Sebastiao I, Desiderio JA. 2019. Synergism of the *Bacillus thuringiensis* Cry1, Cry2, and Vip3 Proteins in *Spodoptera frugiperda* Control. Biotechnol Appl Biochem 188, 798–809.
- Fiuza LM, Polanczyk RA, Crickmore N. 2017a. *Bacillus thuringiensis* and *Lysinibacillus sphaericus* Characterization and use in the field of biocontrol. In Chapter 1: *Bacillus thuringiensis* Characterization: Morphology, Physiology, Biochemistry, Pathotype, Cellular, and Molecular Aspects. Springer Nature, Switzerland, pp. 3–5.
- Fiuza LM, Polanczyk RA, Crickmore N. 2017b. *Bacillus thuringiensis* and *Lysinibacillus sphaericus* Characterization and use in the field of biocontrol. In Chapter 3: *Bacillus thuringiensis* Toxin Classification. Springer Nature, Switzerland, pp. 41–52.
- Fiuza LM. 2001. *Bacillus thuringiensis*: características e potencial no manejo de insetos. Acta Biol Leopoldensia 23, 141–156.
- Flannagan RD, Yu CG, Mathis JP, Meyer TE, Shi X, Siqueira HA, Siegfried BD. 2005. Identification, cloning and expression of a Cry1Ab cadherin receptor from European corn borer, *Ostrinia nubilalis* (Hubner) (Lepidoptera: Crambidae). Insect Biochem Mol Biol 35, 33–40.
- Florez AM, Osorio C, Alzate O. 2012. Protein engineering of *Bacillus thuringiensis* δ-endotoxins. In: Sansinenea E. (Eds.), *Bacillus thuringiensis* Biotechnology. Springer Netherlands, Netherlands, pp. 93–113.
- Franco-Rivera A, Benintende G, Cozzi J, Baizabal-Aguirre VM, Valdez-Alarcón JJ, López-Meza JE. 2004. Molecular characterization of *Bacillus thuringiensis* strains from Argentina. Antoine Van Leeuwenhoek 86, 87–92.
- Frankenhuyzen KV. 2013. Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. J Invertebr Pathol 114, 76–85.
- Gahan LJ, Gould F, Heckel DG. 2001 Identification of a gene associated with Bt resistance in *Heliothis virescens*. Science 293, 857–860.
- Gahan LJ, Pauchet Y, Vogel H, Heckel DG. 2010. An ABC transporter mutation is correlated with insect resistance to *Bacillus thuringiensis* Cry1Ac toxin. PLoS Genet 6, e1001248.
- Galitsky N, Cody V, Wojtczak A, Ghosh D, Luft JR, Pangborn W, English L. 2001. Structure of the insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. Acta Crystallogr Sect D 57, 1101–1109.
- Garczynski SF, Adang MJ. 1995. *Bacillus thuringiensis* CryIA(c) δ-endotoxin binding aminopeptidase in the *Manduca sexta* midgut has a glycosyl-phosphatidylinositol anchor. Insect Biochem Mol Biol 25, 409–415.
- Gayen S, Hossain MA, Sen SK. 2012. Identification of the bioactive core component of the insecticidal Vip3A toxin peptide of *Bacillus thuringiensis*. J Plant Biochem Biotechnol 21, S128–S135.
- Gayen S, Samant MK, Hossain MA, Mandal CC, Sen SK. 2015. A deletion mutant ndv200 of the *Bacillus thuringiensis* vip3BR insecticidal toxin gene is a prospective candidate for the next generation of genetically modified crop plants resistant to lepidopteran insect damage. Planta 242, 269–281.
- Gazit E, La Rocca P, Sansom MSP, Shai Y. 1998. The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* δ-endotoxin are consistent with an “umbrella-like” structure of the pore. Proc Natl Acad Sci U S A 95, 12289–12294.

- Ge AZ, Pfister RM, Dean DH. 1991. Functional domains of *Bacillus thuringiensis* insecticidal crystal proteins: refinement of *Heliothis virescens* and *Trichoplusia ni* specificity domains on CryIA(c). J Biol Chem 266, 17954–17958.
- Gill S, Cowles EA, Francis V. 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* CryIAc toxin-binding protein from the midgut of the lepidopteran insect *Heliothis virescens*. J Biol Chem 270, 27277–27282.
- Girard F, Vachon V, Prefontaine G, Marceau L, Schwartz JL, Masson L, Laprade R. 2009. Helix alpha 4 of the *Bacillus thuringiensis* Cry1Aa toxin plays a critical role in the post-binding steps of pore formation. Appl Environ Microbiol 75, 359–365.
- Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J, Marrone P, Morin L, Stewart A. 2012. Have biopesticides come of age? Trends Biotechnol 30, 250–258.
- Gómez I, Pardo-López L, Muñoz-Garay C, Fernández LE, Pérez C, Sénchez J, Soberón M, Bravo A. 2007. Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. Peptides 28, 169–173.
- Gomis-Cebolla J, Ferreira Dos Santos R, Wang Y, Caballero J, Caballero P, He K, Jurat-Fuentes J, Ferré J. 2020. Domain shuffling between Vip3Aa and Vip3Ca: Chimera stability and insecticidal activity against European, American, African and Asian pests. Toxins (Basel) 12, 99.
- Gomis-Cebolla J, Ruiz de Escudero I, Vera-Velasco NM, Hernandez-Martinez P, Hernandez-Rodriguez CS, Ceballos T, Palma L, Escriche B, Caballero P, Ferré J. 2017. Insecticidal spectrum and mode of action of the *Bacillus thuringiensis* Vip3Ca insecticidal protein. J Invertebr Pathol 142, 60–67.
- Gomis-Cebolla J, Wang Y, Quan Y, He K, Walsh T, James B, .Downes S, Kain W, Wang P, Leonard K, Morgan T, Oppert B, Ferré J. 2018. Analysis of cross-resistance to Vip3 proteins in eight insect colonies, from four insect species, selected for resistance to *Bacillus thuringiensis* insecticidal proteins. J Invertebr Pathol 155, 64–70.
- González-Cabrera J, García M, Hernández-Crespo P, Farinós GP, Ortego F, Castañera P. 2013. Resistance to Bt maize in *Mythimna unipuncta* (lepidoptera: Noctuidae) is mediated by alteration in Cry1Ab protein activation. Insect Biochem Mol Biol 43, 635–643.
- Gouffon C, Van Vliet A, Van Rie J, Jansens S, Jurat-Fuentes JL. 2011. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl Environ Microbiol 77, 3182–3188.
- Granero F, Ballester V, Ferre J. 1996. *Bacillus thuringiensis* crystal proteins Cry1Ab and Cry1Fa share a high affinity binding site in *Plutella xylostella* (L.). Biochem Bioph Res Commun 224, 779–783.
- Graser G, Walters FS, Burns A, Sauve A, Raybould A. 2017. A general approach to test for interaction among mixtures of insecticidal proteins which target different orders of insect pests. J Insect Sci 17, 532–536.
- Greene GL, Leppla NC, Dickerson WA. 1976. Velvetbean caterpillar: A rearing procedure and artificial medium. J Econ Entomol 69, 487–488.
- Griffitts JS, Haslam SM, Yang T, Garczynski SF, Mulloy B, Morris H, Cremer PS, Dell A, Adang MJ, Aroian RV. 2005. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science 307, 922–925.

