DANIELE HELOÍSA PINHEIRO

IDENTIFICAÇÃO DO MECANISMO CELULAR DE ABSORÇÃO DE dsRNA EM *Diabrotica virgifera virgifera* (LeConte) E SELEÇÃO DE CEPAS DE *Bacillus thuringiensis* TÓXICAS CONTRA *Helicoverpa armigera* (Hübner)

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Biotecnologia Vegetal, área de concentração em Biologia Molecular, para a obtenção do título de Doutor.

Dr. Fernando Hercos Valicente

Orientador

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2017

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Pinheiro, Daniele Heloísa.
Identificação do mecanismo celular de absorção de dsRNA em *Diabrotica virgifera virgifera* (LeConte) e seleção de cepas de *Bacillus thuringiensis* tóxicas contra *Helicoverpa armigera* (Hübner) / Daniele Heloísa Pinheiro. – Lavras: UFLA, 2017.
136p. : il.

Tese(doutorado)–Universidade Federal de Lavras, 2017.
Orientador(a): Fernando Hercos Valicente.
Bibliografia.

1. RNA interferente. 2. SID-1. 3. Endocitose. 4. Bactéria entomopatogênica. 5. Controle biológico. I. Universidade Federal de Lavras. II. Título.

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IDENTIFICATION OF THE CELLULAR MECHANISM OF dsRNA UPTAKE IN *Diabrotica virgifera virgifera* (LeConte) AND SELECTION OF *Bacillus thuringiensis* STRAINS TOXIC AGAINST *Helicoverpa armigera* (Hübner)

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APROVADA em 16 de setembro de 2016.

Dra. Andréa Almeida Carneiro	EMBRAPA
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Orientador

LAVRAS – MG
2017

A Deus,
por ter permitido a realização de mais este sonho.

Aos meus pais, Sandra e Tarley,
por todo amor e apoio.

Aos meus irmãos, Douglas e Daniel,
pelo incentivo.
Dedico

AGRADECIMENTOS

Primeiramente agradeço a Deus por ter permitido que eu tivesse a oportunidade de estudar e chegado ao nível de doutorado. Por ter estado ao meu lado durante toda jornada acadêmica, dando-me força nos momentos mais difíceis e também abençoando-me com momentos tão felizes e especiais.

À Universidade Federal de Lavras pela oportunidade de estudo.

À Emprapa Milho e Sorgo e à Universidade de Nebraska pela oportunidade de desenvolver este trabalho.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e ao Conselho Nacional de Pesquisa (CNPq) pela concessão das bolsas de estudo.

Ao meu orientador Dr. Fernando Hercos Valicente, pela orientação, confiança e paciência.

Ao meu co-orientador Dr. Blair D. Siegfried, pela oportunidade de desenvolver parte deste trabalho em seu laboratório na Universidade de Nebraska, orientação, confiança, paciência e exemplo de amor e dedicação `a ciência.

À Dra. Ana Maria Veléz e ao Dr. Haichuan Wang, com os quais pude aprender muito e sempre contar para a realização deste trabalho.

À Natalie Matz pela paciência e valiosos ensinamentos.

Aos laboratoristas Celso e Osmar pela ajuda e ensinamentos.

Aos colegas do Laboratório de Controle Biológico pelos momentos de descontração e pela ajuda nos inúmeros bioensaios.

Aos colegas do Núcleo de Biologia Aplicada pela ajuda quando precisei.

Aos meus amados pais Sandra e Tarley pelo amor incondicional, por sempre terem estado ao meu lado fazendo o possível e impossível pela minha felicidade.

Aos meus amados irmãos Douglas e Daniel por sempre acreditarem no meu potencial e torcerem pelo meu sucesso.

Às minhas queridas amigas Débora, Catarina, Fabiana e Suzana pelas palavras amigas e pelos inúmeros momentos que fizeram da minha “estadia” em Lincoln muito mais fácil e divertida.

À Mayara pela amizade e momentos de descontração.

À minha família que sempre torceu por mim.

A todos que contribuíram de alguma forma para que este sonho se realizasse.

MUITO OBRIGADA!

‘‘...Você não consegue conectar os fatos olhando para frente. Você só os conecta quando olha para trás. Então tem que acreditar que, de alguma forma, eles vão se conectar no futuro. Você tem que acreditar em alguma coisa – sua garra, destino, vida, karma ou o que quer que seja. Essa maneira de encarar a vida nunca me decepcionou e tem feito toda a diferença para mim... Tenha coragem de seguir o seu próprio coração e a sua intuição. Eles de alguma maneira já sabem o que você realmente quer se tornar. Todo o resto é secundário.’’ (Steve Jobs)

‘‘*Acredite na espera. Abrace a incerteza. Aprecie a beleza de tornar. Quando nada é certo, tudo é possível.****’’ (*** *Mandy Hale)*

RESUMO

RNA interferente (RNAi) é um mecanismo de silenciamento gênico desencadeado por moléculas de RNA dupla fita (*double-stranded RNA - dsRNA*), amplamente utilizado na pesquisa entomológica, especialmente para a análise funcional de genes. Seu potencial para o controle de insetos pragas tem sido demonstrado através de vários estudos. Contudo, para a efetiva aplicação do RNAi no manejo de insetos pragas é necessário que o dsRNA após ser ingerido pelo inseto seja absorvido do lúmen intestinal e o sinal de silenciamento transportado sistemicamente de uma célula para outra. *Diabrotica virgifera virgifera* LeConte (larva do milho ocidental) é um dos insetos pragas mais importantes do milho nos Estados Unidos. Com o objetivo de identificar o mecanismo envolvido na absorção celular de dsRNA em adultos de *D. virgifera virgifera*, uma abordagem de "RNAi de RNAi" foi utilizada. Para avaliar se a proteína transmembranar SID-1 (SID-1-like ou SIL) participa da absorção de dsRNA em adultos de *D. virgifera virgifera*, os insetos foram injetados com *silA* dsRNA, *silC* dsRNA ou uma mistura de *silA* e *silC* dsRNAs. Posteriormente, dsRNA do gene marcador *vATPase-A*, foi oferecido aos insetos através da alimentação e a mortalidade dos insetos e expressão do gene *vATPase-A* avaliadas. Além disso, o envolvimento da endocitose dependente da clatrina na absorção de dsRNA foi analizado através do efeito do silenciamento de cinco importantes genes associados com a endocitose (*Clath*, *Vha16*, *AP50*, *Arf72A* e *Rab7*) sobre o RNAi do gene marcador *laccase2*. Os resultados demonstraram que a expressão do gene *vATPase-A* não foi significativamente aumentada pelo silenciamento dos genes *silA* e *silC* em comparação com o tratamento controle no qual os insetos foram injetados com *GFP* dsRNA e depois alimentados com *vATPase-A* dsRNA. O silenciamento do gene *silC* também não afetou a mortalidade da *D. virgifera virgifera*, sugerindo que a proteína SILC não participa da absorção de dsRNA neste inseto. A supressão do gene *silA* afetou significativamente a mortalidade da *D. virgifera virgifera* em relação ao controle, mas não foram observados efeitos na expressão do gene *vATPase-A*, indicando que a proteína SILA não tem um papel fundamental na internalização de dsRNA. O silenciamento dos genes *Clath*, *Vha16* e *AP50* inibiu a absorção do *laccase2* dsRNA e indução do silenciamento gênico, sugerindo que a endocitose desempenha um papel fundamental na absorção de dsRNA em adultos de *D. virgifera virgifera*. Entretanto, a supressão dos genes *Arf72A* e *Rab7* não afetou o silenciamento do gene *laccase2*. Ao contrário do RNAi, o qual é considerado uma técnica relativamente recente, bioinseticidas `a base de *Bacillus thuringiensis* e plantas geneticamente modificadas expressando proteínas desta bactéria já vem sendo empregados como uma eficiente estratégia no controle de insetos pragas ao longo de muitos anos. Contudo, a seleção de

insetos resistentes `as toxinas do *B. thuringiensis* impulsiona a identificação de novas cepas e genes a serem utilizados com esta finalidade. Afim de selecionar cepas de *B. thuringiensis* com atividade inseticida contra *Helicoverpa armigera* (Hübner), bioensaios de toxicidade foram realizados. Oito cepas, 426, 520B, 1636, 1641, 1644, 1648, 1657 e 1658, causaram mortalidade superior `a 75% em *H. armigera* e apresentaram valores de $CL_{50}$ entre 150,1 e 1543,3 ng/cm$^2$. Através de análises moleculares foi demonstrado que estas cepas contêm diferentes genes *cry* e *vip* além de apresentarem um perfil proteico com bandas principais correspondentes `a 140 e 55 KDa. A atividade inseticida das cepas 426, 520B, 1636, 1641, 1644, 1648, 1657 e 1658 também foi avaliada através de bioensaios com *Anticarsia gemmatalis* (Hübner), *Diatraea saccharalis* (Fabricius), *Spodoptera cosmioide* (Walker) e *Pseudoplusia includes* (Walker), demonstrando que algumas destas cepas também causaram altos níveis de mortalidade nestes insetos. Os resultados obtidos indicam que as cepas de *B. thuringiensis* selecionadas apresentam grande potencial para serem utilizadas no controle da *H. armigera* e de outros importantes lepidópteros-praga.

Palavras-chave: Larva do milho ocidental. RNA interferente. SID-1. Endocitose. Bactéria entomopatogênica. Lepidoptera. Controle biológico.

ABSTRACT

RNA interference (RNAi) is a gene silencing mechanism triggered by double-stranded RNA (dsRNA) molecules, widely used in the entomological research, especially for the functional analysis of genes. Its potential for insect pest control has been demonstrated through several studies. However, for the effective application of the RNAi in the insect managemen, it is necessary the uptake of dsRNA from the intestinal lumen after it to be ingested by the insect and the RNAi signal needs to be systemically spread from one cell to another. *Diabrotica virgifera virgifera* LeConte (western corn rootworm) is one of the most important insect pest of maize in United States. In order to identify the mechanism involved in the cellular uptake of dsRNA in *D. virgifera virgifera* adults an "RNAi on RNAi" approach was used. To assess whether the transmembrane SID-1 protein (SID-1-like or SIL) participates of the dsRNA uptake in *D. virgifera virgifera* adults, the insects were injected with *silA* dsRNA, *silC* dsRNA or a mixture of *silA* dsRNA and *silC* dsRNA. Posteriorly, dsRNA of the marker gene *vATPase-A* was offered to the insects through feeding, and then the insect mortality and *vATPase-A* gene expression were evaluated. In addition, the participation of the clathrin-dependent endocytosis in dsRNA uptake was analyzed through the silencing effect of five important genes associated with the endocytosis (*Clath*, *Vha16*, *AP50*, *Arf72A* and *Rab7*) on RNAi of the marker gene *laccase2*. The results demonstrated that the expression of *vATPase-A* gene was not significantly increased by *silA* and *silC* genes silencing compared to the control treatment, in which the insects were injected with GFP dsRNA and then fed with *vATPase-A* dsRNA. The silencing of *silC* gene did not affect the mortality of *D. virgifera virgifera* adults, suggesting that SILC protein does not participate of the dsRNA uptake in this insect. The suppression of the *silA* gene affected significantly the *D. virgifera virgifera* adults mortality compared to control, but no effects on *vATPase-A* gene expression were observed suggesting that the SILA protein does not play a key role in dsRNA uptake. The silencing of *Clath*, *Vha16* and *AP50* genes inhibited the internalization of *laccase2* dsRNA and induction of gene silencing suggesting that endocytosis plays a critical role in dsRNA uptake in *D. virgifera virgifera* adults. However, the suppression of the *Arf72A* and *Rab7* genes did not affect the silencing of *laccase2* gene. Unlike the RNAi which is considered a relatively recent technique, *Bacillus thuringiensis* based biopesticides and genetically modified plants expressing genes of this bacteria have been used as an efficient strategy for insect pest control over many years. However, the selection of resistant insects to *B. thuringiensis* toxins lead to identify new strains and genes to be used with this purpose. Toxicity bioassays were performed to select *B. thuringiensis* strains with insecticide activity against

Helicoverpa armigera (Hübner). Eight strains, 426, 520B, 1636, 1644, 1648, 1657 and 1658, caused mortality higher than 75% in *H. armigera* larvae and showed LC_{50} beetwen 150.1 e 1543.3 ng/cm^2. Through molecular analysis it was demonstrated that these strains harbor different *cry* and *vip* genes. In addition, they showed protein profile with major bands of 140 and 55 kDa. The insecticidal activity of the strains 426, 520B, 1636, 1641, 1644, 1648, 1657 and 1658 was also evaluated through bioassays with *Anticarsia gemmatalis* (Hübner), *Diatraea saccharalis* (Fabricius), *Spodoptera cosmioide* (Walker) and *Pseudoplusia includes* (Walker), demonstrating that some of these strains cause high levels of mortality in these insects. The results indicate that the selected *B. thuringiensis* strains have great potential to be used in the control of *H. armigera* and other important lepidopteran pests.

Keywords: Western corn rootworm. RNA interference. SID-1. Endocytosis. Entomopathogenic bacteria. Lepidoptera. Biological control.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

O ataque de insetos pragas às lavouras constitui uma das maiores causas de perdas econômicas no campo. O controle destes insetos tem sido realizado ao longo de muitos anos através da utilização de inseticidas químicos. No entanto, muitos problemas têm surgido em decorrência da aplicação dos inseticidas químicos, como poluição ambiental, efeitos colaterais nocivos sobre organismos não-alvo, ressurgimento de populações de pragas devido ao rompimento do controle natural, evolução de resistência nos insetos-alvo e o aumento do custo de produção (XUE et al., 2012).

Afim de substituir ou pelo menos reduzir a ampla utilização dos inseticidas químicos algumas estratégias de controle alternativas têm sido empregadas. Estas estratégias incluem o uso de inimigos naturais, parasitóides, entomopatógenos como o *Bacillus thuringiensis* (Bt) e culturas geneticamente modificadas expressando toxinas de Bt (LACEY et al., 2015). Recentemente, a técnica de RNA de interferência tem-se demonstrado promissora para o desenvolvimento de tecnologias que possam ser empregadas no manejo de insetos pragas (GU; KNIPPLE, 2013).

O mecanismo de silenciamento gênico pós-transcricional pelo qual moléculas de RNA de fita dupla chamadas *double-stranded RNA* (dsRNA) induzem a degradação de mRNAs com sequências homólogas, conhecido como RNA de interferência, foi inicialmente descrito em *Caenorhabditis elegans* e posteriormente demonstrado em outros organismos eucarióticos incluindo insetos (FIRE et al., 1998; MELLO; CONTE, 2004). No mecanismo de RNAi, as moléculas de dsRNA endógenas ou exógenas são clivadas por uma ribonuclease III chamada DICER, em fragmentos de 20-25 pb denominados

small interfering RNAs (siRNAs). As fitas anti-senso dos siRNAs são incorporadas ao complexo de silenciamento induzido por RNA (*RNA-induced silencing complex* – RISC), e usadas como guia para reconhecer mRNAs complementares que são clivados pela enzima Argonauta (FIRE et al., 1998; ELBASHIR et al., 2001; MEISTER; TUSCHL, 2004).

Para que o RNAi seja eficientemente usado como uma ferramenta no controle de insetos pragas, o organismo deve ser capaz de absorver o dsRNA a partir do ambiente e o sinal do RNAi se espalhar por todo o sistema biológico movendo-se através das células (HUVENNE; SMAGGHE, 2010). Pelo menos dois mecanismos de absorção de dsRNA têm sido descritos em invertebrados, por meio do canal transmembranar formado pela proteína SID-1 (*Systemic RNA interference-deficient 1*) (FEINBERG; HUNTER, 2003) e através da endocitose dependente da clatrina (SALEH et al., 2006; ULVILA et al., 2006).

Diabrotica virgifera virgifera (LeConte) (Coleoptera: Chrysomelidae), também conhecida como larva do milho ocidental (western corn rootworm - WCR), é um dos insetos pragas mais importantes economicamente da cultura do milho nos Estados Unidos (TUSKA et al., 2002). A compreensão dos mecanismos associados com a internalização celular de dsRNA e o RNAi sistêmico em *D. virgifera virgifera*, poderá ajudar no desenvolvimento de métodos para o controle desta praga, uma vez que este é um fator limitante da eficiência do RNAi, além de ajudar a desvendar potenciais mecanismos de resistência ao RNAi (MIYATA et al., 2014).

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) é considerada um inseto praga de inúmeras culturas agrícolas e apresenta ampla distribuição em todo o mundo, inclusive no continente americano. No Brasil, os primeiros registros da presença da *H. armigera* com base em caracterizações morfológicas e comprovados através de análises moleculares, foram feitos em 2013 (CZEPAK et al., 2013; EMBRAPA, 2013; TAY et al., 2013). As altas infestações desta

praga causaram enormes perdas econômicas aos agricultores brasileiros devido `a perdas diretas de produtividade e aos recursos gastos com produtos fitossanitários para seu controle em cultivos de soja, algodão e milho. Atualmente, a *H. armigera* é considerada uma das pragas mais importantes da agricultura no Brasil (LEITE et al., 2014). Culturas Bt e biopesticidas a base de Bt têm sido utilizados como estratégias eficientes no controle da *H. armigera* e de outros insetos pragas (SANAHUJA et al., 2011; DOWNES; MAHON, 2012; BLANCO et al., 2016). Contudo, com a ampla adoção destas culturas, principalmente quando não são realizadas as devidas práticas de manejo de resistência, a seleção de insetos resistentes às toxinas do *B. thuringiensis* pode ocorrer, tornando os transgênicos Bt ineficientes (TABASHNIK, BRÉVAULT, CARRIÈRE, 2013).

Desta forma, a busca por cepas de *B. thuringiensis* que possam ser utilizadas no manejo integrado da *H. armigera* é de extrema importância. Para garantir a eficácia do biopesticida no campo, a seleção de cepas com alta toxicidade contra o inseto-alvo é um requisito primordial. Além disso, as cepas selecionadas podem ser uma fonte de novos genes que codifiquem proteínas inseticidas com maior toxicidade ou com diferentes modos de ação das já conhecidas e que possam ser utilizados no desenvolvimento de culturas Bt (AZZOUZ et al., 2015).

2 REFERENCIAL TEÓRICO

2.1 RNA interferente

RNA interferente (RNAi) consiste em um mecanismo envolvido na regulação gênica pós-transcricional através da degradação do mRNA, repressão da tradução ou através de modificações epigenéticas que causam repressão da transcrição, mediado por pequenas moléculas de RNA (CASTEL; MARTIENSSEN, 2013). Este fenômeno primeiramente identificado como co-supressão foi demonstrado em eucariotos através da observação de que a introdução de uma cópia extra do gene *chalcona sintase* (*CHS*) em plantas de petúnia, visando obter pétalas mais pigmentadas, resultou em plantas transgênicas com flores cujas pétalas eram variegadas ou até mesmo completamente brancas. O efeito oposto ao esperado ocorreu devido ao fato de que as cópias do gene da própria petúnia e do transgene foram suprimidos (NAPOLI et al., 1990).

O fenômeno de silenciamento gênico através de RNA foi descrito em um estudo com o nematóide *Caenorhabditis elegans* realizado por Fire e colaboradores em 1998, no qual o termo RNAi foi criado. Neste trabalho, foi demonstrado que a injeção das fitas senso e anti-senso correspondentes a um fragmento do gene muscular *unc22* juntas, resultou em uma alteração fenotípica dos *C. elegans* que passaram a exibir um movimento descordenado, enquanto nos *C. elegans* expostos `as fitas senso e anti-senso separadamente não foi observado mudança no fenótipo (FIRE et al., 1998).

São conhecidas três vias do RNAi, mediadas por siRNAs (*small-interfering RNAs*), miRNAs (*micro-RNAs*) ou piRNAs (*Piwi-interacting RNAs*). A via de miRNAs utiliza principalmente transcritos endógenos a partir do genoma da célula, com estrutura de um dsRNA, para regular processos de

desenvolvimento. Já a via de siRNAs está envolvida principalmente com respostas de defesa contra dsRNAs exógenos, de vírus, por exemplo (TOMARI; DU; ZAMORE, 2007). A via dos piRNAs promove o silenciamento de transposons de células germinativas garantindo assim a estabilidade destas células (BRENNECKE et al 2007).

O mecanismo de RNAi pela via dos siRNAs é desencadeado por moléculas dupla fita de RNA denominadas *double-stranded RNA* (dsRNA). No interior da célula estas moléculas são processadas por RNAses do tipo III (DICER) em pequenos fragmentos de RNA (siRNAs) com aproximadamente 20-25 nucleotídeos (FIRE et al., 1998; ELBASHIR et al., 2001). O siRNA é incorporado ao complexo de silenciamento induzido por RNA (*RNA-induced silencing complex* - RISC), cujos principais componentes são proteínas da família das Argonautas. Durante este processo as fitas do siRNA são separadas e a fita senso (*passenger strand*) é eliminada do complexo RISC, enquanto a fita anti-senso (*guide strand*) é mantida e então usada como guia pelo complexo RISC para encontrar os mRNAs alvos que apresentam homologia de sequência e mediar a sua degradação através da proteína Argonauta (MEISTER; TUSCHL, 2004).

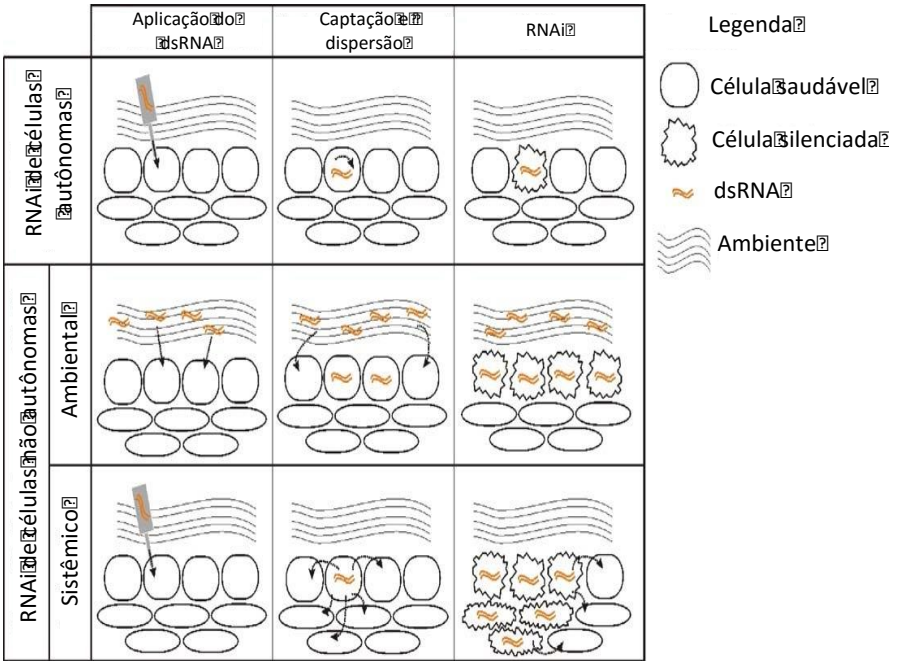
2.2 RNAi de células-autônomas e RNAi de células-não-autônomas

O mecanismo de RNAi pode ser dividido em RNAi de células-autônomas e RNAi de células-não-autônomas. Quando o silenciamento é limitado apenas à célula na qual o dsRNA é introduzido ou expresso, de forma que o efeito do RNAi abrange apenas o interior de células individuais, o processo é chamado RNAi de células-autônomas. Já no caso do RNAi de células-não-autônomas, o silenciamento ocorre em diferentes células ou tecidos

a partir do local que foi introduzido ou produzido o dsRNA (WHANGBO; HUNTER, 2008) (Figura 1).

Existem dois tipos diferentes de RNAi de células-não-autônomas: RNAi ambiental e RNAi sistêmico (Figura 1). O RNAi ambiental refere-se a todos os processos pelos quais o dsRNA é absorvido por uma célula a partir do ambiente. Portanto, este processo também pode ser observado em organismos unicelulares. RNAi sistêmico ocorre apenas em organismos multicelulares uma vez que inclui processos em que o sinal de silenciamento é transportado a partir de uma célula para outra ou de um tecido para outro. Em organismos multicelulares, RNAi ambiental pode ser seguido por RNAi sistêmico e RNAi de células-não-autônomas sempre será seguido por RNAi de células autônomas (JOSE; HUNTER, 2007; WHANGBO; HUNTER, 2008; HUVENNE; SMAGGHE, 2010).

Figura 1 - Esquema representativo dos diferentes mecanismos de RNAi.



No mecanismo de RNAi de células-autônomas o sinal de silenciamento é limitado à célula em que o dsRNA é aplicado. No RNAi ambiental, o dsRNA é absorvido pela célula a partir do ambiente e o sinal de silenciamento é observado em todas as células que absorvem o dsRNA. O RNAi sistêmico envolve todos os processos pelos quais o sinal de silenciamento se dispersa a partir de uma célula na qual o dsRNA é aplicado ou absorvido para outras células ou tecidos.
Fonte: Adaptado de Huvenne e Smagghe, 2010.

Para a eficiente aplicação do RNAi no controle de insetos, especial atenção deve ser dada ao RNAi de células-não-autônomas, visto que o inseto primeiramente terá que ingerir o dsRNA do gene alvo através da alimentação e posteriormente o dsRNA deve ser absorvido do lúmen intestinal pelas células do intestino para que o silenciamento do gene alvo ocorra, representando portanto o RNAi ambiental. Se o gene alvo é expresso em um tecido localizado fora do intestino, o sinal de silenciamento terá que se dispersar através das células e tecidos, representando o RNAi sistêmico (HUVENNE; SMAGGHE, 2010).

2.3 Aplicações do RNAi no controle de insetos pragas

Com o advento das técnicas de sequenciamento de segunda geração, genomas e transcriptomas de inúmeras espécies de insetos puderam ser sequenciados de forma rápida e eficiente, revelando uma grande variedade de genes com funções desconhecidas. Desta forma o RNAi tem sido amplamente usado em estudos de genômica funcional em insetos através da indução de um fenótipo decorrente da supressão do nível de transcritos do gene estudado (BELLÉS, 2010).

Depois dos primeiros estudos demonstrando que o silenciamento gênico em insetos pode ser induzido através da injestão de dsRNA e tornado claro o potencial do RNAi para ser utilizado no controle de insetos pragas, diversos outros trabalhos têm sido realizados com espécies de insetos de diferentes ordens, abordando o mecanismo de resposta de RNAi de células-não-autônomas (ambiental e sistêmico) (ARAÚJO et al., 2006; TURNER et al., 2006; BELLÉS, 2010; MITO et al., 2011; GU; KNIPPLE, 2013).

Pensando em uma maneira prática e de baixo custo para entrega do dsRNA em campo, alguns sistemas de entrega *in vivo* têm sido propostos, por exemplo, através da construção de vetores contendo uma sequência do gene alvo expressos em bactérias (TIAN et al., 2009; LI; ZHANG; ZHANG et al., 2011; ZHU et al., 2011), plantas (BAUM et al., 2007; MAO et al., 2007; PITINO et al., 2011; ZHA et al., 2011; KUMAR; PANDIT; BALDWIN et al., 2012) e vírus hospedeiros de plantas (KUMAR; PANDIT; BALDWIN et al., 2012).

A aplicação tópica do dsRNA também pode ser capaz de induzir o RNAi em alguns insetos. Foi demonstrado que a expressão do gene *AaeIAP1*, responsável pela codificação de uma proteína inibidora da apoptose, em fêmeas do mosquito *Aedes aegypti*, foi suprimida através da aplicação de dsRNA diluído em acetona na região dorsal do tórax, causando significativa mortalidade

(PRIDGEON et al., 2008). Em um outro estudo, o silenciamento dos genes *DS10*, responsável pela codificação da serino-protease C3, e *DS28* o qual codifica uma proteína de função desconhecida, foi induzido através da pulverização de uma solução aquosa de dsRNA diretamente sobre lagartas de primeiro ínstar de *Ostrinia furnacalis*, causando a inibição do crescimento ou morte dos insetos (WANG et al., 2011). A aplicação tópica de dsRNA para cinco genes *CYP4* (genes da família 4 do complexo de citocromos P450 monooxigenases, associados com a resistência a inseticidas) em *Diaphorina citri*, também mostrou ser capaz de causar o silenciamento gênico (KILLINY et al., 2014). Alguns métodos alternativos de entrega do RNAi similares aos inseticidas tradicionais têm sido propostos, tal qual o desenvolvimento de iscas para pragas urbanas, como formigas, baratas e cupins (ZHOU et al., 2008), nanopartículas contendo dsRNA para larvas aquáticas de mosquitos (ZHANG; ZHANG; ZHU, 2010) ou dsRNA envolvido por lipossomos para o controle de *Blattella germanica* (LIN et al., 2016).

A aplicação foliar do dsRNA foi demonstrada como uma maneira viável de utilização do RNAi no controle de insetos pragas. Resultados de um recente trabalho mostraram que o dsRNA é estável como uma aplicação foliar durante pelo menos 28 dias em casa de vegetação, e após o dsRNA secar sobre as folhas, ele não é facilmente removido com água. Neste estudo foi avalido dsRNA do gene *actina* para o controle de *Leptinotarsa decemlineata* em plantas de batata (SAN MIGUEL; SCOTT, 2016).

As plantas transgênicas expressando alguma proteína inseticida de *Bacillus thuringiensis* demonstraram ótimos resultados na proteção de uma ampla categoria de culturas, e portanto têm sido adotadas como uma ferramenta no controle de diversos insetos pragas. No entanto, nem todas culturas apresentam eventos transgênicos Bt que possam ser utilizados pelos agricultores, além do fato de que ao longo do tempo, sob a forte pressão de seleção, insetos

com resistência ao transgênico Bt podem ser selecionados. Além disso, os insetos sugadores, como pulgões, moscas brancas e percevejos, que antes eram considerados pragas secundárias, tornaram-se as principais pragas de algumas culturas, mas tem sido descritas poucas toxinas Bt com efeito inseticida eficiente sobre estas categorias de insetos (CHOUGULE; BONNING, 2012).

Neste contexto, o silenciamento gênico através do RNAi têm sido apontado como uma promissora técnica para complementar os atuais eventos transgênicos Bt e ser a base da nova geração de culturas geneticamente modificadas resistentes a insetos (GATEHOUSE; PRICE, 2011; GU; KNIPPLE, 2013). O primeiro trabalho envolvendo transgênicos baseados em RNAi consistiu no desenvolvimento de um milho transgênico expressando dsRNA do gene alvo *vacuolar ATPase-A* (*vATPase-A*) de *Diabrotica virgifera virgifera*. Este gene codifica a subunidade A do complexo multienzimático vATPase, responsável pela acidificação de organelas celulares e o transporte de prótons H^+ através das membranas celulares. Neste estudo foi demonstrado que após as larvas se alimentarem das plantas geneticamente modificadas, uma alta mortalidade foi observada em decorrência da supressão da expressão do gene *vATPase-A* e consequentemente, redução dos danos causados nas plantas de milho (BAUM et al., 2007).

Mao et al. (2007), desenvolveram plantas de *Arabdopsis thaliana* e *Nicotinum tabacum* geneticamente modificadas capazes de sintetizar dsRNAs do gene *citocromo P450* (*CYP6AE14*) de *Helicoverpa armigera*, cuja proteína é responsável pela desintoxicação do metabólito secundário gossipol, comumente encontrado em plantas de algodão. Desta forma, as lagartas de *H. armigera*, ao ingerir as plantas transgênicas tiveram o gene *CYP6AE14* silenciado, tornando-as incapazes de realizar a desintoxicação do gossipol e causando a inibição do seu crescimento. Posteriormente, foi demonstrado que plantas transgênicas de algodão expressando dsRNAs do gene *CYP6AE14* também foram capazes de

retardar o crescimento das lagartas de *H. armigera*, minimizando os danos nas plantas de algodão transgênicas quando comparado com plantas controles (MAO et al., 2011).

Estes trabalhos demonstram que a alimentação de insetos com plantas transgênicas expressando dsRNAs de algum gene alvo pode ser uma maneira eficaz de ativação do mecanismo de RNAi e pode, portanto, ser utilizada no controle de insetos pragas no campo.