- Grochulski, P, Masson L, Borisova S, Pusztai-Carey M, Schwartz JL, Brousseau R, Cygler C. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. J Mol Biol 254, 447–464.
- Guennelon G, Audemard H, Fremond JC, Abdelmajid El M, FREMOND Mohamed Abdelmajid, JC, EL, Phytiatrie, D.B., RESUMEF econdite, R., 1981. Progresrealises danslelevage permanent du Carpocapse (Laspeyresia pomonella L.) sur milieuartificiel Progres realises danslelevage permanent du Car-pocapse (Laspeyresia pomonella L.) sur milieu artificielA. 84740.
- Guo S, Ye S, Liu Y, Wei L, Xue J, Wu H, Song F, Zhang J, Wu X, Huang D, Rao Z. 2009. Crystal structure of *Bacillus thuringiensis* Cry8Ea1: an insecticidal toxin toxic to underground pests, the larvae of *Holotrichia parallela*. J Struct Biol 168, 259–266.
- Guo Z, Kang S, Chen D, Wu Q, Wang S, Xie W, Shu X, Baxter S, Zhou X, Jurat-Fuentes JL, Zhang Y. 2015. MAPK signaling pathway alters expression of midgut ALP and ABCC genes and causes resistance to *Bacillus thuringiensis* Cry1Ac toxin in diamondback moth. PLOS Genet 11, e1005124.
- Hannay CL. 1953. Crystalline inclusions in aerobic sporeforming Bacteria. Nature 172, 1004.
- He K, Wang Z, Wen L, Bai S, Ma X, Yao Z. 2005. Determination of baseline susceptibility to Cry1Ab protein for Asian corn borer (Lep., Crambidae). J Appl Entomol 129, 407–412.
- He KL, Wang ZY, Zhou DR, Wen LP, Song YY, Yao ZY. 2003. Evaluation of transgenic Bt corn for resistance to the Asian corn borer (Lepidoptera: Pyralidae). J Econ Entomol 96, 935–940.
- Heckel, DG. 2012. Learning the ABCs of Bt: ABC transporters and insect resistance to *Bacillus thuringiensis* provide clues to a crucial step in toxin mode of action. Pestic Bio Chem Physiol 104, 103–110.
- Hernández CS, Ferré J. 2005. Common receptor for *Bacillus thuringiensis toxins* Cry1Ac, Cry1Fa, and Cry1Ja in *Helicoverpa armigera*, *Helicoverpa zea*, and *Spodoptera exigua*. Appl Environ Microbiol 71, 5627–5629.
- Hernández-Martínez P, Gomis-Cebolla J, Ferré J, Escriche B. 2017. Changes in gene expression and apoptotic response in *Spodoptera exigua* larvae exposed to sublethal concentrations of Vip3 insecticidal proteins. Sci Rep 7, 16245.
- Hernández-Martínez P, Hernández-Rodríguez CS, Van Rie J, Escriche B, Ferré J. 2013. Insecticidal activity of Vip3Aa, Vip3Ad, Vip3Ae, and Vip3Af from *Bacillus thuringiensis* against lepidopteran corn pests. J Invertebr Pathol 113, 78–81.
- Hernández-Rodríguez CS, Boets A, Van Rie J, Ferré J. 2009. Screening and identification of *vip* genes in *Bacillus thuringiensis* strains. J Appl Microbiol 107, 219–225.
- Hernández-Rodríguez CS, Ruiz de Escudero I, Asensio AC, Ferré J, Caballero P. 2013. Encapsulation of the *Bacillus thuringiensis* secretable toxins Vip3Aa and Cry1Ia in *Pseudomonas fluorescens*. Biol Control 66, 159–165.
- Herrero S, Gechev T, Bakker PL, Moar WJ, de Maagd RA. 2005. *Bacillus thuringiensis* Cry1Ca-resistant *Spodoptera exigua* lacks expression of one of four aminopeptidase N genes. BMC Genomics 6, 96.
- Herrero S, Gonzalez-Cabrera J, Ferré J, Bakker PL, de Maagd RA. 2004. Mutations in the *Bacillus thuringiensis* Cry1Ca toxin demonstrate the role of domains II and III in specificity towards *Spodoptera exigua* larvae. Biochem J 384, 507–513.
- Herrero S, Oppert B, Ferré J. 2001. Different mechanisms of resistance to *Bacillus thuringiensis* toxins in the Indianmeal moth. Appl Environ Microbiol 67, 1085–1089.

- Horikoshi RJ, Bernandi D, Bernandi O, Malaquias JB, Okuma DM, Miraldo LL, Amaral FS, Omoto C. 2016. Effective dominance of resistance of *Spodoptera frugiperda* to Bt maize and cotton varieties: implications for resistance management. Sci Rep 10, 34864.
- Hossain DM, Shitomi Y, Moriyama K, Higuchi M, Hayakawa T, Mitsui T, Sato R, Hori H. 2004. Characterization of a novel plasma membrane protein, expressed in the midgut epithelia of *Bombyx mori*, that binds to Cry1A toxins. Appl Environ Microbiol 70, 4604–4612.
- Hou X, Han L, An B, Zhang Y, Cao Z, Zhan Y, Cai X, Yan B, Cai J. 2020. Mitochondria and Lysosomes participate in Vip3Aa induced *Spodoptera frugiperda* Sf9 cell. Toxins 12, 116.
- Huang F, Qureshi JA, Meagher Jr RL, Reisig DD, Head GP, Andow DA, Ni X, Kerns D, Buntin D, Niu Y, Yang F, Dangal V. 2014. Cry1F resistance in fall armyworm *Spopdoptera frugiperda*: single genes versus pyramided Bt maize. Plos One 9, e112958.
- Hurley TM, Secchi S, Babcok BA, Hellmich RL. 2002. Managing the risk of European corn borer resistance to Bt corn. Environ Resour Econ 22, 537–558.
- Iatsenko I, Boichenko I, Somme RJ. 2014. *Bacillus thuringiensis* DB27 produces two novel Protoxins, Cry21Fa1 and Cry21Ha1, which act synergistically against nematodes. Appl Environ Microbiol 80, 3266–3275.
- Iatsenko I, Nikolov A, Sommer RJ. 2014. Identification of distinct *Bacillus thuringiensis* 4A4 nematicidal factors using the model nematodes *Pristionchus pacificus* and *Caenorhabditis elegans*. Toxins 6, 2050–2063.
- Ibargutxi MA, Muñoz D, Escudero IRD, Caballero P. 2008. Interactions between Cry1Ac, Cry2Ab, and Cry1Fa *Bacillus thuringiensis* toxins in the cotton pests *Helicoverpa armigera* (Hübner) and *Earias insulana* (Boisduval). Biol Control 47, 89–96.
- Ibrahim MA, Griko N, Junker M, Bulla LA. 2010. *Bacillus thuringiensis*. A genomics and proteomics perspective. Bioengineered Bugs 1, 31–50.
- ISAA, n.d. ISAA GM Approval Database [WWW Document]. URL http://www.isaaa.org/gmapprovaldatabase/ (accessed 4.24.17).
- ISAAA GM Approval Database. Available online: http://www.isaaa.org/gmapprovaldatabase/default.asp (accessed on 20 September 2018).
- ISAAA. 2017. Global status of commercialized biotech/GM crops in 2017: biotech crop adoption surges as economic benefits accumulate in 22 years. ISAAA Brief No. 53. ISAAA, Ithaca, NY. See http://www.isaaa.org/purchasepublications/itemdescription.asp?ItemType=BRIEFS&Control=IB053-2017.
- Jackson RE, Marcus MA, Gould F, Bradley JR, Van Duyn JW. 2007. Cross-resistance responses of Cry1Ac-selected *Heliothis virescens* (Lepidoptera: Noctuidae) to the *Bacillus thuringiensis* protein Vip3A. J Econ Entomol 100, 180–186.
- Jakka SR, Ferré J, Jurat-Fuentes JL. 2015a. Cry toxin binding sites and their use in strategies to delay resistance evolution. In Bt Resistance: Characterization and Strategies for GM Crops Producing *Bacillus thuringiensis* Toxins., in: Soberón M, Gao Y, Bravo A, Boston: CAB Int, pp. 138–49.
- Jakka SR, Gong L, Hasler J, Banerjee R, Sheets JJ, Narva K, Blanco CA, Jurat-Fuentes JL. 2015b. Field devolved mode 1 fall armyworm resistance to Bt corn associated with reduced Cry1Fa toxin binding and midgut alkaline phosphatase expression. Appl Environ Microbiol 82, 1023–1034.

- James C. 2017. Global status of commercialization of biotech/GM crops in 2017: Biotech crop adoption surges as economic benefits accumulate in 22 years. In ISAAA Brief No. 53, ed. ISAAA, pp. 1–4. Ithaca, NY: ISAAA.
- Jenkins JL, Lee MK, Valaitis AP, Curtiss A, Dean DH. 2000. Bivalent sequential binding model of a *Bacillus thuringiensis* toxin to gypsy moth aminopeptidase N receptor. J Biol Chem 275, 14423–14431.
- Jiang K, Hou X, Han L, Tan T, Cao Z, Cai J. 2018b. Fibroblast growth factor receptor, a novel receptor for vegetative insecticidal protein Vip3Aa. Toxins 10, 546.
- Jiang K, Hou XY, Tan TT, Cao ZL, Mei SQ, Yan B, Chang J, Han L, Zhao A, Cai J. 2018a. Scavenger receptor-C acts as a receptor for *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa and mediates the internalization of Vip3Aa via endocytosis. PLOS Pathog 14, e1007347.
- Jiang K, Mei SQ, Wang TT, Pan JH, Chen YH, Cai J. 2016. Vip3Aa induces apoptosis in cultured *Spodoptera frugiperda* (Sf9) cells. Toxicon 120, 49–56.
- Jiang K, Zhang Y, Chen Z, Wu D, Cai J, Gao X. 2020. Structural and functional insights into the C-terminal fragment of insecticidal Vip3A toxin of *Bacillus thuringiensis*. Toxins 12, 438.
- Jiang XF, Luo LZ, Zhang L, Sappington TW, Hu Y. 2011. Regulation of migration in *Mythimna separata* (Walker) in China: A review integrating environmental, physiological, hormonal, genetic, and molecular factors. Environ Entomol 40, 516–533.
- Jiang YY, Li CG, Zen J, Liu J. 2014. Population dynamics of the armyworm in China: A review of the past 60 years' research. Chin J Appl Entomol 51, 890–898.
- Jiménez-Juárez N, Mun˜oz-Garay C, Gomez I, Saab-Rincon G, Damian-Almazo JY, Gill SS, Sobero´n M, Bravo A. 2007. *Bacillus thuringiensis* Cry1Ab mutants affecting oligomer formation are non-toxic to *Manduca sexta* larvae. J Biol Chem 282, 21222–21229.
- Jin TT, Chang X, Gatehouse AMR, Wang ZY, Edwards MG, He KL. 2014. Down regulation and mutation of a *cadherin* gene associated with Cry1Ac resistance in the Asian corn borer, *Ostrinia furnacalis* (Gueneé). Toxins 6, 2676–2693.
- Jouzani GS, Valijanian E, Sharafi R. 2017. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. Appl Microbiol Biotechnol 101, 2691-2711.
- Jurat-Fuentes JL, Adang MJ. 2001. Importance of Cry1 delta-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). Appl Environ Microbiol 67, 323–329.
- Jurat-Fuentes JL, Adang MJ. 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur J Biochem 271, 3127–3135.
- Jurat-Fuentes JL, Crickmore N. 2017. Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. J Invertebr Pathol 142, 5–10.
- Jurat-Fuentes JL, Heckel DG, Ferré J. 2021. Mechanism of resistance to insecticidal proteins of *Bacillus thuringiensis*. Annu Rev Entomol 66, 121–140.
- Jurat-Fuentes JL, Karumbaiah L, Jakka SR, Ning C, Liu C, Wu K, Jackson J, Gould F, Blanco C, Portilla M, Perera O, Adang M. 2011. Reduced levels of membrane-bound alkaline phosphatase are common to lepidopteran strains resistant to Cry toxins from *Bacillus thuringiensis*. PLoS One 6, e17606.
- Kahn TW, Chakroun M, Williams J, Walsh T, James B, Monserrate J, Ferré J. 2018. Efficacy and resistance management potential of a modified Vip3C protein for control of *Spodoptera frugiperda* in maize. Sci Rep 8, 16204.

- Kain WC, Zhao JZ, Janmaat AF, Myers J, Shelton AM, Wang P. 2004. Inheritance of resistance to *Bacillus thuringiensis* Cry1Ac toxin in a greenhouse-derived strain of cabbage looper (Lepidoptera: Noctuidae). J Econ Entomol 97, 2073–2078.
- Karabörklü S, Azizoglu U, Azizoglu ZB. 2018. Recombinant entomopathogenic agents: a review of biotechnological approaches to pest insect control. World J Microbiol Biotechnol 34, 14.
- Kauer R, Sharma A, Gupta D, Kalita M, Bhatnagar RK. 2014. *Bacillus thuringiensis* toxin, Cry1C interacts with 128HLHFHLP134 region of aminopeptidase N of agricultural pest, *Spodoptera litura*. Process Biochem 49, 688–696.
- Khajuria C, Buschman LL, Chen M-S, Siegfried BD, Zhu KY. 2011. Identification of a novel Aminopeptidase P-like gene (*OnAPP*) possibly involved in Bt toxicity and resistance in a major corn pest (*Ostrinia nubilalis*). PLoS ONE 6, e23983
- Knight PJ, Knowles BH, Ellar DJ. 1994. The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane is aminopepidase N. Mol Microbiol 11, 429–436.
- Knight PJ, Knowles BH, Ellar DJ. 1995. Molecular cloning of an insect Aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. J Biol Chem 270, 17765–17770.
- Knowles BH. 1994. Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. Adv Insect Physiol 24, 275–308.
- Kole RK, Banerjee H, Bhattacharyya A. 2001. Monitoring of market fish samples for endosulfan and hexachlorocyclohexane residues in and around Calcutta. Bull Environ Contam Toxicol 67, 554–559.
- Kouassi LN, Tsuda K, Goto C. 2009. Biological activity and identification of nucleopolyhedroviruses isolated from *Mythimna separata* and *Spodoptera litura* in Japan. Biol Control 54, 537–548.
- Kumar A, Chaturvedi AK, Yadav K, Arunkumar KP, Malyan SK, Raja MP, Kumar R, Khan SA, Yadav KK, Rana KL, Kour D, Yadav N, Yadav AN. 2019a. Fungal phytoremediation of heavy metal-contaminated resources: current scenario and future prospects. In: Yadav AN, Singh S, Mishra S, Gupta A. (Eds.), Recent advancement in white biotechnology through fungi: Volume 3: Perspective for Sustainable Environments. Springer International Publishing, Cham, pp. 437–461.
- Kumar M, Kour D, Yadav AN, Saxena R, Rai PK, Jyoti A, Tomar RS. 2019b. Biodiversity of methylotrophic microbial communities and their potential role in mitigation of abiotic stresses in plants. Biologia 74, 287–308.
- Kunthic K, Watanabe H, Kawano R, Tanaka Y, Promdonkoy B, Yao M, Boonserm P. 2017a. pH regulates pore formation of a protease activated Vip3Aa from *Bacillus thuringiensis*. Biochim Biophys Acta Biomembr 1859, 2234–2241.
- Kunthic T, Surya W, Promdonkoy B, Torres J. Boonserm P. 2017b. Conditions for homogeneous preparation of stable monomeric and oligomeric forms of activated Vip3A toxin from *Bacillus thuringiensis*. Eur Biophys J 46, 257–264.
- Kurtz RW, McCaffery A, O'Reilly D. 2007. Insect resistance management for Syngenta's VipCot transgenic cotton. J Invertebr Pathol 95, 227–230.
- Kurtz RW. 2010. A review of Vip3A mode of action and effects on Bt Cry protein-resistant colonies of lepidopteran larvae. Southwest Entomol 35, 391–394.
- La Merrill M, Emond C, Kim MJ, Antignac JP, Le Bizec B, Clément K, Birnbaum LS, Robert Barouki R. 2013. Toxicological function of adipose tissue: focus on persistent organic pollutants. Environ. Health Perspect 121, 162–169.

- Lacey LA, Grzywacz D, Shapiro-llan DI, Frutos R, Brownbridge M, Goettel MS. 2015. Insect pathogens as biological control agents: back to the further. J Invertebr Pathol 132, 1–41.
- Leahy J, Mendelsohn M, Kough J, Jones R, Berckes N. 2014. Biopesticide oversight and registration at the US Environmental Protection Agency, Gross AD, Coats JR, Duke SO, Seiber JN, Biopesticides: State of the Art and Future Opportunities, pp. 3–18.
- Lee MK, Jenkins JL, You TH, Curtiss A, Son JJ, Adang MJ, Dean DH. 2001. Mutations at the arginine residues in alpha8 loop of *Bacillus thuringiensis* delta-endotoxin Cry1Ac affect toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N. FEBS Lett 497, 108–112.
- Lee MK, Miles P, Chen JS. 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem Biophys Res Commun 339, 1043–1047.
- Lee MK, Rajamohan F, Jenkins JL, Curtiss AS, Dean DH. 2000. Role of two arginine residues in domain II, loop 2 of Cry1Ab and Cry1Ac *Bacillus thuringiensis* delta-endotoxin in toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N. Mol Microbiol 38, 289–298.
- Lee MK, Walters FS, Hart H, Palekar N, Chen JS. 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin. Appl Environ Microbiol 69, 4648–4657.
- Lee MK, Young BA, Dean DH. 1995. Domain III exchanges of *Bacillus thuringiensis* Cry1A toxins affect binding to different gypsy moth midgut receptors. Biochem Biophys Res Commun 216, 306–312.
- Lemes ARN, Davolos CC, Legori PCBC, Fernandes OA, Ferré J, Lemos MVF, Desiderio JA. 2014. Synergism and antagonism between *Bacillus thuringiensis* Vip3A and Cry1 proteins in *Heliothis virescens*, *Diatraea saccharalis* and *Spodoptera frugiperda*. Plos One 9, e107196.
- Li C, Xu N, Huang X, Wang W, Cheng J, Wu K, Shen Z. 2007. *Bacillus thuringiensis* Vip3 mutant proteins: Insecticidal activity and trypsin sensitivity. Biocontrol Sci and Techn 17, 699–708.
- Li J, Derbyshire DJ, Promdonkoy B, Ellar JD. 2001. Structural implications for the transformation of the *Bacillus thuringiensis* δ-endotoxins from water-soluble to membrane-inserted forms. Biochem Soc Trans 29, 571–577.
- Li JD, Carroll J, Ellar DJ. 1991. Crystal structure of insecticidal deltaendotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature 353, 815–821.
- Liao C, Heckel DG, Akhurst R. 2002. Toxicity of *Bacillus thuringiensis* insecticidal proteins for *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae), major pests of cotton. J Invertebr Pathol 80, 55–63.
- Liu CX, Xiao YT, Li XC, Oppert B, Tabashnik BE, Wu KM. 2014. Cis-mediated down-regulation of a trypsin gene associated with Bt resistance in cotton bollworm. Sci Rep 4, 7219.
- Liu J, Song F, Zhang J, Liu R, He K, Tan J, Huang D. 2007. Identifi-cation of *vip3A*-type genes from *Bacillus thuringiensis* strains and characterization of a novel vip3A-type gene. Lett Appl Microbiol 45, 432–438.
- Liu JG, Yang AZ, Shen XH, Hua BG, Shi GL. 2011. Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation. J Invertebr Pathol 108, 92–97.
- Liu YJ, Liu J, Ying SH, Liu SS, Feng MG. 2013. A fungal insecticide engineered for fast *Per Os* killing of caterpillars has high field efficacy and safety in full-season control of cabbage insect pests. Appl Environ Microbiol 79, 6452–6458.