2.4 SID-1 (*Systemic RNA interference-deficient-1*)

Em *C. elegans* o mecanismo celular de absorção de dsRNA do ambiente e dispersão do sinal de silenciamento ocorre através do canal transmembranar SID-1. O gene *sid-1* (*systemic RNA interference-deficient 1*) codifica a proteína SID-1 que contém um longo domínio amino-terminal extracelular seguido por uma série de domínios transmembranares que formam um canal pelo qual as moléculas de dsRNA passivamente atravessam (WINSTON; MOLODOWITCH; HUNTER, 2002; FEINBERG; HUNTER, 2003).

Genes homólogos de *sid-1* foram identificados em vários insetos, porém, apesar de esse gene estar geralmente associado com a internalização de dsRNA, uma correlação direta entre a presença deste gene e a capacidade de absorção e disseminação do dsRNA pelas células não pode ser estabelecida (HUVENNE; SMAGGHE, 2010). *Drosophila melanogaster* não tem homólogos do gene *sid-1* e também não apresenta uma forte resposta sistêmica do RNAi, contudo alguns trabalhos mostram que determinados tecidos, tanto em Drosophila como em outros dípteras, são capazes de absorver dsRNA (DZITOYEVA; DIMITRIJEVIC; MANEV, 2001; BLANDIN et al., 2002; GOTO et al., 2003; ZHU; CHEN; RAIKHEL, 2003; BOISSON et al., 2006).

Alguns lepidópteros, apesar de apresentarem uma ou mais cópias do gene *sid-1*, exibem uma fraca resposta sistêmica do RNAi. Cultura de células de *Bombyx mori* quando expostas ao meio contendo dsRNA apresentaram baixo nível de internalização destas moléculas mesmo que o gene *sid-1* estivesse sendo expresso pelas células. Porém, a superexpressão do gene *sid-1* de *C. elegans* nestas células foi capaz de aumentar significativamente a capacidade de absorção do dsRNA do meio de cultura, sugerindo diferenças funcionais entre os genes *sid-1* de insetos e *C. elegans* (KOBAYASHI et al., 2012; MON et al., 2012).

Outras proteínas SID envolvidas na absorção de dsRNA têm sido descritas em *C. elengans*, dentre elas SID-2 que é responsável pela absorção de dsRNA exclusivamente pelas células intestinais, SID-3 o qual é expressa na maioria dos tecidos e requerida para eficiente internalização de dsRNA, além de SID-5, uma proteína associada com endossomos, que promove o transporte de dsRNA entre as células (WINSTON et al., 2007; HINAS et al., 2012; JOSE et al., 2012).

2.5 Endocitose

Endocitose é um importante processo celular envolvido na absorção de nutrientes e no controle da densidade de receptores na superfície das células. Muitos mecanismos de endocitose requerem depressões ou vesículas revestidas por proteínas, sendo geralmente clatrina a principal proteína. A endocitose pode ser classificada em dois tipos, endocitose dependente da clatrina e endocitose independente da clatrina, o qual não requer esta proteína para desempenhar as suas atividades. A proteína clatrina é constituída de três cadeias pesadas (190 KDa) e três cadeias leves (35 KDa). Multimerizadas, as subunidades de clatrina formam uma estrutura poliédrica que reveste as vesículas formadas durante a

endocitose (MUKHERJEE; GHOSH; MAXFIELD, 1997; ROYLE, 2006; MCMAHON; BOUCROT, 2011).

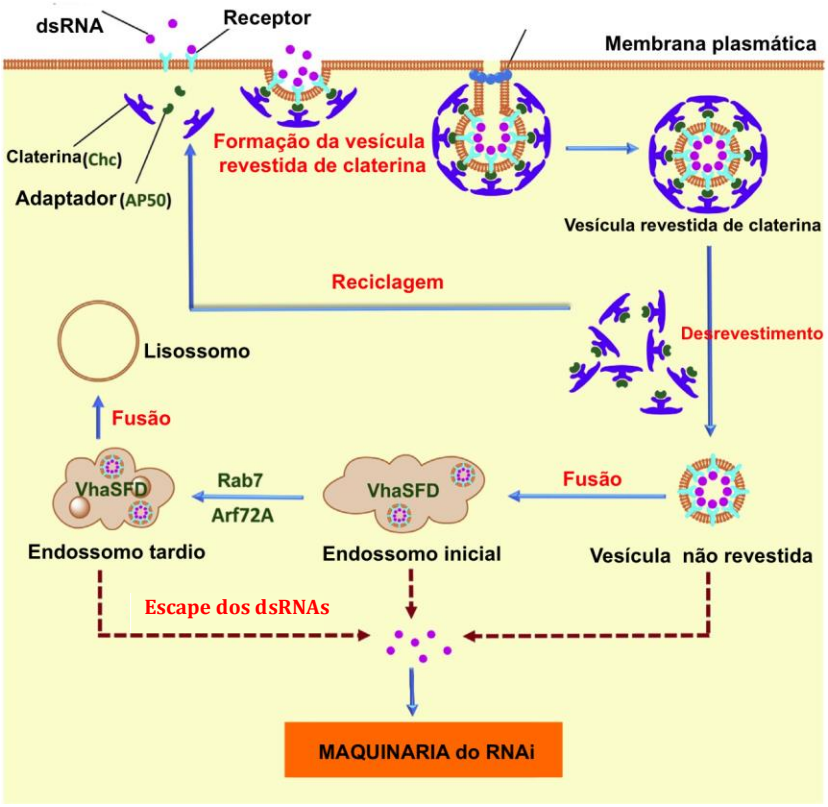
O envolvimento da endocitose dependente da clatrina na internalização de dsRNA tem sido demonstrado em várias espécies de insetos. Ulvila et al. (2006) analisaram 2000 fragmentos de dsRNA, isolados a partir de uma biblioteca de cDNA de células S2 de *D. melanogaster*, para avaliar se estes fragmentos teriam algum efeito inibitório sobre a letalidade induzida pelo RNAi de *Ubi-p63E*, um gene essencial que codifica uma ubiquitina responsável pela viabilidade celular. Eles identificaram quatro genes, dentre eles o responsável pela codificação da cadeia pesada da clatrina. Quando a expressão do gene *clatrina* em células S2 foi suprimida pelo RNAi, as células não morreram mesmo expostas ao tratamento com Ubi-p63E dsRNA, sugerindo que as moléculas são internalizadas pelo mecanismo de endocitose dependente da clatrina.

Em outro estudo, Saleh et al. (2006) rastrearam o genoma de células S2 de *D. melanogaster* e identificaram 23 genes que podiam estar envolvidos na via endocítica e necessários para a incorporação celular de dsRNA. Alguns desses genes têm sido reconhecidos como diretamente e/ou indiretamente envolvidos com a endocitose.

A participação da endocitose na absorção de dsRNA tem sido também demonstrada em carrapato (*Haemaphysalis longicornis*) (AUNG et al., 2011), gafanhoto do deserto (*Schistocera gregaria*) (WYNANT et al., 2014), ácaro predador (*Metaseiulus occidentalis*) (WU; HOY, 2014), besouro castanho (*Tribolium castaneum*) (XIAO et al., 2015), mosca da fruta (*Bactrocera dorsalis*) (LI et al., 2015), e besouro da batata do Colorado (*Leptinotarsa decemlineata*) (CAPPELLE et al., 2016). No entanto, o envolvimento da endocitose na absorção de dsRNA em vários outros insetos, incluindo *Diabrotica virgifera virgifera*, não são bem compreendidos.

Xiao e colaboradores (2015) propuseram um modelo para o mecanismo celular de absorção de dsRNA através da via da endocitose dependente da clatrina consistindo de 5 etapas: 1) Primeiramente, as moléculas de dsRNA ligam-se à proteínas receptoras da membrana plasmática da célula; 2) o dsRNA e o complexo de proteínas receptoras interagem com o complexo da proteína adaptadora 2 (AP-2) formando uma depressão revestida de clatrina na superfície interna da membrana plasmática; 3) em seguida, a depressão vai para dentro da célula formando uma vesícula revestida por clatrina; 4) a vesícula é desrevestida e se funde com um endossomo inicial; 5) o endossomo inicial amadurece, transformando-se no endossomo maduro e posteriormente funde-se ao lisossomo. Nesta via, as moléculas de dsRNA devem ser liberadas dos endossomos para dentro do citosol, onde elas poderão desencadear o processo de RNAi através da maquinaria nuclear do RNAi (Figura 2) (XIAO et al., 2015).

Figura 2 - Mecanismo da endocitose dependente da clatrina envolvido na absorção celular de dsRNA.



A proteína clatrina reveste as depressões na superfície interna da membrana plasmática da célula. Em seguida, a depressão passa para dentro da célula formando uma vesícula revestida de clatrina. A formação da vesícula revestida pela clatrina requer o complexo da proteína adaptadora 2 (AP-2), do qual a subunidade tipo-mu, conhecida como AP50, faz parte. Após as moléculas de dsRNA extracelulares serem internalizadas pela célula, lisossomos e endossomos são acidificados pela bomba de prótons, H (+)-vATPase, uma enzima heteromultimérica constituída por várias subunidades, dentre elas, as subunidades VhaSFD e Vha16. Rab7 é uma proteína relacionada a ras (uma pequena GTPase) e desempenha um papel chave na regulação do tráfico endo-lisossomal e na maturação endossomal. As moléculas de dsRNA devem escapar dos endossomos para o citosol onde irão desencadear o processo de RNAi através da maquinaria nuclear do RNAi.

Fonte: Adaptado de Xiao et al., 2015.

2.6 *Diabrotica virgifera virgifera*

Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae), comumente conhecida como larva do milho ocidental (western corn rootworm - WCR), é um dos insetos pragas mais importantes da cultura do milho nos Estados Unidos. As larvas se alimentam das raízes do milho levando `a diminuição da produtividade da cultura através da redução da absorção de nutrientes e água, além de interfererir na estabilidade da planta. A maior parte dos danos nas plantas é causada por larvas de segundo e terceiro ínstar, mas adultos que se alimentam do estigma ou da espiga também podem ser prejudiciais `a produção de sementes e milho doce (TUSKA et al., 2002).

Inseticidas de solo, tratamentos de sementes contra larvas e aplicações de inseticidas foliares dirigidos aos adultos são alguns dos métodos de controle comuns da *D. virgifera virgifera* (DILLEN et al., 2010). Relatos de evolução de resistência a várias classes de inseticidas, incluindo hidrocarbonetos clorados, carbamatos e organofosfatos, por larvas e adultos de *D. virgifera virgifera* têm sido feitos ao longo dos anos nos Estados Unidos. Este fato somado ao aumento da degradação dos inseticidas pelos micróbios do solo têm reduzido a eficácia dos inseticidas (ROZEN; ESTER, 2010).

Uma prática muito comum adotada pelos agricultores dentro do manejo integrado da *D. virgifera virgifera* consiste na rotação de culturas. Tipicamente, estes insetos requerem campos plantados com milho em anos consecutivos para completar seu ciclo de vida. Os ovos eclodem no final do inverno e as larvas se alimentam das raízes de milho. Os adultos emergem em pleno verão e as fêmeas põem os ovos no solo onde o milho é plantado, e ficam em diapausa obrigatório. Teoricamente, a única cultura onde as larvas podem sobreviver é o milho, então a rotação de culturas anuais, ou seja, a alternância anual de milho com outra

cultura na mesma área, tem sido um importante método de controle (LEVINE; OLOUMI-SADEGHI, 1996).

No entanto, *D. virgifera virgifera* se adaptou `a esta estratégia, apresentando resistência comportamental `a rotação de culturas. Nesse caso, as fêmeas põem ovos em campos que não sejam de milho, geralmente de soja (*Glycine max*), que é plantada após o milho na primavera seguinte (LEVINE; OLOUMI-SADEGHI, 1996). Assim, a *D. virgifera virgifera* conseguiu adaptar-se `as rotações semestrais de milho-soja por meio da ovoposição em soja ou produzindo ovos que permanecem em diapausa por mais de um inverno (LEVINE et al., 2002; MILLER et al., 2009).

Híbridos de milho geneticamente modificados que expressam proteínas inseticidas de *B. thuringiensis* dirigidas `a *D. virgifera virgifera* são amplamente usados para o controle desta praga. Em 2003, o milho expressando Cry3Bb1 começou a ser produzido comercialmente nos Estados Unidos (VAUGHN et al., 2005). Desde então, híbridos de milho expressando as proteínas Cry3Bb1, mCry3A, eCry3.1Ab e Cry34/35Ab1 individulamente ou piramidadas têm sido utilizados comercialmente (DEITLOFF et al., 2015).

No entanto, vários casos de resistência a estes híbridos transgênicos têm sido reportados (NARVA; SIEGFRIED; STORER, 2013; DEVOS et al., 2013). Em decorrência dos problemas de resistência aos atuais métodos empregados no manejo da *D. virgifera virgifera*, novas alternativas precisam ser criadas e o RNAi surge como uma promissora estratégia no combate a esta praga, tanto por meio do desenvolvimento de transgênicos expressando dsRNA de algum gene de interesse, quanto através de outras tecnologias que sejam baseadas no RNAi.

Atualmente o evento transgênico de milho MON 87411 da empresa Monsanto, expressando a proteína Cry3Bb1 de *B. thuringiensis* e dsRNA do gene *Snf7* de *D. virgifera virgifera*, está passando por extensivas avaliações de segurança ambiental e alimentar, para que possa ser liberado comercialmente.

Esta tecnologia `a base do RNAi poderá estar disponível para os produtores em um futuro próximo, sendo portanto, mais uma ferramenta no controle da *D. virgifera virgifera* (FISHILEVICH et al., 2016). O gene *snf7* codifica um componente do complexo ESCRT-III (Complexo de triagem endossomal III) que é essencial para vários processos biológicos, incluindo a ordenação de receptores da membrana celular (KIM et al., 2011).

2.7 *Bacillus thuringiensis*

Uma grande quantidade de espécies bacterianas associadas aos insetos pragas e benéficos tem sido descrita, entretanto poucas bactérias entomopatogênicas são empregadas comercialmente para o controle de insetos pragas de culturas agrícolas, florestas e insetos vetores de doenças. Algumas espécies usadas para este fim são *B. thuringiensis, Lysinibacillus* (*Bacillus*) *sphaericus*, *Paenibacillus spp*. e *Serratia entomophila*, com destaque para várias subespécies de *B. thuringiensis* a partir das quais são produzidas algumas formulações de biopesticidas (JURAT-FUENTES; JACKSON, 2012; LACEY et al., 2015).

Bacillus thuringiensis é uma bactéria Gram-positiva pertencente ao grupo *Bacillus cereus*, capaz de produzir durante a esporulação inclusões cristalinas com propriedades inseticidas, o que a distingue das espécies *B. cereus*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides* e *B. weihenstephanensi* que também estão inclusas neste grupo (DIDELOT et al., 2009). Esta bactéria é amplamente distribuída pelo mundo, podendo ser isolada de diferentes ambientes como solo, água, insetos mortos, poeira de silos de armazenagem de grãos, rizosfera e filoplano (RAYMOND et al., 2010).

O *B. thuringiensis* apresenta atividade tóxica contra uma ampla gama de ordens de insetos, dentre elas Lepidoptera, Diptera, Coleoptera, Hemiptera,

Hymenoptera, Homoptera e Orthoptera (VAN FRANKENHUYZEN, 2009), incluindo diversas pragas agrícolas como *Spodoptera frugiperda*, *Diatraea saccharalis*, *Anticarsia gemmatalis* e *Helicoverpa armigera* (Lepidoptera) e importantes vetores de doenças pertencente aos gêneros Culex, Aedes e Anopheles (Diptera) (LERECLUS; DELECLUSES; LECADET, 1993).