- Loguercio LL, Barreto ML, Rocha TL, Santos CG, Teixeira FF, Paiva E. 2002. Combined analysis of supernatant-based feeding bioassays and PCR as a first-tier screening strategy for Vip-derived activities in *Bacillus thuringiensis* strains effective against tropical fall armyworm. J Appl Microbiol 93, 269–277.
- Lu H, Rajamohan F, Dean DH. 1994. Identification of amino acid residues of *Bacillus thuringiensis* δ-endotoxin CryIAa associated with membrane binding and toxicity to *Bombyx mori*. J Bacteriol 176, 5554–5559.
- Luo K, Sangadala S, Masson L, Mazza A, Brousseau R, Adang MJ. 1997. The *Heliothis virescens* 170-kDa aminopeptidase functions as 'Receptor A' by mediating specific *Bacillus thuringiensis* Cry1A δ-endotoxin binding and pore formation. Insect Biochem Mol Biol 27, 735–743.
- Mahadeva Swamy HM, Asokan R. 2013. *Bacillus thuringiensis* as 'nanoparticles' – a perspective for crop protection. Nanosci. Nanotechnol.-Asia. https://doi.org/10.2174/22106812112029990006.
- Mahon RJ, Downes SJ, James B. 2012. Vip3A resistance alleles exist at high levels in Australian targets before release of cotton expressing this toxin. PLoS One 7, e39192.
- Mahon RJ, Olsen KM, Downes S, Addison S. 2007. Frequency of alleles conferring resistance to the Bt toxins Cry1Ac and Cry2Ab in Australian populations of *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 100, 1844–1853.
- Martínez-Solís M, Pinos P, Endo H, Portugal L, Sato R, Ferré J, Herrero S, Hernández-Martínez P. 2018. Role of *Bacillus thuringiensis* Cry1A toxins domains in the binding to the ABCC2 receptor from *Spodoptera exigua*. Insect Biochem Molec 101, 47-56.
- Martins ES, Monnerat RG, Queiroz PR, Dumas VF, Braz SV, de Souza Aguiar RW, Gomes AC, Sanchez J, Bravo A, Ribeiro BM. 2010. Midgut GPIanchored proteins with alkaline phosphatase activity from the cotton boll weevil (*Anthonomus grandis*) are putative receptors for the Cry1B protein of *Bacillus thuringiensis*. Insect Biochem Mol Biol 40, 138–145.
- Masson L, Lu YJ, Mazza A, Brousseau R, Adang MJ. 1995. The CryIA(c) receptor purified from *Manduca sexta* displays multiple specificities. J Biol Chem 270, 20309–20315.
- Masson L, Tabashnik BE, Liu YB, Schwartz JL. 1999. Helix 4 of the *Bacillus thuringiensis* Cry1Aa toxin lines the lumen of the ion channel. J Biol Chem 274, 31996–32000.
- Mathew LG, Ponnuraj J, Mallappa B, Chowdary LR, Zhang J, Tay WT, Walsh TK, Gordon KHJ, Heckel DG, Downes S, Carriere Y, Li X, Tabashnik BE, Fabrick JA. 2018. ABC transporter mis-splicing associated with resistance to Bt toxin Cry2Ab in laboratory- and field-selected pink bollworm. Sci Rep 8, 13531.
- Mesrati LA, Tounsi S, Jaoua S. 2005a. Characterization of a novel *vip3*-type gene from *Bacillus thuringiensis* and evidence of its presence on a large plasmid. FEMS Microbiol Lett 244, 353–358.
- Mesrati LA, Tounsi S, Kamoun F, Jaoua S. 2005b. Identification of a promoter for the vegetative insecticidal protein-encoding gene vip3LB from *Bacillus thuringiensis*. FEMS Microbiol Lett 247, 101–104.
- Moar WJ, Berry C, Narva KE. 2017. The structure/function of new insecticidal proteins and regulatory challenges for commercialization. J Invertebr Pathol 142, 1–4.
- Monnerat R, Martins E, Macedo C, Queiroz P, Praça L, Soares CM, Moreira H, Grisi I, Sila J, Soberón M. 2015. Evidence of field-evolved resistance of *Spodoptera frugiperda* to Bt corn expressing Cry1F in Brazil that is still sensitive to modified Bt toxins. PLoS ONE 10, e0119544.

- Morin S, Biggs RW, Sisteron MS, Shriver L, Ellers-Kirk C, Higginson D, Holley D, Gahan LJ, Heckel DG, Carriere Y, Dennehy TJ, Brown JK, Tabashnik BE. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc Natl Acad Sci U S A 100, 5004–5009.
- Morse RJ, Yamamoto T, Stroud RM. 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure (Cambridge) 9, 409–417.
- Munson P, Rodbard D. 1980. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107, 220–239.
- Murawska E, Fiedoruk K, Bideshi DK, Swiecicka I. 2013. Complete genome sequence of *Bacillus thuringiensis* subsp. *thuringiensis* strain IS5056, an isolate highly toxic to *Trichoplusia ni*. Genome Announc 1, e00108–13.
- Nagamatsu Y, Koike T, Sasaki K, Yoshimoto A, Furukawa Y. 1999. The cadherinlike protein is essential to specificity determination and cytotoxic action of the *Bacillus thuringiensis* insecticidal CryIAa toxin. FEBS Lett 460, 385–390.
- NCBI, n.d. NCBI C Toolkit Cross Reference – BLOSUM 80 [WWW Document]. URL https ://www.ncbi.nlm.nih.gov/IEB/ToolBox/C_DOC/lxr/source/data/BLOSUM80 (accessed 4.16.17).
- Ni M, Ma W, Wang, X, Gao M, Dai Y, Wei X, Zhang L, Peng Y, Chen S, Ding L, Tian Y, Li J, Wang H, Wang X, Xu G, Guo W, Yang Y, Wu Y, Heuberger S, Tabashnik BE, Zhang T, Zhu Z. 2017. Next generation transgenic cotton: pyramiding RNAi and Bt counters insect resistance. Plant Biotechnol. J. https://doi.org/10.1111/pbi.12709.
- Nielsen MM, Andersen KK, Westh P, Otzen DE, Mascher E, Lundahl P, Almgren M, Pastore A. 2007. Unfolding of β-sheet proteins in SDS. Biophys J 92, 3674–3685.
- Nimsanor S, Srisaisup M, Jammor P, Promdonkoy B, Boonserm P. 2020. Intracellular localization and cytotoxicity of *Bacillus thuringiensis* Vip3Aa against *Spodoptera frugiperda* (Sf9) cells. J Invertebr Pathol 171, 107340.
- Ning CM, Wu KM, Liu CX, Gao YL, Juratfuentes JL, Gao XW. 2010. Characterization of a Cry1Ac toxinbinding alkaline phosphatase in the midgut from *Helicoverpa armigera* (Hu¨bner) larvae. J Insect Physiol 56, 666–672.
- Núñez-Ramírez R, Huesa J, Bel Y, Ferré J, Casino J, Arias-Palomo E. 2020. Molecular architecture and activation of the insecticidal protein Vip3Aa from *Bacillus thuringiensis*. Nat Commun 11, 3974.
- Obata F, Kitami M, Inoue Y, Atsumi S, Yoshizawa Y, Sato R. 2009. Analysis of the region for receptor binding and triggering of oligomerization on *Bacillus thuringiensis* Cry1Aa toxin. FEBS J 276, 5949–5959.
- Oerke EC. 2006. Crop losses to pests. J Agric Sci 144, 31–43.
- Olson S. 2015. An analysis of the biopesticide market now and where it is going. Outlooks Pest Manag. 26, 203–206.
- Omoto C, Bernardi O, Salmeron E, Sorgatto RJ, Dourado PM, Crivellari A, Carvalho RA, Willse A, Martinelli S, Head GP. 2016. Field-evolved resistance to Cry1Ab maize by *Spodoptera frugiperda* in Brazil. Pest Manag Sci 72, 1727–1736.
- Oppert B, Tracey Ellis R, Babcock J. 2010. Effects of Cry1F and Cry34Ab1/35Ab1 on storage pests. J Stored Prod Res 46, 143–148.

- Osman G, Altaf J, Saleh AS, Soltane R, Abulreesh H, Arif I, Ramadan A, Osman Y. 2017. First report of detection of the putative receptor of *Bacillus thuringiensis* toxin Vip3Aa from black cutworm (*Agrotis ipsilon*). Saudi J Biol Sci 25, 441–445.
- Osman GH, Soltane R, Saleh I, Abulreesh HH, Gazi KS, Arif IA, Ramadan AM, Alameldin HF, Osman YA, Idriss M. 2019. Isolation, characterization, cloning and bioinformatics analysis of a novel receptor from black cut worm (*Agrotis ipsilon*) of *Bacillus thuringiensis* Vip3Aa toxins. Saudi J Biol Sci 26, 1078–1083.
- Palma L, Berry C. 2016. Understanding the structure and function of *Bacillus thuringiensis* toxins. Toxicon 109, 1–3.
- Palma L, Hernández-Rodríguez CS, Maeztu M, Hernández-Martínez P, Ruiz de Escudero I, Escriche B, Muñoz D, Van Rie J, Ferré J, Caballero P. 2012.Vip3C, a novel class of vegetative insecticidal proteins from *Bacillus thuringiensis.* Appl Environ Microb 78, 7163–7165.
- Palma L, Muñoz D, Berry C, Murillo J, Caballero P. 2014. *Bacillus thuringiensis* toxins: an overview of their biocidal activity. Toxins (Basel) 6, 3296–3325.
- Palma L, Scott D, Harris G, Din SU, Williams T, Roberts O, Young, Caballero P, Berry C. 2017. The Vip3Ag4 insecticidal protoxin from *Bacillus thuringiensis* adopts a tetrameric configuration that is maintained on proteolysis. Toxins (Basel) 9, 165.
- Pandian GN, Ishikawa T, Togashi M, Shitomi Y, Haginoya K, Yamamoto S, Nishiumi T, Hori H. 2008. *Bombyx mori* midgut membrane protein P252, which binds to *Bacillus thuringiensis* Cry1A, is a chlorophyllide-binding protein, and the resulting complex has antimicrobial activity. Appl Environ Microbiol 74, 1324–1331.
- Panetta JD. 1993. Engineered microbes: the Cell Cap system. In: Kim L. (Ed). (Ed.), Advanced Engineered Pesticides. Marcel Decker, pp. 379–382.
- Pardo-López L, Gómez I, Rausell C, Sanchez J, Soberón M, Bravo A. 2006. Structural changes of the Cry1Ac oligomeric pre-pore from *Bacillus thuringiensis* induced by N-acetylgalactosamine facilitates toxin membrane insertion. Biochemistry 45, 10329–10336.
- Pardo-López L, Muñoz-Garay C, Porta H, Rodríguez-Almazán C, Soberón M, Bravo A. 2009. Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*. Peptides 30, 589–595.
- Pardo-López L, Soberón M, Bravo A. 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiol 37, 3–22.
- Park Y, González-Martínez RM, Navarro-Cerrillo G, Chakroun M, Kim Y, Ziarsolo P, Blanca J, Cañizares J, Ferré J, Herrero S. 2014. ABCC transporters mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis. BMC Biol 12, 46.
- Parker MW, Pattus F, Tucker AD, Tsernoglou D. 1989. Structure of the membrane pore-forming fragment of colicin A. Nature 337, 93–96.
- Parker W, Song PS. 1992. Protein structures in SDS micelle-protein complexes. Biophys J 61, 1435–1439.
- Pauchet Y, Bretschneider A, Augustin S, Heckel DG. 2016. A P-glycoprotein is linked to resistance to the *Bacillus thuringiensis* Cry3Aa toxin in a leaf beetle. Toxins 8, 362.
- Pereira EJG, Storer NP, Siegfried BD. 2011. Fitness costs of Cry1F resistance in laboratory-selected European corn borer (Lepidoptera: Crambidae). J Appl Entomol 135, 17–24.

- Pickett BR, Gulzar A, Ferré J, Wright DJ. 2017. *Bacillus thuringiensis* Vip3Aa toxin resistance in *Heliothis virescens* (Lepidoptera: Noctuidae). Appl Environ Microbiol 83, e03506–16.
- Pigott CR, Ellar DJ. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol Mol Biol Rev 71, 255–281.
- Pimentel D. 2005. Environmental and economic costs of the application of pesticides primarily in the United States. Environ Dev Sustain 7, 229–252.
- Pinos D, Andrés-Garrido A, Ferré J, Hernández-Martínez P. 2021. Response mechanisms of invertebrates to *Bacillus thuringiensis* and its pesticidal proteins. Microbiol Mol Biol Rev 85, e00007–20.
- Pinos D, Chakroun M, Millán-Leiva A, Jurat-Fuentes JL, Wright DJ, Hernández-Martínez P, Ferré J. 2020. Reduced membrane-bound alkaline phosphatase does not affect binding of Vip3Aa in a *Heliothis virescens* resistant colony. Toxins 12, 409.
- Qian Z, Li-Zhen C, Qiong L, Yan Z, Ge-mei L. 2015. Toxicity and binding analyses of *Bacillus thuringiensis* toxin Vip3Aa in Cry1Ac-resistant and –susceptible strains of *Helicoverpa armigera* (Hübner). J Invertebr Agricul 14, 347–354.
- Qin Y, Ying SH, Chen Y, Shen ZC, Feng MG. 2010. Integration of insecticidal protein Vip3Aa1 into *Beauveria bassiana* enhances fungal virulence to *Spodoptera litura* larvae by cuticle and *Per Os* infection. Appl Environ Microbiol 76, 4611–4618.
- Qiu L, Wang P, Wu T, Li B, Wang X, Lei C, Lin Y, Zhao J, Ma W. 2018. Down regulation of *Chilo suppressalis* alkaline phosphatase genes associated with resistance to three transgenic *Bacillus thuringiensis* rice lines. Insect Mol Biol 27, 83–89.
- Quan Y, Ferré J. 2019. Structural domains of the *Bacillus thuringiensis* Vip3Af protein unraveled by tryptic digestion of alanine mutants. Toxins 11, 368.
- Quan Y, Yang J, Wang Y, Hernández-Martínez P, Ferré J, He K. 2021. The rapid evolution of resistance to Vip3Aa insecticidal protein in *Mythimna separata* (Walker) is not related to altered binding to midgut receptors. Toxins 13, 364.
- Rajagopal R, Arora N, Sivakumar S, Rao NG, Nimbalkar SA, Bhatnagar RK. 2009. Resistance of *Helicoverpa armigera* to Cry1Ac toxin from *Bacillus thuringiensis* is due to improper processing of the protoxin. Biochem J 419, 309–316.
- Rajamohan F, Alcantara E, Lee MK, Chen XJ, Curtiss A, Dean DH. 1995. Single amino acid changes in domain II of *Bacillus thuringiensis* CryIAb δ-endotoxin affect irreversible binding to *Manduca sexta* midgut membrane vesicles. J Bacteriol 177, 2276–2282.
- Rajamohan F, Cotrill JA, Gould F, Dean DH. 1996a. Role of domain II, loop 2 residues of *Bacillus thuringiensis* CryIAb δ-endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J Biol Chem 271, 2390–2396.
- Rajamohan F, Hussain SR, Cotrill JA, Gould F, Dean DH. 1996b. Mutations at domain II, loop 3, of *Bacillus thuringiensis* CryIAa and CryIAb delta-endotoxins suggest loop 3 is involved in initial binding to lepidopteran midguts. J Biol Chem 271, 25220–25226.
- Rajput VS, Jayshree Jhala J, Acharya VS. 2020. Biopesticides and their mode of action against insect pests: a review. Int J Chem Stud 8, 2856–2862.
- Rang C, Gil P, Neisner N, Van Rie J, Frutos R. 2005. Novel Vip3-related protein from *Bacillus thuringiensis*. Appl Environ Microbiol 71, 6276 –6281.