Durante a fase de esporulação o *B. thuringiensis* produz inclusões cristalinas formadas por proteínas Cry e/ou Cyt denominadas δ-endotoxinas que são liberadas juntamente com o esporo após a lise celular (LERECLUS et al., 1989; DE MAAGD et al., 2003) e são as principais responsáveis pela atividade entomopatogênica (HÖFTE; WITELEY, 1989; GLARE; O' CALLANGHAM, 2000). Além das proteínas Cry e Cyt, o *B. thuringiensis* pode produzir uma série de outras proteínas e metabólitos com atividade inseticida como enterotoxinas, quitinases, α-exotoxinas, β-exotoxinas, hemolisinas, fosfolipases (HANSEN; SALAMITOU, 2000) e as Vips (proteínas inseticidas vegetativas) (ESTRUCH et al., 1996). Os esporos também podem contribuir com a patogenicidade através da ação sinérgica realizada juntamente com as δ-endotoxinas (MIYASONO et al., 1994; JOHNSON; McGAUGHEY, 1996).

Dentre os produtos disponíveis no mercado, as formulações `a base de *B. thuringiensis* subesp. *kurstaki* são efetivas contra lepidópteros que atacam importantes culturas agrícolas e florestas. Já as formulações `a base de Bt *aizawai* são principalmente ativas contra lagartas de lepidópteros que se alimentam de grãos estocados. Aquelas `a base de Bt *tenebrionis* e Bt *san diego* para o controle de coleópteros e por último, `a base de Bt *israelensis* são geralmente utilizadas contra dípteros vetores de doenças humanas como *Aedes aegypti* e *Anopheles* sp. (SOBERON; GILL; BRAVO, 2009).

O *B. thuringiensis* tem sido também amplamente utilizado no controle de insetos pragas através do desenvolvimento de culturas transgênicas expressando uma ou mais proteína Cry ou Vip. Devido `a grande aceitação por

parte dos produtores, a área de cultivo em todo o mundo de lavouras Bt tem aumentado enormemente ao longo das últimas duas décadas, ocupando mais de 84 milhões de hectares em 2015 (JAMES, 2015), o que resultou em significante redução do uso de inseticidas químicos e consequentemente dos danos ambientais decorrentes da sua aplicação (CANNON, 2000; JAMES, 2015). Além disto, as toxinas do *B. thuringiensis* por apresentarem especificidade no modo de ação, não apresentam significantes efeitos adversos aos organismos não-alvo (KUMAR; CHANDRA; PANDEY, 2008).

Embora o *B. thuringiensis* seja um efetivo agente de controle biológico, a seleção de insetos resistentes à suas toxinas persiste como um problema causado pela forte pressão de seleção decorrente do intensivo uso destas toxinas, seja através dos biopesticidas ou de plantas transgênicas (Mc GAUGHEY, 1985; TABASHINIK, 1994; JANMAAT; MYERS et al., 2003; TABASHNIK et al., 2008; KRUGER; VAN RENSBURG; VAN DEN BERG, 2009; LIU et al., 2010; STORER et al., 2010; ZHANG et al., 2011; DHURUA; GUJAR, 2011; GASSMANN et al., 2011; YANG et al., 2011; ZHANG et al., 2012). Assim, é de extrema importância identificar e caracterizar novas cepas de *B. thuringiensis* que expressem toxinas ativas contra o inseto-alvo para serem utilizadas no desenvolvimento de novos biopesticidas ou para a identificação e isolamento de genes que possam ser empregados na transformação genética de plantas (BOUKEDI et al., 2016).

2.8 δ-endotoxinas (Cry e Cyt)

As δ-endotoxinas pertencem a uma classe de toxinas bacterianas conhecidas como toxinas formadoras de poros (TFP). Estas toxinas são caracterizadas por serem solúveis em água e sofrerem alterações conformacionais que permitem sua inserção ou translocamento na membrana

celular através da formação de poros que alteram a homeostase iônica e causam a morte do hospedeiro. As TFP são classificadas em dois grupos principais. As toxinas α-helicoidais, sendo que a α-hélice é responsável por formar o poro na membrana celular, e as β-barril, que se inserem na membrana formando um β-barril composto de monômeros de grampos de folhas β-pregueadas. As toxinas Cry são classificadas como α-helicoidais e as Cyt como β-barril (PARKER; FEIL, 2005).

As δ-endotoxinas compreendem duas famílias multigênicas, *cry* e *cyt*, atualmente mais de 770 genes *cry* e 37 genes *cyt* já foram identificados. As proteínas Cry estão classificadas em 74 grupos organizados em diferentes subgrupos, além de existirem 3 grupos de toxinas Cyt (CRICKMORE et al., 2016). Esta classificação é realizada com base na identidade das sequências de aminoácidos destas proteínas (CRICKMORE et al., 1998).

Grande parte das cepas de *B. thuringiensis* produzem mais de um tipo de cristal que pode ser formado por diferentes proteínas Cry e/ou Cyt, como ocorre por exemplo em *B. thuringiensis* subesp. *israelensis*, o qual apresenta 5 genes codificadores de proteínas Cry e um outro gene que codifica uma proteína Cyt, todos localizados em um mesmo plasmídeo de 72 MDa (GONZALEZ; CARLTON, 1984; LERECLUS et al., 1989).

A composição da inclusão cristaliana e as estruturas das toxinas determinam a forma do cristal, podendo ser bipiramidal, cubóide, rombóide, ovóide, esférico, retangular ou ainda sem forma definida (HABID; ANDRADE, 1998). As proteínas são mantidas associadas de forma estável na inclusão parasporal devido `a complexas interações como ligações de hidrogênio, pontes dissulfeto e a hidrofobicidade estabelecidas entre elas (GILL; COWLES; PIETRANTONIO, 1992). Acredita-se que esta capacidade de formar cristais das δ-endotoxinas pode diminuir sua susceptibilidade `a degradação por proteases do hospedeiro (SCHNEPF et al., 1998).

2.9 Estrutura das proteínas Cry

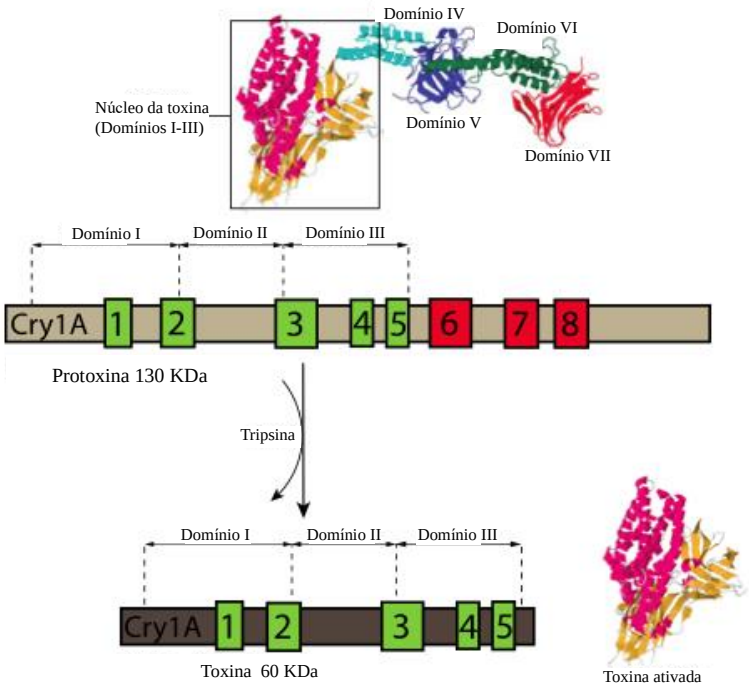
A maioria das proteínas Cry descritas, pertencem `a família de toxinas de três domínios que são caracterizadas por apresentarem uma estrutura de três domínios dentro do núcleo da toxina (DE MAAGD; BRAVO; CRICKMORE, 2001; DE MAAG et al., 2003; BRAVO; GILL; SOBERON, 2007). As toxinas Cry de três domínios apresentam protoxinas com tamanho de 65 ou 130 KDa, o que distingue as protoxinas de 130 KDa das menores é a presença de uma extensão C-terminal que é clivada durante a ativação proteolítica no intestino do inseto (DE MAAGD; BRAVO; CRICKMORE, 2001).

O domínio I (domínio perfurante), localizado na extremidade N-terminal, é formado por um conjunto de sete α-hélices. Durante a ativação da toxina estas estruturas são clivadas proteoliticamente em todas as toxinas Cry de três domínios e isto pode ser responsável pela inserção da toxina na membrane celular e a formação dos poros (SCHNEPF et al., 1998; BEN-DOV, 2014; XU et al., 2014; PALMA et al., 2014). Já o domínio II (domínio central) é composto por três folhas β-antiparalelas e desempenha um importante papel nas interações receptor-toxina (JENKINS; DEAN, 2000; XU et al., 2014). O domínio III (domínio de ligação de galactose) é clivado proteoliticamente em algumas toxinas Cry de três domínios, sendo constituído por um sanduíche de folhas-β e também está envolvido na ligação ao receptor e na formação de poros (XU et al., 2014).

A análise da estrutura da protoxina Cry1Ac, por exemplo, revelou a presença de 7 domínios e 8 blocos de aminoácidos conservados. A região da toxina é dobrada em três domínios enquanto que a pro-região prolongada forma 4 domínios adicionais. Os domínios IV e VI são α-helicoidais enquanto os domínios V e VII têm uma topologia do tipo β-barril (EVDOKIMOV et al., 2014).

A presença de cinco blocos de aminoácidos conservados, localizados no núcleo de atividade tóxica destas proteínas (domínio I, II e III) e três outros blocos conservados localizados fora do núcleo de atividade e na região C-terminal da protoxina foram revelados através do alinhamento de múltiplas sequências de diferentes proteínas Cry (Figura 3).

Figura 3 - Blocos de aminoácidos conservados e domínios de proteínas Cry de três domínios.



As caixas verdes representam os cinco blocos de aminoácidos conservados localizados no núcleo tóxico da proteína Cry (HÖFTE & WHITELEY, 1989). As caixas vermelhas indicam os três blocos de aminoácidos conservados presentes na extensão C-terminal (SCHNEPF et al., 1998).
Fonte: Adaptado de Palma et al., 2014.

2.10 Receptores

A interação das toxinas Cry com as microvilosidades da membrana apical das células do intestino médio do inseto envolve diferentes proteínas que atuam como receptores, incluindo aminopeptidase-N (APN), fosfatase alcalina (ALP), proteínas do tipo caderina (CADR), um glicoconjugado (GCR) de 270 KDa e proteínas transportadoras da família ABCC (BRAVO; GILL; SOBERON, 2007; PIGOTT; ELLAR, 2007; SOBERON; GILL; BRAVO, 2009; TANAKA et al., 2013; PARK; KIM, 2013; PARK et al., 2014). Glicolipídeos ou proteínas intracelulares, tais como v-ATPase subunidade A e actina também tem demonstrado ser capaz de interagir com as toxinas Cry (McNALL; ADANG, 2003; GRIFFITS, 2005; KRISHNAMOORTHY et al., 2007; BAYYAREDDY et al., 2009).

As caderinas constituem uma superfamília de proteínas altamente diversas, envolvidas em muitas funções, dentre elas adesão intracelular (ANGST; MARCOZZI; MAGEE, 2001). A interação das toxinas Cry com as caderinas foi demostrada inicialmente através de uma glicoproteína de 210 KDa identificada nas vesículas da borda escovada da membrana apical (BBMVs) de *Manduca sexta* (VADLAMUDI; JI; BULLA, 1993). Posteriormente, esta glicoproteína foi clonada, expressa e caracterizada como uma proteína do tipo caderina e denominada BT-R_1, sendo então validada como um receptor da toxina Cry1Ab (VADLAMUTI et al., 1995).

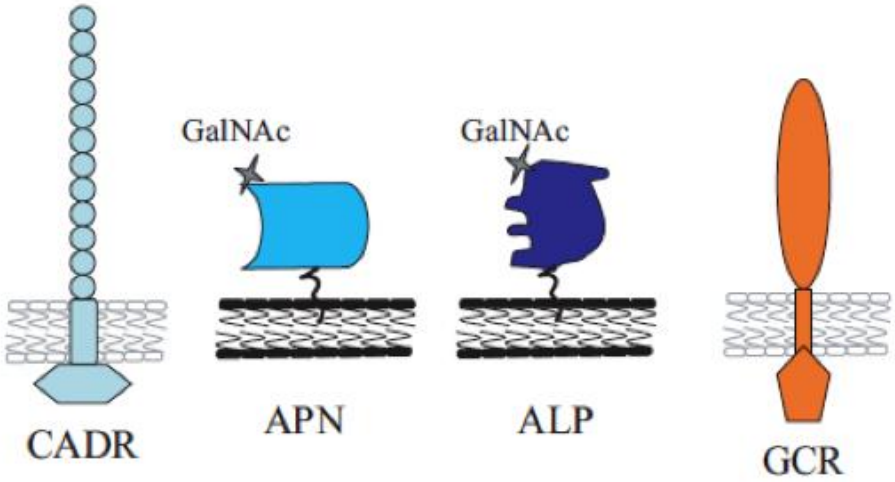
Desde então, tem sido demonstrado que as caderinas atuam como receptores de diferentes toxinas Cry em espécies de pelo menos três ordens de insetos, incluindo Lepidoptera, *Bombyx mori*, *Heliothis virescens*, *Helicoverpa armigera*, *Pectinophora gossypiella*, *Spodoptera exigua*, *Ostrinia nubilalis*; Diptera, *Anopheles gambiae*, *Alphitobius diaperinus*, *Aedes aegypti*; e Coleoptera, *Tenebrio molitor*, *Henosepilachna vigintioctomaculata* e *Tribolium*

castaneum (PIGOT; ELLAR et al., 2007; HUA et al., 2008, 2013; CHEN et al., 2009, 2014; FABRICK et al., 2009; PARK et al., 2009; BRAVO et al., 2011; LIKITVIVATANAVONG et al., 2011; SONG et al., 2012; CONTRERAS et al., 2013; PARK; KIM, 2013; REN et al., 2013; HUA; PARK; ADANG, 2014). Em Lepidoptera, os receptores caderina que se ligam `as toxinas Cry são constituídos por cinco domínios, o peptídeo sinal, 8 a 12 repetições de caderina, o domínio extracelular proximal da membrana, a região transmembrana e o domínio citoplasmático interno (BEL; ESCRICHE, 2006).

Os receptores do tipo APN e ALP são proteínas ancoradas ao glicosilfosfatidil-inositol (GPI) e estão localizados no interior de plataformas de lipídeos (*Lipid Rafts*) que constituem pequenos domínios na membrana celular. Essas estruturas apresentam altas concentrações de colesterol e glicoesfingolipídeos, estando provavelmente envolvidas em vias de transdução de sinal (GRIFFITS, 2005).

As ALPs e APNs também tem sido amplamente estudadas como receptores das toxinas Cry em diversos insetos, tais como *Manduca sexta, Bombix mori, Helicoverpa armigera, Heliothis virerescens, Lymantria dispar, Plutella xylostella, Spodoptera litura, Epiphyas postvi*, os quais pertencem à ordem Lepidoptera, além de *Aedes aegypty, Anopheles aebimanus, Anopheles quadrimaculatus, Anopheles gambiae* da ordem Diptera e *Anthonomus grandis* da ordem Coleoptera (PIGOT; ELLAR et al., 2007; ZÚÑIGA-NAVARRETE, et al., 2012).

Figura 4 - Moléculas receptoras de proteínas Cry1A.



CADR (caderina), APN (aminopeptidase-N), ALP (fosfatase alcalina), GCR (glicoconjugado).
Fonte: Bravo; Gill; Soberon, 2007.

2.11 Modo de ação das proteínas Cry

O modo de ação das proteínas Cry tem sido proposto com base em diversos estudos realizados com proteínas principalmente pertencentes à classe Cry1 em Lepidoptera (HÖFTE; WHITELEY, 1989; BRAVO; GILL; SOBERON, 2007). Através do modelo de ligação sequencial, o qual é o mais aceito, a toxina ativada passa por complexos eventos de ligações sequenciais `a diferentes receptores presentes nas células do intestino médio da lagarta, resultando na formação de poros na membrana celular (BRAVO; GILL; SOBERON, 2007).