- Rashid M, Khan RA, Zhang YL. 2013. Physiological and population responses of armyworm *Mythimna separata* (Lepidoptera: Noctuidae) to a sublethal dose of cantharidin-AC. J Econ Entomol 106, 2177–2182.
- Raymond B, Johnston PR, Nielsen-LeRoux C, Lereclus D, Crickmore N. 2010. *Bacillus thuringiensis*: an impotent pathogen? Trends Microbiol 18, 189–94.
- Roush RT. 1997. Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? Pestic Sci 51, 328–334.
- Roush RT. 1998. Two-toxin strategies for management of insecticidal transgenic crops: Can pyramiding succeed where pesticide mixtures have not? Philos Trans R Soc Lond Ser B Biol Sci 353, 1777–1786.
- Ruiz de Escudero I, Banyuls N, Bel Y, Maeztu M, Escriche B, Muñoz D, Caballero P, Ferré J. 2014. A screening of five *Bacillus thuringiensis* Vip3A proteins for their activity against lepidopteran pests. J Invertebr Pathol 117, 51–55.
- Russell RB, Betts MJ, Barnes MR. Amino acid properties [WWW Document]. URL http://www.russelllab.org/aas/ (accessed 4.15.17).
- Russell RM, Roberston JL, Savin NE. 1977. POLO: a new computer program for Probit analysis. Bull Entomol Soc Am 23, 209–213.
- Sahin B, Gomis-Cebolla J, Günes H, Ferré J. 2018. Characterization of *Bacillus thuringiensis* isolates by their insecticidal activity and their production of Cry and Vip3 proteins. PLoS ONE 13, e0206813.
- Sanchis V. 2011. From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis*. A review. Agron Sustain Dev 31, 217–231.
- Sangadala S, Walters FS, English LH, Adang MJ. 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and 86Rb(+)-K+ efflux in vitro. J Biol Chem 269, 10088–10092.
- Saraswathy N, Nain V, Sushmita K, Kumar PA. 2008. A fusion gene encoding two different insecticidal proteins of *Bacillus thuringiensis*. Indian J Biotechnol 7, 204–209.
- Sato R, Adegawa S, Li X, Tanaka S, Endo H. 2019. Function and role of ATP-binding cassette transporters as receptors for 3D-Cry toxins. Toxins 11, 124.
- Sattar S, Biswas PK, Hossain MA, Maiti MK, Sen SK, Basu A. 2008. Search for vegetative insecticidal proteins (VIPs) from local isolates of *Bacillus thuringiensis* effective against lepidopteran and homopteran insect pests. J Biopest 1, 216–222.
- Savary S, Bregaglio S, Willocquet L, Gustafson D, Mason D'Croz D, Sparks A, Castilla, N, Djurle A, Allinne C, Sharma M, Rossi V, Amorim L, Bergamin A, Yuen J, Esker P, McRoberts, N, Avelino J, Duveiller E, Koo J, Garrett K. 2017. Crop health and its global impacts on the components of food security. Food Secur 9, pp. 311-327.
- Savary S, Teng PS, Willocquet L, Nutter FW. 2006. Quantifcation and modeling of crop losses: a review of purposes. Annu Rev Phytopathol 44, 89–112.
- Savary S, Willocquet L, Pethybridge SJ, Esker P, McRoberts N, Nelson A. 2019. The global burden of pathogens and pests on major food crops. Nat Ecol Evol 3, 430–439.
- Sawaya MR, Cascio D, Gingery M, Rodriguez J, Goldschmidt L, Colletier J, Messerschmidt MM, Boutet S, Koglin JE, Williams GJ, Brewster AS, Nass K, Hattne J, Botha S, Doak RB, Shoeman RL, DePonte DP, Park H, Federici BA, Sauter NK, Schlichting I, Eisenberg DS. 2014. Protein crystal structure obtained at 2.9 Å resolution from injecting bacterial cells into an

X-ray free-electron laser beam. PNSA 111, 12769–12774.

- Schnepf E, Crickmore N, Rie JV, Lereclus D, Baum J, Feitelson J. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol 62, 775–806.
- Sellami S, Cherif M, Abdelkefi-Mesrati L, Tounsi S, Jamoussi K. 2015. Toxicity, activation process, and histopathological effect of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 on *Tuta absoluta*. Appl Biochem Biotechnol 175, 1992–1999.
- Sellami S, Jemli S, Abdelmalek N, Cherif M, Abdelkefi-Mesrati L, Tounsi S, Jamoussi K. 2018. A novel Vip3Aa16-Cry1Ac chimera toxin: Enhancement of toxicity against *Ephestia kuehniella*, structural study and molecular docking. Int J Biol Macromol 117, 752–761.
- Selvapandiyan A, Arora N, Rajagopal R, Jalali SK, Venkatesan T, Singh SP, Bhatnagar RK. 2001. Toxicity analysis of N- and C-terminus deleted vegetative insecticidal protein from *Bacillus thuringiensis*. Appl Environ Microbiol 67, 5855–5858.
- Sena JA, Hernández-Rodríguez CS, Ferré J. 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3A proteins with *Spodoptera frugiperda* midgut binding sites. Appl Environ Microbiol 75, 2236–2237.
- Sengupta A, Sarkar A, Priya P, Ghosh Dastidar S, Das S. 2013. New insight to structure-function relationship of GalNAc mediated primary interaction between insecticidal Cry1Ac toxin and HaALP receptor of *Helicoverpa armigera*. PLoS One 8, e78249.
- Shabbir MZ, Quan Y, Wang Z. 2018. Characterization of the Cry1Ah resistance in Asian corn Borer and its cross-resistance to other *Bacillus thuringiensis* toxins. Sci Rep 8, 234.
- Sharma A, Shukla A, Attri K, Kumar M, Kumar P, Suttee A, Singh G, Barnwal RP, Singla N. 2020. Global trends in pesticides: A looming threat and viable alternatives. Ecotox Environ Safe 201, 110812.
- Sharma HC, Davies JC. 1983. The Oriental Armyworm*, Mythimna separata* (Wal.) *d*istribution, biology and control: a literature review; Center for Oversea Pest Research, ODA Miscellaneous Report 59; Center for Oversea Pest: London, UK.
- Sharma HC, Sullivan DJ, Bhatnagar VS. 2002. Population dynamics and natural mortality factors of the oriental armyworm, *Mythimna separata* (Lepidoptera: Noctuidae), in South Central India. Crop Prot 21, 721–732.
- Shelton AM, Zhao JZ, Roush RT. 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu Rev Entomol 47, 845–881.
- Shi Y, Mowery RA, Ashley J, Hentz M, Ramirez AJ, Bilgicer B, Slunt-Brown H, Borchelt DR, Shaw BF. 2012. Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding. Protein Sci 21, 1197–1209.
- Siebert MW, Babock JM, Nolting A, Santos AC, Adamczyk JJ, Neese PA, King JE, Jenkins JN, McCarty J, Lorenz GM, Fromme DD, Lassiter RB. 2008. Efficacy of Cry1F insecticidal protein in maize and cotton for control of fall armyworm (Lepidoptera: Noctuidae). Fla Entomol 81, 555–565.
- Siegfried BD, Spencer T, Crespo A, Pereira E, Marcon P. 2006. Ten years of Bt resistance monitoring in the European corn borer: What we know, what we don't know, and what we can do better. Am Entomol 53, 208–214.
- Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7, 539.

- Singh G, Sachdev B, Sharma N, Seth R, Bhatnagar RK. 2010. Interaction of *Bacillus thuringiensis* vegetative insecticidal protein with ribosomal S2 protein triggers larvicidal activity in *Spodoptera frugiperda*. Appl Environ Microbiol 76, 7202–7209.
- Smedley DP, Ellar DJ. 1996. Mutagenesis of three surface-exposed loops of a *Bacillus thuringiensis* insecticidal toxin reveals residues important for toxicity, receptor recognition and possibly membrane insertion. Microbiology 142, 1617–1624.
- Software L. 1987. Polo-PC a user's guide to probit or logit analysis.
- Song F, Chen C, Wu S, Shao E, Li M, Guan X, Huang Z. 2016. Transcriptional profiling analysis of *Spodoptera litura* larvae challenged with Vip3Aa toxin and possible involvement of trypsin in the toxin activation. Sci Rep 6, 23861.
- Song F, Lin Y, Chen C, Shao E, Guan X, Huang Z. 2016.Insecticidal activity and histopathological effects of Vip3Aa protein from *Bacillus thuringiensis* on *Spodoptera litura*. J Microbiol Biotechnol 26, 1774–1780.
- Song FP, Zhang J, Gu AX, Wu Y, Han LL, He KL, Chen ZY, Yao J, Hu YQ, Li GX, Huang DF. 2020. Identification of *cry1I*-Type genes from *Bacillus thuringiensis* strains and characterization of a novel *cry1I*-type gene. Appl Environ Microbiol 69, 5207–5211.
- Song R, Peng DH, Yu ZN, Sun M. 2008. Carboxy-terminal half of Cry1C can help vegetative insecticidal protein to form inclusion bodies in the mother cell of *Bacillus thuringiensis*. Appl Microbiol Biot 80, 647–654.
- Song X, Kain W, Cassidy D, Wang P. 2015. Resistance to Bacillus thuringiensis Toxin Cry2Ab in *Trichoplusia ni* is conferred by a novel genetic mechanism. Appl Environ Microbiol 81, 5184–5195.
- Song Y, Wang H, Chen Y, Wang S, Sun H. 2017. Cross-resistance and biochemical resistance mechanisms of emamectin benzoate resistant population of *Mythimna separate*. Chin J Pesticide Sci 19, 18–24.
- Song Y, Zhou D, He K. 1999. Studies on mass rearing of Asian corn borer: development of a satisfactory non-agar semi-artificial diet and its use. Acta Phytophylacica Sin 26, 324–328.
- Soonsanga S, Rungrod A, Audtho M, Promdonkoy B. 2019. Tyrosine-776 of Vip3Aa64 from *Bacillus thuringiensis* is important for retained larvicidal activity during high-temperature storage. Curr Microbiol 76, 15–21.
- Sopko MS, Narva KE, Bowling AJ, Pence HE, Hasler JJ, Letherer TJ, Larsen CM, Zack MD. 2019. Modification of Vip3Ab1 C-Terminus Confers Broadened Plant Protection from Lepidopteran Pests. Toxins 11, 316.
- Srinivasan A, Giri AP, Gupta VS. 2006. Structural and functional diversities in lepidopteran serine proteases. Cell Mol Biol Lett 11, 132–154.
- Stockert JC, Blázquez-Castro A, Cañete M, Horobin RW, Villanueva Á. 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets. Acta Histochem. 114, 785-796.
- Storer NP, Babcock JM, Schlenz M, Meade T, Thompson GD, Bing JW, Huckaba RM. 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J Econ Entomol 103, 1031–1038.
- Strange R, Scott PR. 2005. Plant disease: a threat to global food security. Annu Rev Phytopathol 43, 83–116.