Após serem ingeridas, as inclusões cristalinas são solubilizadas no intestino médio alcalino das lagartas susceptíveis e então ativadas por proteases intestinais que realizam a clivagem de pequenos fragmentos das extremidades N e C terminais (BRAVO et al., 2004). Posteriormente, ocorrem sucessivas ligações das toxinas Cry aos receptores intestinais das lagartas, envolvendo dois

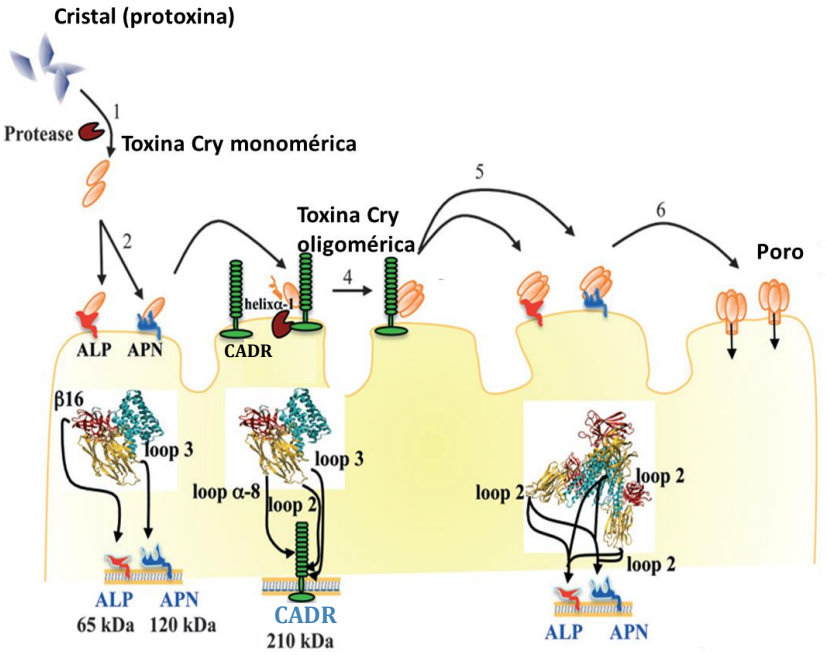
passos, um reversível e outro irreversível, sendo portanto bifásica. Na etapa reversível, ocorre o reconhecimento da proteína pelo receptor mas a separação do complexo toxina-receptor ainda é possível, enquanto na segunda etapa, ocorre a inserção da toxina na membrana apical da célula tornando a ligação irreversível (VAN RIE et al., 1990; SCHENEPF et al., 1998).

Em lagartas, as toxinas ativas Cry1A primeiramente se ligam aos receptores ALP e APN através de uma interação de baixa afinidade (MASSON et al., 1995; PACHECO et al., 2009; ARENAS et al., 2010). A interação com ALP e APN concentra a toxina ativada nas microvilosidades da membrana das células do intestino médio tornando-as aptas a se ligarem através de uma interação de alta afinidade com os receptores CADR (VADLAMUDI et al., 1995; GOMEZ et al., 2006; PACHECO et al., 2009; ARENAS et al., 2010).

A interação das proteínas Cry com a CADR é complexa e envolve três epítopos da CADR, correspondentes às regiões extracelulares denominadas CR7, CR11 e CR12. Estes epítopos interagem com as alças expostas 2, 3 e α-8 do domínio II da toxina Cry, promovendo uma mudança conformacional da CADR através da clivagem proteolítica da extremidade N-terminal, incluindo a α-hélice 1 do domínio I (GOMEZ et al., 2002; ATSUMI et al., 2008). Esta clivagem expõe regiões hidrofóbicas do domínio I, necessárias para a oligomerização das toxinas Cry (na forma de um tetrâmero) que corresponde à estrutura chamada pré-poro da toxina (GOMEZ et al., 2002; PACHECO et al., 2009; ARENAS et al., 2010).

A estrutura oligomérica Cry apresenta maior afinidade pelos receptores ALP e APN, assim o oligômero se liga a estes receptores e em seguida ocorre a inserção da toxina ativa na membrana de forma irreversível, induzindo a abertura ou formação de poros que provocam uma quebra no balanço osmótico da célula e consequente lise celular (PARDO-LÓPEZ et al., 2006; ARENAS et al., 2010; PARDO-LÓPEZ et al., 2013).

Figura 5 - Representação esquemática do mecanismo de ação das toxinas Cry em Lepidoptera.



1- A protoxina após ser ingerida pelo inseto é solubilizada no lúmen do intestino médio das lagartas devido ao elevado pH e às condições de redução. Posteriormente ela é ativada por proteases do intestino, gerando o fragmento de toxina. 2- A toxina Cry monomérica liga-se aos receptores ALP e APN, numa interação de baixa afinidade, a toxina fica então localizada em estreita proximidade com a membrana. 3- A toxina Cry monomérica se liga ao receptor CADR numa interação de alta afinidade e esta interação induz a clivagem proteolítica da extremidade N-terminal da toxina, incluindo a hélice α-1 do domínio I. 4- A toxina Cry clivada torna-se capaz de formar um oligômero com as outras toxinas constituindo uma estrutura de pré-poro. 5- A estrutura oligomérica Cry liga-se com alta afinidade aos receptores ALP e APN. 6- O pré-poro insere-se na membrana promovendo a formação de poros.
Fonte: Adaptado de Pardo-Lopez et al., 2013.

2.12 *Helicoverpa armigera*

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) é uma praga altamente polífaga que ataca várias culturas agrícolas importantes como o algodão, tabaco, tomate, pimentão, milho, soja, além de plantas ornamentais e

flores, causando grandes perdas econômicas (EPPO, 2007). Ela apresenta ciclo holometábolo passando pelas fases de ovo, lagarta, pré-pupa, pupa, adulta e o seu período larval é completado com o desenvolvimento de seis ínstares. Após as lagartas interromperem sua alimentação até o momento em que elas empupam caracteriza a fase de pré-pupa. A fase de pupa da *H. armigera* perdura normalmente entre 10 e 14 dias (ALI; CHOUDHURY, 2009). O desenvolvimento pupal ocorre no solo e de acordo com as condições climáticas pode entrar em diapausa (KARIM, 2000). Os adultos fêmeas têm uma longevidade média de 11,7 dias, um pouco superior `a longevidade dos machos, o qual é de 9,2 dias (ALI; CHOUDHURY, 2009). Uma única fêmea é capaz de pôr entre 2200 e 3000 ovos sobre as plantas hospedeiras durante o período de oviposição de aproximadamente 5,3 dias (NASERI et al., 2011), caracterizando o elevado potencial reprodutivo deste inseto.

Esta praga é a mais importante da agricultura na Ásia, Europa, África e Austrália, os danos causados `as culturas são estimados em mais de 2 bilhões de dólares por ano, excluindo os custos sócio-econômicos e ambientais associados ao seu controle (TAY et al., 2013). A *H. armigera* foi reconhecida no Brasil como uma praga quarentenária desde 1999 e foi incluida na categoria de praga quarentenária A1 em 2008 (OLIVEIRA et al., 2003; AGROPEC CONSULTORIA, 2013). Em 2013 foram feitos os primeiros registros da ocorrência da *H. armigera* no Brasil, sendo reconhecida como uma grave ameaça para a segurança biológica não só deste país mas de todo continente Americano que até então não apresentava registros desta praga (CZEPAK et al., 2013; TAY et al., 2013; SPECHT et al., 2013).

No mesmo ano esta praga teve registros no Paraguai (SENAVE, 2013) e em 2014 apresentou relatos também na Argentina (MURÚA et al., 2014), Bolívia, Uruguai (KRITICOS et al., 2015) e Porto Rico (NORTH AMERICAN PLANT PROTECTION ORGANIZATION [NAPPO] 2014). Dados publicados

em um recente trabalho, no qual foi utilizado um modelo de distribuição potencial para avaliar a ameaça de invasão global desta praga, com ênfase nos riscos para os Estados Unidos, sugeriram que a dispersão da *H. armigera* para a América do Norte é uma questão de tempo (KRITICOS et al., 2015). Já em 2015, três indivíduos machos de *H. armigera* foram coletados através de uma armadilha de feromônio em uma plantação de tomate em Bradenton, Flórida, sendo o primeiro relato desta praga nos Estados Unidos (HAYDEN; BRAMBILA, 2015).

Nas safras 2012/2013 e 2013/2014, altas infestações de lagartas de *Helicoverpa* spp. foram detectadas em diferentes regiões do Brasil. O ataque destes insetos `a várias culturas causaram significantes perdas econômicas (POMARI-FERNANDES; DE FREITAS BUENO; SOSA-GÓMEZ, 2015). Somente na safra 2012/2013 após a ocorrência da *H. armigera* no Brasil os danos causados por ela foram de aproximadamente 800 milhões de dólares (BUENO et al. 2014).

O controle da *H. armigera* é baseado no uso de inseticidas sintéticos, entretanto como resultado de sucessivas aplicações, trinta por cento de todos os pesticidas usados no mundo diretamente contra *H. armigera* tornaram-se ineficazes devido `a seleção de populações resistentes (KRANTHI et al., 2002; UDAMALE et al., 2013). Biopesticidas `a base de *B. thuringiensis* e principalmente plantas transgênicas expressando toxinas de *B. thuringiensis* têm sido utilizados como estratégias alternativas aos pesticidas sintéticos no manejo integrado de muitos insetos pragas, incluindo a *H. armigera* (SHARMA, 2005; HÉMA et al., 2009; KNIGHT; HEAD; ROGERS, 2015; YANG; LI; WU, 2013).

O plantio de algodão Bt aliado `a estratégias de manejo da resistência para o controle da *H. armigera* tem sido muito bem sucedido na Austrália (DOWNES; MAHON, 2012). No Brasil, as culturas geneticamente modificadas

expressando toxinas Cry incluem milho, soja e algodão. Embora elas tenham sido introduzidas para controlar pragas de lepidópteros nativas, como *Helicoverpa zea*, *Heliothis virescens* e *Spodoptera frugiperda*, as experiências australianas sugerem que elas podem ser eficazes também contra *H. armigera* quando combinado com uma estratégia de manejo adequada (KRITICOS et al., 2015). Contudo, como estas culturas expressam toxinas Cry pertencentes `a mesma classe, no caso Cry1, somado ao fato de que extensas áreas são cultivadas com estes transgênicos, muitas vezes sem a adoção de áreas de refúgio, expõe as populações de *H. armigera* `a forte pressão de seleção, podendo tornar estes eventos transgênicos ineficazes no combate desta praga em um curto período de tempo se táticas de manejo de resistências não forem adotadas (TAY et al., 2013).

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Normas de formatação da Revista Insect Biochemistry and Molecular Biology (Versão Preliminar)

Endocytic pathway is associated with double-stranded RNA uptake in the western corn rootworm (*Diabrotica virgifera virgifera* LeConte)

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ABSTRACT

RNA interference (RNAi) is a technique used to silence specific target genes in a variety of eukaryotes and has recently been suggested as a potential tool for insect pest management. The uptake of dsRNA after dietary exposure is critical for RNAi efficiency however the exact mechanism of uptake remains to be defined in many insects. In this study we evaluated the role of putative SID-1-like transmembrane proteins and genes of the endocytic pathway in dsRNA uptake after oral exposure in the western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte). For this purpose, we used a two-stage dsRNA exposure bioassay in which WCR adults were first exposed to dsRNA targeting the putative *sid-1*-like (*sil*) or endocytic genes followed by exposure of a secondary dsRNA (*vATPase-A* or *laccase2*). We first evaluated the *sid-1*-like genes (*silA* and *silC*) with a secondary exposure to the lethal *vATPase-A* dsRNA. Our data indicated that the silencing of *silC* gene did not yield a significant reduction in mortality of WCR adults or increase in *vATPase-A* expression. While the silencing of *silA* significantly decreased the mortality and changes in *vATPase-A* expression could not be detected. Subsequently, we evaluated genes involved in clathrin-dependent endocytosis including *Clath*, *Vha16*, *AP50*, *Arf72A*, and *Rab7*, followed by exposure to *laccase2* dsRNA that affects integumental pigmentation. *Arf72A* and *Rab7* knockdown did not suppress the RNAi response of *laccase2*, but the silencing of *AP50* led to a significant decrease in RNAi response to *laccase2* dsRNA. Additionally, both *Clath* and *Vha16* knockdown reduced *laccase2* dsRNA uptake, although the trend was not as clear as for *AP50*. Taken together, the results of this study suggest that SID-1-like proteins are not critical for cellular dsRNA uptake, however endocytosis is an important mechanism required for dsRNA uptake in WCR adults.

Keywords: RNA interference, SID-1, endocytic pathway, dsRNA uptake, *Diabrotica virgifera virgifera*.

1 INTRODUCTION

RNA interference (RNAi) is a biological mechanism of post-transcriptional gene silencing triggered by double-stranded RNA (dsRNA) molecules (Hammond et al., 2001). In the cell, the dsRNA is first processed by the RNase III enzyme, called Dicer, into small interfering RNAs (siRNAs) of approximately 20-25 nucleotides. The siRNA strands are separated and one of the strands, known as guide strand, is incorporated into the RNA-induced silencing complex (RISC). The Argonaute 2 protein, which is the main component of the RISC, uses the guide strand to recognize the target mRNAs with complementary sequences and catalyze their cleavage (Hammond et al., 2001; Hammond, 2005; Shabalina and Koonin, 2008).

Experiments conducted with a variety of insect orders have shown that RNAi can be used as a potential tool for insect pests management by induction of a RNAi response after ingestion of dsRNA (Baum et al., 2007; Mao et al., 2007; Bélles, 2010; Mito et al., 2011; Terenius et al., 2011). The mechanism that allows the dsRNA uptake from the gut lumen by midgut cells and the systemic spread from cell to cell has a significant influence in the RNAi efficiency. A better understanding of these processes could aid in the development and improvement of RNAi technologies for insect pests management.

The systemic RNA interference-defective-1 (SID-1) protein is perhaps the best studied of those proteins required for dsRNA uptake and systemic RNAi (Winston et al., 2002; Feinberg and Hunter, 2003). In *Caenorhabditis elegans*, the SID-1 multi-transmembrane domain protein is expressed on the cell membrane and functions as a channel for the passive transport of dsRNA between cells (Feinberg and Hunter, 2003). Besides SID-1, other proteins are involved in dsRNA uptake in *C. elegans*, including SID-2, SID-3, and SID-5. SID-2 is responsible for the active import of environmental dsRNA from the

intestinal lumen (Winston et al., 2007; McEwan et al., 2012), while SID-3 is a conserved tyrosine kinase which allows the entry of dsRNA into cells (Jose et al., 2012). In contrast, SID-5 is an endosome-associated protein, believed to be involved with the spread of the RNAi signal (Hinas et al., 2012; Rocheleau, 2012).

Homologous sequences of the *sid-1* gene have been identified in many species from different orders with the exception of Diptera (Tomoyasu et al., 2008). An association was initially proposed between the presence of *sid-1*-like (*sil*) genes and systemic spread of dsRNA since in *Drosophila melanogaster* and other dipterans, *sid-1*-like genes are absent and these species do not display a robust systemic RNAi response. However, subsequent studies with other insect orders demonstrated that the presence of *sid-1*-like genes does not necessarily result in a robust systemic RNAi response. For example, the silkworm, *Bombyx mori*, harbors three *sid-1*-like genes, but it does not exhibit a strong systemic RNAi response (Tomoyasu et al., 2008).

Endocytosis has been suggested as an alternative dsRNA uptake mechanism in *D. melanogaster* S2 cells. The silencing of the *clathrin heavy chain* (*Clath*) gene, which plays a critical role in clathrin-dependent endocytosis, reduced the lethality of S2 cells after exposure to the dsRNA of *Ubi-p63E*, an important gene for cell viability. In addition, the dual knockdown of the scavenger receptors, SR-CI and Eater, which mediate endocytosis, reduced the internalization of dsRNA in the S2 cells up to 90% (Ulvila et al., 2006). Saleh et al. (2006) performed a functional screening of a dsRNA library of *D. melanogaster* S2 cells and found several genes involved in the endocytic pathway that have been shown to be necessary for cellular uptake of dsRNA. The requirement of the endocytosis pathway for cell entry of dsRNA has since been demonstrated in other insects such as *Schistocerca gregaria* (Wynant et al.,

2014), *Bactrocera dorsalis* (Li et al., 2015), *Tribolium castaneum* (Xiao et al., 2015), and *Leptinotarsa decemlineata* (Cappelle et al., 2016).

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte is one of the most important insect pests of corn throughout the U.S. Corn Belt. Besides, WCR is projected to be the first insect that will be managed with RNAi by plants expressing dsRNA. Control failures of WCR with synthetic insecticides, crop rotation, and transgenic plants expressing *Bacillus thuringiensis* toxins have become a serious problem and new management strategies are urgently needed (Metcalf, 1983; Meinke et al., 1998; Wangila et al., 2015; Devos et al., 2013; Levine et al., 2002; Rozen and Ester, 2010; Gassmann et al., 2014).

WCR exhibits a robust systemic RNAi response induced by direct injection (Alves et al., 2010) or feeding of dsRNA provided in artificial diet (Rangsamy and Siegfried, 2012; Li et al., 2015; Hu et al., 2016) or in transgenic plants expressing dsRNA (Baum et al., 2007; Li et al., 2015; Hu et al., 2016). Transgenic maize events based on RNAi for WCR management are likely to be deployed by the end of the decade to complement other management strategies (Fishilevich et al., 2016). Understanding the mechanism of dsRNA uptake and the spread of the RNAi signal will contribute to the improvement of RNAi efficiency for the management of WCR and other insect pests. Furthermore, these studies could provide information on potential mechanisms of resistance to RNAi based technologies.

In the present study, we report that the RNAi response to dsRNA of the marker gene, *vATPase-A*, reduced when the *silA* gene was silenced, but not when the *silC* gene or both genes were silenced simultaneously. This finding suggests that dsRNA entry and dispersion is not predominantly linked to SIL proteins in WCR adults. However, the interruption of the endocytic pathway by the silencing of *AP50*, *Clath* and *Vha16* genes decreased the suppression of a

second marker gene, *laccase2*, suggesting that endocytosis is involved in dsRNA uptake in WCR adults.

2 MATERIAL AND METHODS

2.1 Insects

Non-diapause newly emerged WCR adults were purchased from Crop Characteristics Inc. (Farmington, MN). The artificial diet used in all bioassays was modified from Branson and Jackson (1988). The diet consisted of 6 g of the dry ingredients reported by Branson and Jackson (1988), 12.5 ml of water, 0.365 g of agar, 0.7 ml of glycerol, and 27.5 µl of a solution of 47% propionic acid and 6% phosphoric acid to reduce microbial contamination. The diet was dispensed into a Petri dish, allowed to solidify at room temperature, and diet plugs were cut using a cork borer (4 mm diameter). WCR adults were kept in a growth chamber at 23 ± 1 °C, 75 ± 5% relative humidity with 16:8 photoperiod.

2.2 Identification of putative *sid-1*-like and endocytic pathway genes in the WCR transcriptome

Putative *sid-1*-like (*silA* and *silC*) and endocytic pathway genes were identified by comparing nucleotide sequences reported for *S. gregaria* and *T. castaneum* against the WCR transcriptome described by Eyun et al. (2014). The nucleotide sequences encoding for clathrin heavy chain (Clath) (KJ135005.1) and vacuolar H-ATPase 16 (Vha16) (KJ135006.1) from *S. gregaria* were used as query sequences to search for putative homologs in the WCR transcriptome. The sequences encoding for clathrin coat assembly protein AP50 (AP50) (NM_001293581.1), ras-related protein (Rab7) (KJ476829.1), ADP-ribosylation factor-like protein 1 (Arf72A) (XM_967932.3), systemic RNA interference

defective-1-related A (Sid-1A) (NM_001105542.1), and systemic RNA interference defective-1-related C (Sid-1C) (NM_001105658.1) from *T. castaneum* were used as queries.

2.3 Double stranded RNA (dsRNA) preparation

Total RNA was isolated from the whole body of a single WCR adult using GeneJET RNA Purification Kit (Fermentas - Thermo Scientific, Waltham, MA) following manufacturer`s instructions. First-strand cDNA was generated with 500 ng of total RNA with the Quantitech Reverse Transcription Kit (Qiagen, Valencia, CA). Gene specific primers were designed with Primer3Plus software (Koressaar and Remm, 2007; Untergasser et al., 2012) to amplify *silA*, *silC*, *Clath*, *Vha16*, *AP50*, *Arf72A*, *Rab7*, *laccase2*, and *GFP* genes, all primers contained a T7 promoter at the 5` end. For the amplification of *vATPase-A*, the primers described by Rangasamy and Siegfried (2012) were used (Supplementary Table 1). All amplification products were sequenced to confirm the identity. For negative control, the non-specific green fluorescence protein gene (*GFP*) was amplified from pIZT/V5-His expression vector (Invitrogen, USA).

The amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and used as a template for in vitro dsRNAs synthesis using the MEGAscript high-yield transcription Kit (Applied Biosystems Inc., Foster City, CA). The synthesized dsRNAs were purified using the RNAeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer`s instructions, quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Franklin, MA), and examined by agarose gel electrophoresis to determine purity and integrity.

2.4 Insect bioassay for functional analysis of *silA* and *silC* genes

To determine if the lethal effect of the *vATPase-A* dsRNA would be altered by the knockdown of *sil* genes, a volume of 0.6 µl of *silA* and *silC* dsRNA at 1 µg/µl, and a mixture of *silA* and *silC* dsRNA containing equal amounts of both dsRNAs for a total of 600 ng were injected into individual beetles. Insects were injected between the coxae of the last pair of legs using a glass capillary syringe and then fed with untreated diet. WCR adults were also injected with dsRNA targeting the gene *laccase2*, which is required for cuticular pigmentation and is not lethal to WCR, to determine if competitive inhibition of dsRNA occurs when multiple species-specific dsRNAs were provided to beetles. WCR adults injected with *GFP* dsRNA were used as control and uninjected adults were used to assess mortality associated with the injury caused by the injection. The beetles were anaesthetized with carbon dioxide to facilitate the injections.

For the treatments involving exposure to the *vATPase-A* dsRNA, diet plugs (4 mm diameter x 2 mm height) were surface-treated with 500 ng of dsRNA and provided 3, 5, 7, 9 and 11 days after injection. For the remainder of the assays beetles were provided with untreated diet for a total of 14 days (Figure 1). On day 7, one beetle was collected from each treatment, snap frozen in liquid nitrogen and stored at -80 °C until total RNA extraction. Three replications each with 16 beetles were used for each treatment and mortality was recorded daily until day 14.

2.5 Insect bioassay for functional analysis of endocytic genes

To determine the involvement of endocytosis in dsRNA uptake by the knockdown of endocytic pathway genes, we varied the time between the first

and second dsRNA exposure according to the time necessary for gene silencing and the effect of the gene silencing in the survival of the WCR adults. The bioassays with *Clath* and *Vha16* were performed in 5 days with the first and second dsRNA exposure on day 0 and day 3. The bioassays with *AP50, Arf72A* and *Rab7* were performed over 10 days since these genes had weak knockdown after 3 days of exposure to dsRNA. The first and second dsRNA exposure occurred on days 0 and 8 (Figure 2).

For this experiment, both dsRNA exposures were performed by feeding. During the first dsRNA exposure WCR adults were fed with diet plugs treated with 600 ng of *Clath, Vha16, AP50, Arf72A, Rab7* or *GFP* dsRNA every other day. In the second dsRNA exposure, new diet plugs coated with 600 ng of *laccase2* or *GFP* dsRNA were provided to the WCR adults on day 3 or 8 according to the group treatment. Immediately before the second dsRNA exposure and two days after, WCR adults were collected from all the treatments, flash-frozen in liquid nitrogen and stored at -80 °C until total RNA extraction. Three replications each with 14 beetles per treatment were performed.

2.6 Gene expression profiles

The expression of the *silA, silC, Clath* and *Vha16* genes in WCR were evaluated in 6 different life stages – egg, first instar larva, second instar larva, third instar larva, pupa and adult (female and male) and in different tissues from third instar larvae (head, gut, fat body and integument) and from adults (head, thorax, abdomen and gut). Three replicates were performed, each including 50 eight-day-old eggs, 30 first instar larvae, 25 second instar larvae, 10 third instar larvae, 1 pupa, 1 adult, 15 heads, 15 guts, 10 fat bodies, 10 integuments from larval stage and 3 heads, 3 thoraxes, 3 abdomens, 15 guts from adult stage,

pooled as a biological sample. All samples were flash-frozen in liquid nitrogen and stored at -80 °C until total RNA extraction.

2.7 Real time PCR

cDNAs were synthetized with the Quantitec Reverse Trascription Kit (Qiagen, Valencia, CA) using 500 ng of RNA and following the manufacturer`s instructions. The qPCR reactions included 1 µl of cDNA diluted 50x, 5 µl of Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA), 0.2 µl at 10 µM of each primer and 3.6 µl of nuclease free water, for a total volume of 10 µl. The primers were designed with Primer3Plus (Koressaar and Remm, 2007; Untergasser et al., 2012) and validated by PCR amplification efficiencies (E) and correlation coefficients (R^2) analysis (Supplementary Table 1). Both primer efficiency test and qPCR were performed with a 7500 Fast RT-PCR system (Applied Biosystems, Grand Island, NY). The thermocycler conditions were one cycle at 95 °C for 20s, followed by 40 cycles of denaturation at 95 °C for 3s and annealing/extension at 60 °C for 30s. At the end of each PCR reaction, a melting curve was generated to confirm a single peak and rule out the possibility of primer-dimers and nonspecific product formation. The expression of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and *β-actin* was used as reference gene (Rangasamy and Siegfried, 2012).

2.8 Statistical analysis

Gene expression and mortality were subjected to an analysis of variance (ANOVA) using PROC GLIMMIX and the least-square statement was used for pairwise comparisons among the treatments. All statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC). Data

were expressed as mean ± standard error of mean (SE), and values of $p < 0.05$ were considered statistically significant.

3 RESULTS AND DISCUSSION

3.1 Identification of putative *sid-1*-like and endocytic pathway genes in the WCR transcriptome

The putative *sid-1*-like (*sil*) and endocytic pathway genes were identified by searching a WCR transcriptome database (Eyun et al., 2014) using nucleotide sequences from *S. gregaria* and *T. castaneum* as queries. The Blast search identified putative *silA, silC, Clath, Vha16, AP50, Arf72A* and *Rab7* genes. In addition, the Blastx analysis revealed that the deduced WCR protein sequences displayed high sequence identity with their homologous proteins from different insect species and shared the highest identity with proteins of *T. castaneum* or *L. decemlineata* (above 67%) (Supplementary Table 2).

3.2. The effect of *silA* and *silC* genes silencing in dsRNA uptake

WCR exhibits non-cell autonomous RNAi since the dsRNA is taken up from the intestinal lumen by the cells upon feeding and the RNAi signal is systemically spread from the midgut to other tissues (Bolognesi et al., 2012). To determine if the *D. v. virgifera* SID-1-like protein is responsible for the dsRNA uptake in WCR adults, we performed experiments using a “RNAi on RNAi” approach which has been successfully used to associate the genes Dicer2 and Ago2 with the RNAi pathway (Veléz et al. 2016). In a previous study, feeding artificial diet treated with dsRNA of the *vacuolar-ATPase subunit A* (*vATPase-A*) caused high mortality in WCR adults after 14 days of exposure (Rangasamy

and Siegfried, 2012). Therefore we chose *vATPase-A* as a marker gene to investigate the role of the SILA and SILC proteins in the uptake of dsRNA in WCR adults.

The assay was designed to evaluate the effect of the suppression of *sil* genes on the mortality of WCR adults after *vATPase-A* dsRNA exposure. Seven days after the dsRNA injections we observed a robust reduction in expression of the *silA*, *silC* and *laccase2* genes (Figure 3). In the treatment group SilA+vATPase-A and SilA/SilC+vATPase-A, the expression of silA was significantly ($p<0.05$) reduced compared to the control (adults injected with *GFP* dsRNA and subsequently fed with *vATPase-A* dsRNA) by 89.1% and 87.5%, respectively (Figure 3A). The expression of *silC* in the treatment group SilC+vATPase-A and SilA/SilC+vATPase-A was reduced by 83.9% and 95.5%, respectively, and significantly different from the control (Figure 3B). Compared to the control, the reduction of *laccase2* expression was 98.8% and 99.6% when the WCR adults were injected with *laccase2* dsRNA and posteriorly fed with untreated diet or *vATPase-A* dsRNA, respectively (Figure 3C).

The down-regulation of the *silC* gene did not significantly suppress the mortality of the WCR adults injected with *silC* dsRNA or with the mixture of *silA* and *silC* dsRNAs and subsequently fed with *vATPase-A* dsRNA compared to the control (Figure 4). Furthermore, *vATPase-A* transcript abundance was not significantly influenced by the down-regulation of the *silC* implying that in WCR adults, SILC protein is not required for dsRNA uptake. Although silA dsRNA seemed to affect WCR adult mortality when it was used alone in the injections, the results from the qPCR analysis indicated no significant increase in *vATPase-A* transcript abundance suggesting that the SILA protein may play a small role in cellular uptake of dsRNA.

To determine if competition for the RNAi machinery occurred between the dsRNAs from the first and second exposure, we included a treatment group

in which the WCR adults were injected with *laccase2* dsRNA followed by feeding with *vATPase-A* dsRNA. The knockdown of *laccase2*, a non-lethal gene involved with cuticular tanning (Arakane et al., 2005) and that is not involved in dsRNA uptake, did not affect the knockdown of the *vATPase-A* gene and the mortality of the WCR adults as compared with those of control injected with *GFP* dsRNA and fed with *vATPase-A* dsRNA (Figure 4). These results indicate that the effects on mortality and *vATPase-A* expression of the treatment groups injected with *sil* dsRNA and fed with *vATPAse-A* dsRNA were not due competition between dsRNAs. Furthermore, the silencing of *silA* and *silC* genes at the same time suggests that oversaturation of the RNAi machinery did not occur when a mixture of *silA* and *silC* dsRNAs was injected (Figure 3A and 3B).

Similar results have been reported for other insects including *L. migratoria*, *S. gregaria*, *Plutella xylostella* and *T. castaneum* where the *sil* genes appear not be involved in systemic RNAi (Luo et al., 2012; Wynant et al., 2014; Wang et al., 2014; Tomoyasu et al., 2008). Detailed sequence analysis of the SIL proteins has revealed that in some insects the N-terminal extracellular domain share more identity with the tag-130 protein of *C. elegans*, which is involved with cholesterol internalization, than with the SID-1 (Wynant et al., 2014; Tomoyasu et al., 2008; Luo et al., 2012). The WCR SIL proteins tested in this experiment may be homologs of tag-130 and associated with cholesterol uptake.

The transmembrane protein SID-1 is necessary to promote the uptake of dsRNA in *C. elegans*, allowing the systemic spread of the RNAi effect (Winston et al., 2002). The presence of *C. elegans sid-1* homologues has been confirmed in several insects such as *Schistocerca americana*, *Spodoptera exigua*, *Spodoptera litura*, *Spodoptera frugiperda*, *Aphis glycines*, *Aphis gossypii* and *Anthonomus grandis* (Bansal and Michel, 2013; Gong et al., 2015; Xu and Han, 2008; Firmino et al., 2013; Ghosh et al., 2014; Dong and Friedrich, 2005; Tian et al., 2009). However, as observed in the present study, the presence of *sil*

genes in insects and a systemic RNAi response do not assure the participation of SIL proteins in the internalization of dsRNA, suggesting that other mechanisms exists for dsRNA uptake.

The participation of the *sil* genes in the dsRNA uptake has been reported in *Apis mellifera*, *Nilaparvata lugens* and *L. decemlineata* (Aronstein et al., 2006; Xu et al., 2013; Cappelle et al., 2016). In *L. decemlineata* was suggested that *silA* and *silC* are required for dsRNA uptake however the silencing of *silA* promoted a stronger suppression of marker gene knockdown compared to the silencing of *silC* (Cappelle et al., 2016). In our study only *silA* significantly suppressed the *vATPase-A* phenotype in WCR adults. Accordingly, we speculate that *silA* is more likely to be involved with the import of dsRNA.

Among insects, the number of *sil* genes, the involvement of these genes with the uptake of dsRNA, and its correlation with the effectiveness of RNAi is highly variable (reviewed by Cappelle et al. 2016). Therefore, the precise involvement of the *sil* genes with dsRNA internalization in insects is not clear, seems to be species-specific and needs to be determined case-by-case.