- Su H, Jiang T, Sun Y, Gu H, Wu J, Yang Y. 2020. Effect of three insect-resistant maizes expressing Cry1Ie, Cry1Ab/Cry2Aj and Cry1Ab on the growth and development of armyworm *Mythimna separata* (Walker). J Integr Agric 19, 1842–18491.
- Syed T, Askari M, Meng Z, Li Y, Abid MA, Wei Y, Guo S, Liang C, Zhang R. 2020. Current insights on vegetative insecticidal proteins (Vip) as next generation pest killers. Toxins 12, 522.
- Tabashnik BE, 2015. ABCs of insect resistance to Bt. PloS Genet 11, e1005646.
- Tabashnik BE, Brévault T, Carrière Y. 2013. Insect resistance to Bt crops: Lessons from the first billion acres. Nat Biotechnol 31, 510–521.
- Tabashnik BE, Brévault T, Yves Carrière Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nat Biotechnol 31, 510–520.
- Tabashnik BE, Gassmann AJ, Crowder DW, Carrière Y. 2008. Insect resistance to *Bt* crops: evidence versus theory. Nat Biotechnol 26, 199 –202.
- Tabashnik BE, Van Rensburg JBJ, Carrière Y. 2009. Field-evolved insect resistance to Bt crops: definition, theory and data. J Econ Entomol 102, 2011–2025.
- Tabashnik BE, Carrière Y. 2020. Evaluating cross-resistance between Vip and Cry toxins of *Bacillus thuringiensis*. J Econ Entomol 113, 553-561.
- Tabashnik BE. 1992. Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl Environ Microbiol 58, 3343–3346.
- Tabashnik BE. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu Rev Entomol 39, 47–79.
- Tanaka S, Endo H, Adegawa S, Iizuka A, Imamura K, Kikuta S, Sato R. 2017. *Bombyx mori* ABC transporter C2 structures responsible for the receptor function of *Bacillus thuringiensis* Cry1Aa toxin. Insect Biochem Mol Biol 91, 44–54.
- Tanaka S, Miyamoto K, Noda H, Jurat-Fuentes JL, Yoshizawa Y, Endo H, Sato R. 2013. The ATP-binding cassette transporter subfamily C member 2 in *Bombyx mori* larvae is a functional receptor for Cry toxins from *Bacillus thuringiensis*. FEBS J 280, 1782–1794.
- Tay WT, Mahon RJ, Heckel DG, Walsh TK, Downes S, James WJ, Lee S, Reineke A, Williams AK, Gordon KHJ. 2015. Insect resistance to *Bacillus thuringiensis* toxin Cry2Ab is conferred by mutations in an ABC transporter subfamily a protein. PLoS Genet 11, e1005534.
- Teakle RE, Jensen JM. 1985. *Heliothis punctigera*. In: Singh R, Moore RF (Eds.), Handbook of Insect Rearing Vol 2. Elsevier, Amsterdam, The Netherlands, pp. 312–322.
- Terra WR, Ferreira C, Jordao BP, Dillon RJ. 1996. Digestive enzymes. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. Chapman & Hall, London, pp. 153–194.
- Tiewsiri K, Wang P. 2011 Differential alteration of two aminopeptidases N associated with resistance to *Bacillus thuringiensis* toxin Cry1Ac in cabbage looper. Proc Natl Acad Sci USA 108, 14037-14042.
- Tilman D, Fargione J, Wolff B, D'Antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D. 2001. Forecasting agriculturally driven global environmental change. Science 292, 281–284.
- Vadlamudi RK, Weber E, Ji I, Ji TH, Bulla Jr LA. 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J Biol Chem 270, 5490–5494.
- Valaitis AP, Jenkins JL, Lee MK, Dean DH, Garner KJ. 2001. Isolation and partial characterization of gypsy moth BTR-270, an anionic brush border membrane glycoconjugate that

binds *Bacillus thuringiensis* Cry1A toxins with high affinity. Arch Insect Biochem Physiol 46, 186–200.

- Van Frankenhuyzen K. 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invertebr Pathol 101, 1–16.
- Van Frankenhuyzen K. 2013. Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. J Invertebr Pathol 114, 76–85.
- Van Rie J, Jansens S, Höfte H, Degheele D, Van Mellaert H. 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins: Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur J Biochem 186:239–247.
- Van Rie J. 2000. *Bacillus thuringiensis* and its use in transgenic insect control technologies. Int J Med Microbiol 290, 463–469.
- Vélez AM, Spencer TA, Alves AP, Moellenbeck D, Meagher RL, Chirakkal H, Siegfried BD. 2013. Inheritance of Cry1F resistance and frequency of resistant alleles in Spodoptera frugiperda (Lepidoptera: Noctuidae). Bull Entomol Res 103, 700–713.
- Walsh TK, Downes SJ, Gascoyne J, James W, Parker T, Armstrong J, Mahon RJ. 2014. Dual Cry2Ab and Vip3A resistant strains of *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae); testing linkage between loci and monitoring of allele frequencies. J Econ Entomol 107, 1610–1617.
- Walters FS, de Fontes CM, Hart H, Warren GW, Chen JS. 2010. Lepidopteranactive variable-region sequence imparts coleopteran activity in eCry3.1Ab, an engineered *Bacillus thuringiensis* hybrid insecticidal protein. Appl Environ Microbiol 76, 3082–3088.
- Walters FS, Stacy CM, Lee MK, Palekar N, Chen JS. 2008. An engineered chymotrypsin/cathepsin G site in domain I renders *Bacillus thuringiensis* Cry3A active against Western corn rootworm larvae. Appl Environ Microbiol 74, 367–374.
- Wang J, Wang H, Liu S, Liu L, Tay WT, Walsh TK, Yang Y, Wu Y. 2017. CRISPR/Cas9 mediated genome editing of *Helicoverpa armigera* with mutations of an ABC transporter gene HaABCA2 confers resistance to *Bacillus thuringiensis* Cry2A toxins. Insect Biochem Mol Biol 87, 147–153.
- Wang J, Zhang H, Wang H, Zhao S, Zuo Y, Yang Y, Wu Y. 2016. Functional validation of cadherin as a receptor of Bt toxin Cry1Ac in *Helicoverpa armigera* utilizing the CRISPR/Cas9 system. Insect Biochem Mol Biol 76, 11–17.
- Wang JD, Chen LF, Wang YR, Fu HY, Ali A, Xiao D, Wang R, Gao SJ. 2018. Silence of ryanodine receptor gene decreases susceptibility to chlorantraniliprole in the oriental armyworm, *Mythimna separata* (walker). Pesticide Biochem Physiol 148, 34–41.
- Wang L, Ma Y, Wan P, Liu K, Xiao Y, Wang J, Cong S, Xy D, Wu K, Fabrick FA, Li X, Tabashnik B. 2018a. Resistance to *Bacillus thuringiensis* linked with a cadherin transmembrane mutation affecting cellular trafficking in pink bollworm from China. Insect Biochem Mol Biol 94, 28–35.
- Wang P, Zhao J, Rodrigo-Simón A, Kain W, Janmaat AF, Shelton AM, Ferré J, Myers J. 2007. Mechanism of resistance to *Bacillus thuringiensis* toxin Cry1Ac in a greenhouse population of the cabbage looper, *Trichoplusia ni*. Appl Environ Microbiol 73, 199–1207.
- Wang Y, Wang Y, Wang Z, Bravo A, Soberón M, He K. 2016. Genetic basis of Cry1F-resistance in a laboratory selected Asian corn borer strain and its cross-resistance to other *Bacillus thuringiensis* toxins. PLoS ONE 11, e0161189.
- Wang YN. 1984. Manual of Artificial Insect Diet; Shanghai Scientific and Technical Publishers: Shanghai, China, pp. 44–47.

- Wang ZY, Fang LF, Zhou ZS, Pacheco S, Gomez I, Song FP, Soberon M, Zhang J, Bravo A. 2018b. Specific binding between *Bacillus thuringiensis* Cry9Aa and Vip3Aa toxins synergizes their toxicity against Asiatic rice borer (*Chilo suppressalis*). J Biol Chem 293, 11447–11458.
- Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. 2009. Jalview Version 2–a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191.
- Wei JZ, Hale K, Carta L, Platzer E, Wong C, Fang SC, Aroian RV. 2003. *Bacillus thuringiensis* crystal proteins that target nematodes. Proc Natl Acad Sci USA 100, 2760–2765.
- Welch KL, Unnithan GC, Degain BA, Wei J, Zhang J, Li X, Tabashnik BE, Carrière Y. 2015. Cross-resistance to toxins used in pyramidal Bt crops and reistence to Bt sprays in *Helicoverpa zea*. J Invertebr Pathol 132, 149–156.
- Wheeler MW, Park RM, Bailer AJ. 2006. Comparing median lethal concentration values using confidence interval overlap or ratio tests. Environ Toxicol Chem 25, 1441–1444.
- WHO. 2011. The Use of DDT in Malaria Vector Control. Global Malaria Programme. World Health Organisation, Geneva.
- WHO. 2012. Global Strategy for Dengue Prevention and Control 2012-2020. World Health Organisation Press, Switzerland.
- WHO. 2017. Guidelines for Drinking-Water Quality, fourth ed. WHO, Geneva, pp. 631 incorporating the 1st addendum.
- Wolfersberger MG, Luthy P, Maurer P, Parenti P, Sacchi VF, Giordana B, Hanozet GM. 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp Biochem Physiol 86A, 301–308.
- Wolfersberger MG. 1990. The toxicity of two *Bacillus thuringiensis* deltaendotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experientia 46, 475–477.
- Wolff AS, Engel S, Berkowitz G, Teitelbaum S, Siskind J, Barr DB, Wetmur J. 2007. Prenatal pesticide and PCB exposures and birth outcomes. Pediatr Res 61, 243–250.
- Wu J, Zhao F, Bai J, Deng G, Qin S, Bao Q. 2007. Evidence for positive Darwinian selection of *vip* gene in *Bacillus thuringiensis*. J Genet Genomics 34, 649–660.
- Wu ZL, Guo WY, Qiu JZ, Huang TP, Li XB, Guan X. 2004. Cloning and localization of vip3A gene of *Bacillus thuringiensis*. Biotechnol Lett 26, 1425–1428.
- Xiao Y, Wu K. 2019. Recent progress on the interaction between insects and *Bacillus thuringiensis* crops. Phil Trans R Soc B 374, 20180316.
- Xiao YT Dai Q, Hu R, Pacheco S, Yang Y, Liang G, Soberon M, Bravo A, Liu K, Wu K. 2017. A single point mutation resulting in cadherin mislocalization underpins resistance against *Bacillus thuringiensis* toxin in cotton bollworm. J Biol Chem 292, 2933–2943.
- Xiao YT, Zhang T, Liu CX, Heckel DG, Li XC, Tabashnik BE, Wu KM. 2014. Mis-splicing of the ABCC2 gene linked with Bt toxin resistance in *Helicoverpa armigera*. Sci Rep 4, 6184.
- Xie R, Zhuang M, Ross LS, Gomez I, Oltean DI, Bravo A, Soberon M, Gill SS. 2005. Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J Biol Chem 280, 8416–8425.
- Xu L, Wang Z, Zhang J, He K, Ferry N, Gatehouse AMR. 2010. Cross-resistance of Cry1Ab-selected Asian corn borer to other Cry toxins. J Appl Entomol 5, 429–438.

- Xu X, Yu L, Wu Y. 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Appl Environ Microbiol 71, 948–954.
- Yang F, Morsello S, Head GP, Sansone C, Huang F, Gilreath RT, Kerns DL. 2018a. F2 screen, inheritance and cross-resistance of field-derived Vip3A resistance in *Spodoptera frugiperda* (Lepidoptera: Noctuidae) collected from Louisiana, USA. Pest Manag Sci 74, 1769–1778.
- Yang F, Santiago Gonzalez JC, Little N, Reisig D, Payne G, Dos Santos RF, Jurat-Fuentes JL, Kurtz R, Kerns DL. 2020. First documentation of major Vip3Aa resistance alleles in field populations of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in Texas, USA. Sci Rep 10, 5867.
- Yang F, Santiago-González JC, Little N, Reisig D, Payne G, Ferreira Dos Santos R, Jurat-Fuentes JL, Kurtz R, Kerns DL. 2020. First documentation of major Vip3Aa resistance alleles in field populations of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) in Texas, USA. Sci Rep 10, 5867.
- Yang F, Williams J, Porter P, Huang F, Kerns DL. 2019. F2 screen for resistance to *Bacillus thuringiensis* Vip3Aa51 protein in field populations of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) from Texas, USA. Crop Prot 126, 104915.
- Yang J, Quan Y, Sivaprasath P, Shabbir MS, Wang Z, Ferré J, He K. 2018b. Insecticidal activity and synergistic combinations of ten different Bt toxins against *Mythimna separata* (Walker). Toxins 10, 454.
- Yang J, Quan Y, Sivaprasath P, Shabbir MS, Wang Z, Ferré J, He K. 2018. Insecticidal activity and synergistic combinations of ten different Bt toxins against *Mythimna separata* (Walker). Toxins 10, 454.
- Yang X, Chen W, Song X, Ma X, Cotto-Rivera RO, Kain W, Chu H, Chen Y, Fei Z, Wang P. 2019. Mutation of ABC transporter ABCA2 confers resistance to Bt toxin Cry2Ab in *Trichoplusia ni*. Insect Biochem Mol Biol 112, 103209.
- Yang Y, Zhu YC, Ottea J, Husseneder C, Leonard BR, Abel C, Huang F. 2010. Molecular characterization and RNA interference of three midgut aminopeptidase N isozymes from *Bacillus thuringiensis*-susceptible and -resistant strains of sugarcane borer, *Diatraea saccharalis*. Insect Biochem Mol Biol 40, 592–603.
- Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa lyses midgut epithelium cells of susceptible insects. Appl Environ Microbiol 63, 532–536.
- Yu X, Liu T, Sun Z, Guan P, Zhu J, Wang S, Li S, Deng Q, Wang L, Zheng A, Li P. 2012. Co-expression and synergism analysis of Vip3Aa29 and Cyt2Aa3 insecticidal proteins from *Bacillus thuringiensis*. Curr Microbiol 64, 326–331.
- Yuan XD, Zhao M, Wei JZ, Zhang WN, Wang BJ, Khaing KM, Liang GM. 2017. New insights on the role of alkaline phosphatase 2 from *Spodoptera exigua* (Hu¨bner) in the action mechanism of Bt toxin Cry2Aa. J Insect Physiol 98, 101–107.
- Zack MD, Sopko MS, Frey ML, Wang XJ, Tan SY, Arruda JM, Letherer TT, Narva KE. 2017. Functional characterization of Vip3Ab1 and Vip3Bc1: Two novel insecticidal proteins with differential activity against lepidopteran pests. Sci Rep 7, 11112.
- Zeng J, Jiang YY, Liu J. 2013. Analysis of the armyworm outbreak in 2012 and suggestions of monitoring and forecasting. Plant Prot 39, 117–121. (In Chinese)

- Zhang H, Wu S, Yang Y, Tabashnik BE, Wu Y. 2012. Non-recessive Bt toxin resistance conferred by an intracellular cadherin mutation in field-selected populations of cotton bollworm. PLOS ONE 7, e53418.
- Zhang J, Pan ZZ, Xu L, Liu B, Chen Z, Li Jie, Niu LY, Zhu YJ, Chen QX. 2018. Proteolytic activation of *Bacillus thuringiensis* Vip3Aa protein by *Spodoptera exigua* midgut protease. Int J Biol Macromol 107, 1220–1226.
- Zhang R, Hua G, Andacht TM, Adang MJ. 2008. A 106-kDa aminopeptidase is a putative receptor for *Bacillus thuringiensis* Cry11Ba toxin in the mosquito *Anopheles gambiae*. Biochemistry 47, 11263–11272.
- Zhang S, Cheng H, Gao Y, Wang G, Liang G, Wu K. 2009. Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem Mol Biol 39, 421–429.
- Zhang YW, Liu YJ, Ren Y, Liu Y, Liang GM, Song FP, Bai SX, Wang JH, Wang GY. 2013. Overexpression of a novel *Cry1Ie* gene confers resistance to Cry1Ac-resistant cotton bollworm in transgenic lines of maize. Plant Cell Tissue Organ Cult 115, 151–158.
- Zhang Z, Teng XL, Ma WH, Li F. 2017. Knockdown of two cadherin genes confers resistance to Cry2A and Cry1C in Chilo suppressalis. Sci Rep 7, 5992.
- Zhang Z, Zhang Y, Wang J, Liu J, Tang Q, Li X, Cheng D. 2018. Insecticide resistance of the field populations of oriental armyworm, *Mythimna separata* (Walker) in Shaanxi and Shanxi provinces of China. J Integr Agric 17, 1527–1537.
- Zhao J, Jin L, Yang Y, Wu Y. 2010. Diverse cadherin mutations conferring resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Helicoverpa armigera*. Insect Biochem Mol Biol 40, 113–118.
- Zhao JZ, Cao J, Collins HL, Bates SL, Roush RT, Earle ED. 2005. Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. Proc Natl Acad Sci USA 102, 8426–8430.
- Zhao JZ, Cao J, Li YX, Collins HL, Roush RT, Earle ED, Shelton AM. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21, 1493–1497.
- Zheng L, Baumann U, Reymond JL. 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res 32, e115.
- Zheng M, Evdokimov AG, Moshiri F, Lowder C, Hass F. 2020. Crystal structure of a Vip3B family insecticidal protein reveals a new fold and a unique tetrameric assembly. Protein Sci 29, 824–829.