In contrast to the results with *sil* genes, a recent study performed with WCR larvae demonstrated through a "RNAi on RNAi" approach that the silencing of *silA* and *silC* suppressed the RNAi phenotype of the *ebony* gene suggesting the involvement of both *sil* genes in the uptake of dsRNA. However, the phenotype resulting from ebony RNAi was not very pronounced indicating that these proteins probably are not a unique mechanism associated with the uptake of dsRNA in WCR (Miyata et al., 2014). A possible explanation for these distinct results is that we used a different development stage of WCR and the participation of SILC proteins in dsRNA uptake might be lower in the adult stage than in larval stage. Furthermore, the assay used in our study was different from the assay system used by Miyata et al. (2014) which may have had higher sensitivity to detect RNAi response of the marker gene.

There is some evidence that SID-1 might operate as a homomultimer (Shih et al., 2009). According to the results shown by Kobayashi et al. (2012) in cultured cells of *B. mori* expressing the *sid-1* gene of *C. elengans*, the RNAi effect depends on the concentration of the SID-1 protein, implying that SID-1 is unable to create a functional multimer when its level is lower than a certain threshold concentration. Consequently, it is possible that the SILC level is not high enough in WCR adults to form the functional protein multimer required for uptake of dsRNA. Interestingly, for *silA* we observed a significant reduction in mortality and a high expression of the *silA* gene in the gut where the first step of dsRNA uptake occurs after oral exposure, supporting the idea that the ability of multimer formation is linked to SIL protein availability. Furthermore, in *C. elegans* most of the neurons are refractory to systemic RNAi and SID-1 is not detected in this type of cell but neurons expressing *sid-1* respond efficiently to feeding RNAi suggesting that the weak response to systemic RNAi of the neurons is caused by the low level of SID-1 (Calixto et al., 2010).

RNAi induced knockdown of the *silC* gene was not complete potentially leaving some level of mRNA that could be translated in protein, which may have allowed dsRNA internalization. In addition, the SILC protein might have a long half-life and the protein knockdown may have not occurred at the same level of the mRNA. Therefore, the participation of SILC protein in dsRNA uptake should not be discarded. More detailed studies should be performed in order to provide additional evidence about the role of SIL proteins in dsRNA uptake and spread in WCR adults.

3.3 The effect of knockdown of clathrin-dependent endocytosis elements in dsRNA uptake

To examine the hypothesis that the clathrin-dependent endocytosis is involved in dsRNA uptake in WCR adults, we again utilized a "RNAi on RNAi" approach. For this purpose, genes encoding proteins active in specific steps of the endocytic pathway (Table 1) were silenced and the effect on *laccase2* dsRNA internalization was assessed by quantifying *laccase2* knockdown. Furthermore, we chose the *laccase2* as a marker gene rather than *vATPase-A*, which encodes the subunit A of the v-ATPase multisubunit enzyme complex that plays an important activity in several cellular processes including the endocytic pathway.

We evaluated *Clath*, *Vha16*, *AP50*, *Arf72A*, and *Rab7* gene knockdown triggered by dsRNA feeding of the respective target genes in WCR adults by qPCR of all treatment groups at two time points, before and two days after the second dsRNA exposure, referred to as stage 1 and stage 2 respectively (Figure 5). The expression of all five target genes was strongly reduced compared to the control after the first dsRNA exposure and remained suppressed after the second dsRNA exposure (*laccase2* or *GFP* dsRNA). These results indicate that the expression of the endocytic genes was unaffected by exposure to a second dsRNA and that the knockdown did not wear off during the time that the WCR adults were exposed to the second dsRNA.

When WCR adults were fed with *GFP* dsRNA and later with *laccase2* dsRNA, the *lacasse2* expression decreased by 79.4% relative to the control in which WCR adults were treated at both stages with *GFP* dsRNA. In contrast, when WCR adults were exposed to *AP50* dsRNA followed by *laccase2* dsRNA, the *laccase2* expression was reduced by only 5.9% compared to the control suggesting strongly that the down-regulation of *AP50* in WCR adults

significantly antagonizes the knockdown of *laccase2*, and implicating the endocytosis pathway in dsRNA uptake in WCR adults similar to the role that was described for *T. castaneum* (Xiao et al., 2015).

Interestingly, we observed that both *Clath* and *Vha16* dsRNAs caused a non-specific reduction in *laccase2* transcript levels (65.3% and 40.4%, respectively), compared to GFP+GFP control. Therefore, it appears that knockdown of *Clath* and *Vha16* also affects the expression of non-target genes. Similar effects were observed in *Metaseiulus occidentalis* and *L. migratoria* in which *Clath* silencing reduced the expression of the non-target genes, *cathepsin* and *methoprene-tolerant* genes by 40% and 88%, respectively (Wu and Hoy, 2014; Ren et al., 2014). As a consequence, in order to prevent that the results from the Clath+Laccase2 and Vha16+Laccase2 treatment groups were obscured by this non-specific effect, the Clath+GFP and Vha16+GFP treatment groups were incorporated in the calculations of *laccase2* silencing. The observations made in our study and previous works suggest that when performing this type of experiment is important to use adequate controls that will lead to a correct interpretation of the data.

The gene silencing of *laccase2* in beetles first treated with *GFP* dsRNA and followed by laccase2 dsRNA (GFP+GFP x GFP+Laccase2) was 91.1%. In beetles treated with *Clath* dsRNA and subsequently with *laccase2* dsRNA (Clath+GFP x Clath+Laccase2) the expression of *laccase2* decreased by 62.5%, while in beetles treated with *Vha16* dsRNA and later with *laccase2* dsRNA (Vha16+GFP x Vha16+Laccase2) the expression decreased 43.0% (Figure 6). Although we did not observe statistical difference of *laccase2* expression between the treatment groups GFP+Laccase2 and Clath+Laccase2 or Vha16+Laccase2, these data suggest that *Clath* and *Vha16* transcript suppression reduced the expression of laccase2 by 28.6% and 48.1% respectively, reinforcing our hypothesis that endocytosis is required for dsRNA

uptake in WCR adults. Additional studies that test the effects of selective inhibitors in the knockdown of marker genes, will reinforce the involvement of the endocytosis in dsRNA uptake. *Arf72A* and *Rab7* silencing did not result in significant increase of *laccase2* expression. According to the mechanism of clathrin-dependent endocytosis in cellular uptake of dsRNA proposed by Xiao et al. (2015), *Arf72A* and *Rab7* act in a final stage of the endocytic pathway. After the formation of uncoated vesicles and early endosomes, the dsRNA molecules can escape from the endosomes into the cytosol becoming available to the RNAi core machinery to trigger the RNAi process. It is thus possible that sufficient *laccase2* dsRNA escaped in the earlier steps and the knockdown of *Arf72A* and *Rab7* genes did not significantly affect the expression of *laccase2*.

The involvement of the endocytic pathway in the uptake of dsRNA has been demonstrated in *D. melanogaster* S2 cells, *T. castaneum*, *S. gregaria* and *B. dorsalis*, suggesting that this mechanism of dsRNA internalization might be widespread among insects (Ulvila et al., 2006; Saleh et al., 2006; Wynant et al., 2014; Xiao et al., 2015; Li et al., 2015). However, Cappelle et al. (2016) reported that endocytosis and SIL proteins (SILA and SILC) are implicated in the uptake of dsRNA in *L. decemlineata* showing for the first time that both mechanisms can work together in insects. Another recent study demonstrated that the silencing of *Vha16* in *L. decemlineata* cell line (Lepd-SL1) blocked RNAi suggesting that this gene is essential for RNAi while the silencing of *Rab7*, *Arf72A*, *silA* and *silC* genes caused a partial block in RNAi (Yoon et al., 2016).

3.4. Gene expression profiles

It is possible that the expression level and/or tissue specificity of the genes related with the dsRNA uptake mechanism can influence the susceptibility

to RNAi. Baseline expression of *silA, silC, Clath* and *Vha16* WCR putative genes in different developmental stages and tissues were investigated by qPCR analysis. As indicated in Figure 7, all genes were expressed in every developmental stage and tissue evaluated. The expression of *silA* and *silC* varied significantly between the developmental stages but in general they exhibited lower expression levels in third instar and pupae as compared to other developmental stages (egg, first and second instar; and adult). The highest expression of *silA* was in the egg stage followed by first instar larva and adult males, while *silC* had greater expression in egg and adult stages.

For *T. castaneum*, *silC* was highly expressed in the pupal stage and *silA* showed uniform expression throughout development (Tomoyasu et al., 2008), while in the lepidopterans *P. xylostella* and *S. litura, sil* was highly expressed in adults and eggs, respectively (Wang et al., 2014; Gong et al., 2015). In *A. glycines*, *sil* exhibited similar expression in all life stages (Bansal and Michel, 2013). Taken together these results suggest that expression of *sil* genes does not follow any pattern between insect species.

In terms of different tissues, the expression of *silA* was significantly higher in the gut compared to other tissues in both larva and adult stages, with limited transcript levels in the head and the integument. In the adult stage, *silC* had the highest expression in the thorax compared to the other tissues that presented no significant differences in expression level. The *silC* was expressed uniformly across the tissues evaluated in the larval stage. It is interesting that these two genes which have been described as having similar functions in *C. elegans*, showed strikingly different expression patterns in the tissues tested. Contrary to what we observed for *silA* in WCR, Luo et al. (2012) reported that sil had significantly greater expression in the fat body compared to the midgut in *L. migratoria*. In *S. litura*, higher levels of *sil* were found in midgut and fat body compared to cuticle (Gong et al., 2015) and in *A. glycines* no significant

differential expression of *sil* in epidermis, gut and fat body was observed (Bansal and Michel, 2013). The expression of *Clath* and *Vha16* genes was also lower in third instar larvae and pupae compared to other life stages. In the larval stage the expression of *Clath* in the head and fat body were higher than in the gut and integument, while *Vha16* was uniformly expressed in all tissues. In the adult stage, *Clath* showed the highest expression in the head followed by thorax, abdomen, and the lowest in gut, while a gradual increase of *Vha16* expression was observed in head followed by thorax, abdomen, and gut unlike what we observed for *Clath*. Analysis of *Clath* expression in *T. castaneum* during different developmental stages revealed that it was more abundant in pupae and adults, and it was highly expressed in all tissues examined, including the gut, malpighian tubules, fat body, and carcass from pupae (Xiao et al., 2015).

There are not enough studies to determine if there is a trend in terms of the expression of the genes related to the uptake of dsRNA between different species and the association between their expression level and the efficiency of the systemic RNAi response. Further analysis of gene expression would be interesting to help us understand the factors that interfere in the susceptibility to RNAi in insects.

4 CONCLUSIONS

In summary, the results described in the present paper suggest that SILA and SILC are not essential for dsRNA uptake while the clathrin-dependent endocytosis is more likely to be involved with the import of dsRNA in WCR adults. Our research provides valuable information that can have important implications for the development and efficacy improvement of RNAi-based management strategies directed to WCR.

Competing interests

The authors declare that they have no competing interest.

Acknowledgments

This work was partially supported by CNPq - National Council for Scientific and Technological for DHP's scholarship.

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Figures and tables

Table 1
Genes involved in the clathrin-mediated endocytosis pathway identified in *D. v. virgifera*.

Gene name	Biological function	Reference
Clath	Clathrin heavy chain is the major componet of clathrin-coated pits formed on the inner surface of the cytoplasmic membrane.	McMahon and Boucrot, 2011
Vha16	Vacuolar (H^+)-ATPase16-kDa subunit is involved in endocytic trafficking.	Saleh et al., 2006
AP50	AP50, the medium chain subunit (μ2) of AP2 adaptor protein complex, acts linking the clathrin to cargo.	McMahon and Boucrot, 2011
Arf72A	Arf72A is a member of the ADP-ribosylation factor (Arf) small GTPases proteins and is involved with endosome trafficking.	Saleh et al., 2006
Rab7	Rab7 is a member of the Ras superfamily of GTPases and has an important role in endocytic trafficking between early and late endossomes.	Hyttinen et al., 2013

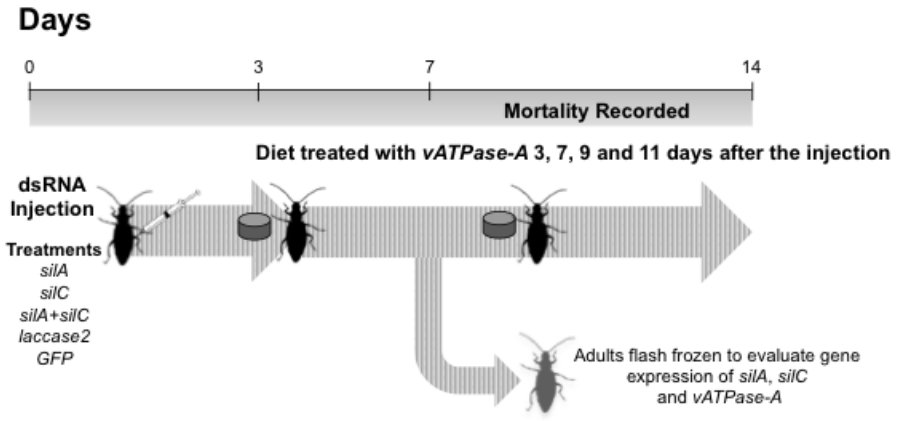

Figure 1 - Scheme of the bioassay to determine the effect of the silencing of the *silA* and *silC* genes in the mortality of the WCR adults and *vATPase-A* expression after *vATPase-A* dsRNA exposure.

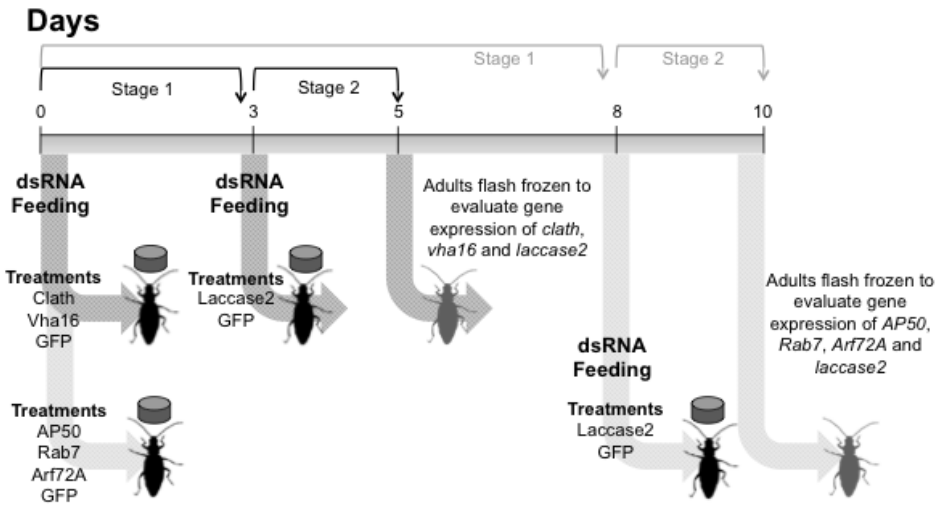

Figure 2 - Scheme of the bioassay to determine the effect of the silencing of the endocytic genes (*clath*, *vha16*, *AP50*, *Rab7* and *Arf72A*) in the *laccase2* expression of WCR adults treated with *laccase2* dsRNA.

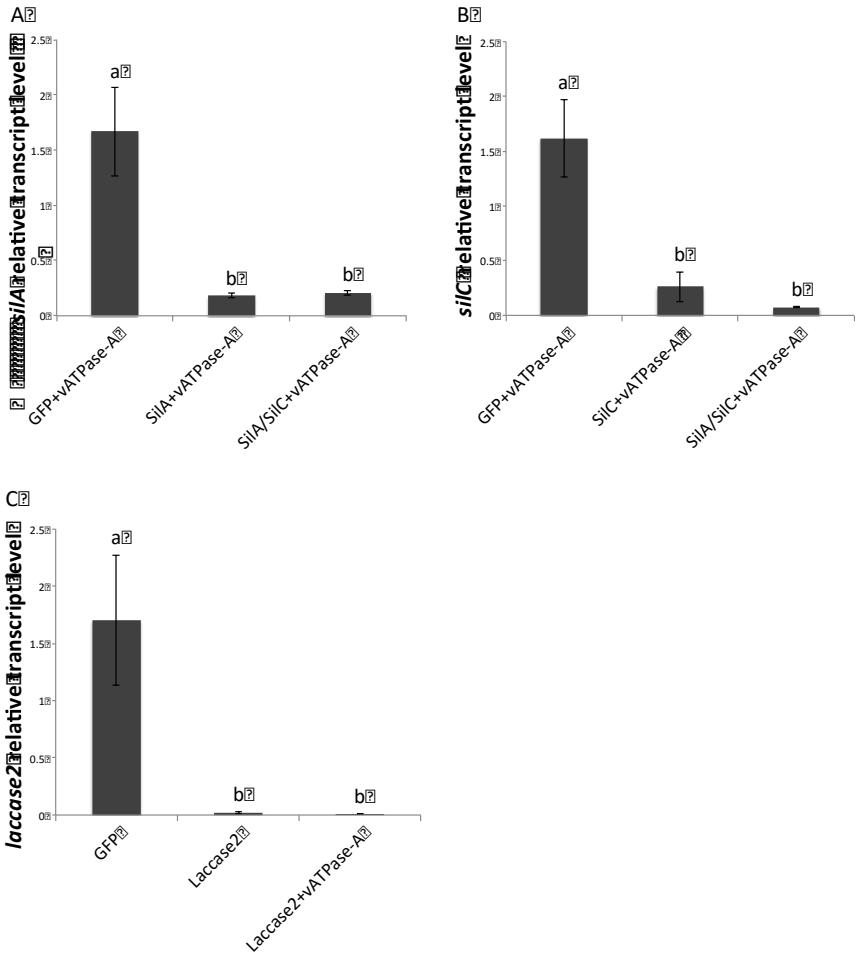

Figure 3 - Silencing of *silA*, *silC* and *laccase2* genes in the whole body of WCR adults. Relative transcript level of *silA* (A), *silC* (B) and *laccase2* (C) evaluated 7 days after the injection of 600 ng of the respective dsRNA or a combination of *silA* and *silC* dsRNAs into WCR adults. Values shown are the means and standard errors (±SE) of three biological replicates each with two technical replicates. Different letters represent significant differences at P value < 0.05.

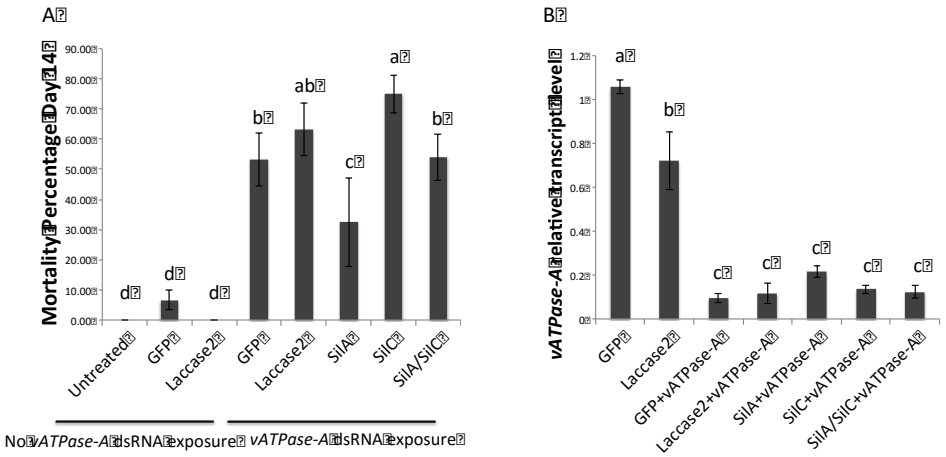

Figure 4 - Percent mortality and expression of *vATPase-A* in WCR adults. (A) Mortality of WCR adults from the different treatment groups after 14 days. WCR adults were injected with 600 ng of *GFP*, *laccase2*, *silA*, *silC* or a combination of *silA* and *silC* dsRNAs and posteriorly fed with *vATPase-A* dsRNA. WCR adults injected with *GFP* and not exposed to *vATPase-A* dsRNA were used as control. (B) Relative *vATPase-A* transcript level evaluated at 7 days after exposed to *vATPase-A* dsRNA determined by qPCR analysis. Values shown are the means and standard errors (±SE) of three biological replicates. Different letters represent significant differences at P value < 0.05.

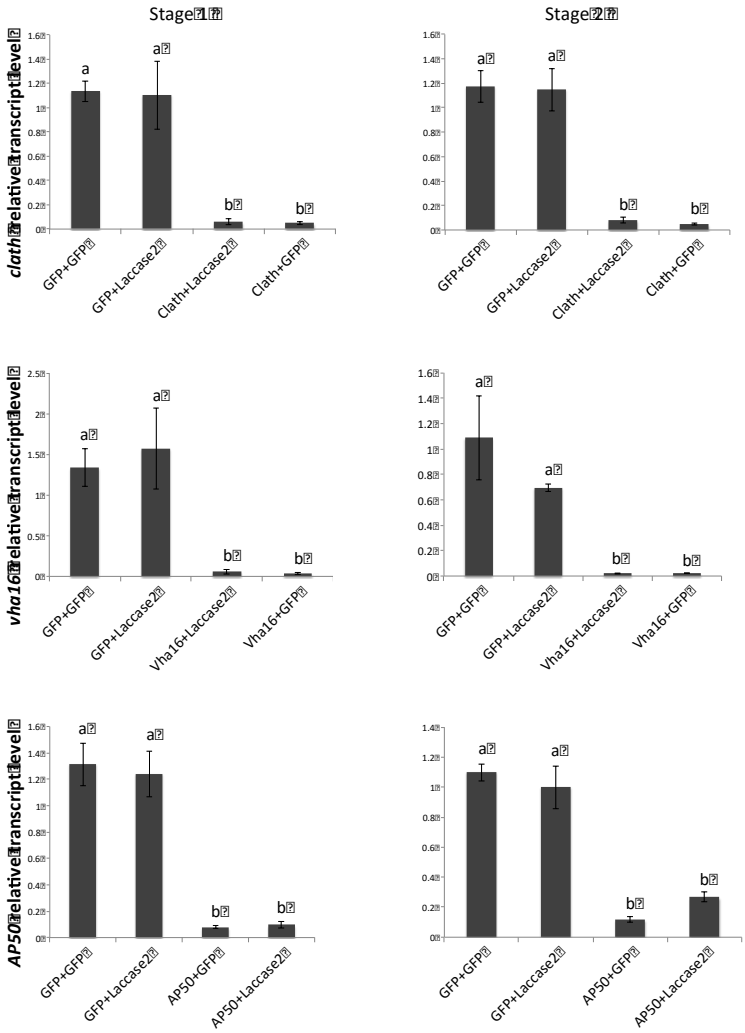

Figure 5 - Relative expression of *Clath*, *Vha16*, *AP50*, *Arf72A* and *Rab7* genes in the whole body of WCR adults. Relative transcript levels of *Clath*, *Vha16*, *AP50*, *Arf72A* and *Rab7* genes from the different treatment groups after the first dsRNA exposure (stage 1) and after the second dsRNA exposure (stage 2) determined by qPCR analysis. Values shown are the means and standard errors (±SE) of three biological replicates each with two technical replicates. Different letters represent significant differences at P value < 0.05. (Continua).

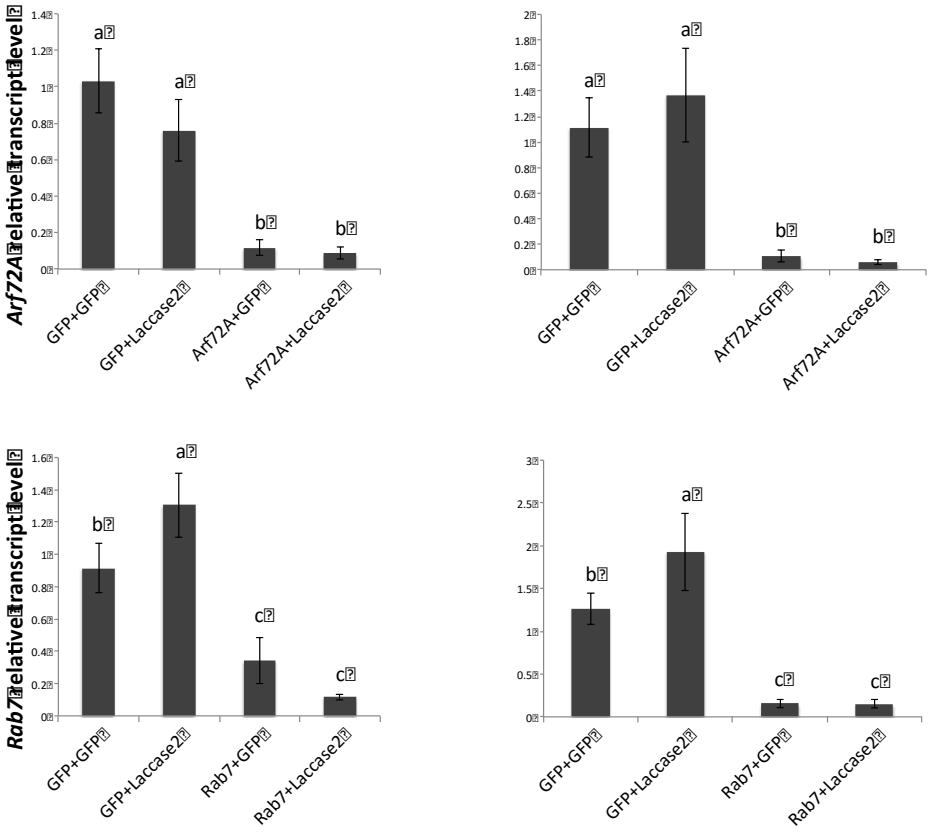

Figure 5 - Relative expression of *Clath*, *Vha16*, *AP50*, *Arf72A* and *Rab7* genes in the whole body of WCR adults. Relative transcript levels of *Clath*, *Vha16*, *AP50*, *Arf72A* and *Rab7* genes from the different treatment groups after the first dsRNA exposure (stage 1) and after the second dsRNA exposure (stage 2) determined by qPCR analysis. Values shown are the means and standard errors (±SE) of three biological replicates each with two technical replicates. Different letters represent significant differences at P value < 0.05. (Conclusão).

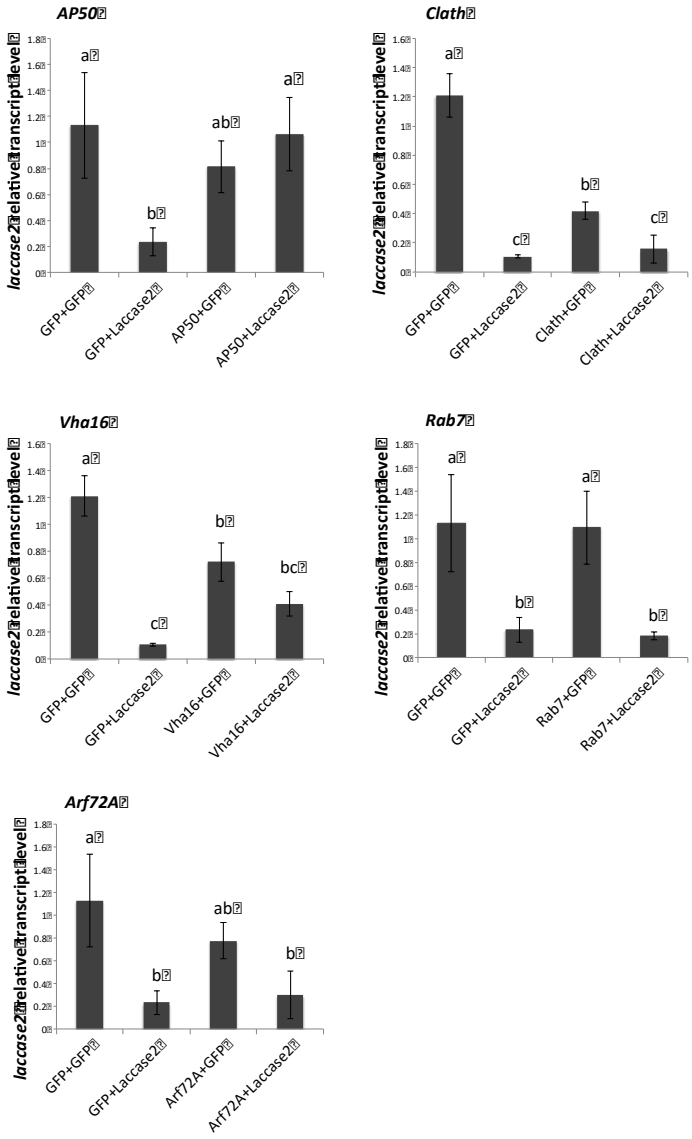

Figure 6 - Effect of endocytosis-related genes knockdown on *laccase2* relative transcript level. The relative transcript level of *laccase2* from the different treatment groups was determined by qPCR analysis. Values shown are the means and standard errors (±SE) of three biological replicates each with two technical replicates. Different letters represent significant differences at P value < 0.05.

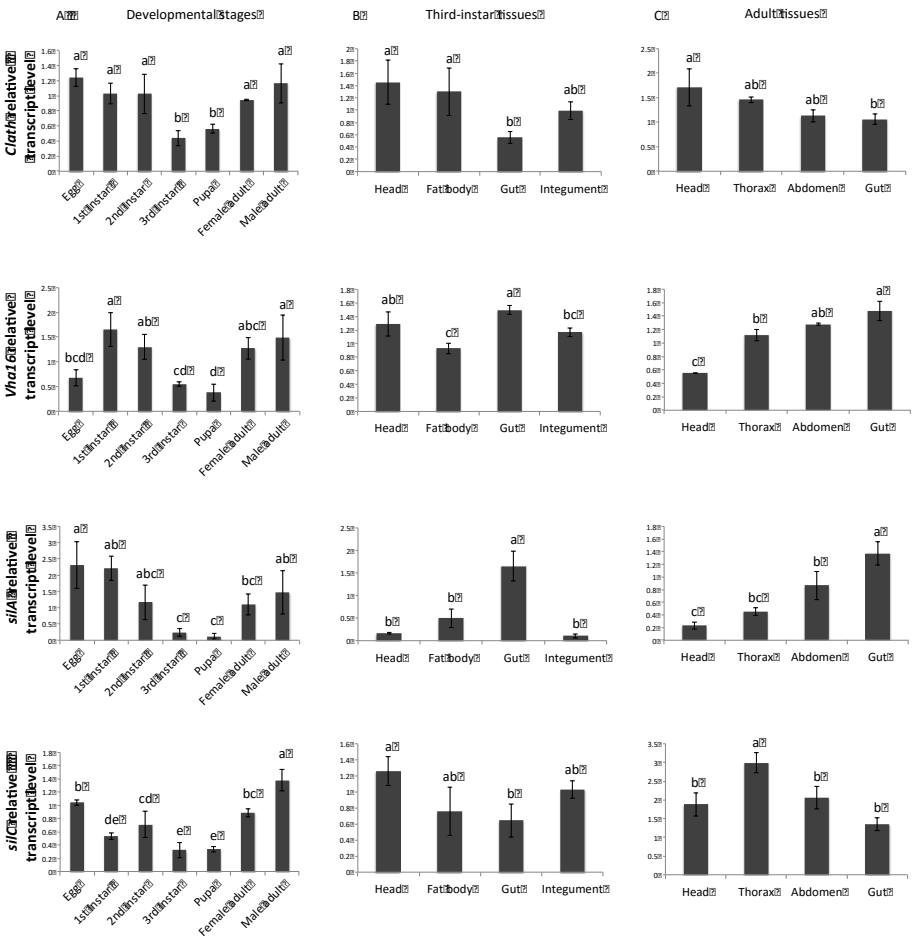

Figure 7 - Developmental stage and tissue expression profile of WCR *silA*, *silC*, *Clath*, and *Cha16* genes. (A) *silA*, *silC*, *Clath*, and *Cha16* gene expression of different WCR developmental stages (egg, first instar larva, second instar larva, third instar larva, female adult, male adult). (B) *silA*, *silC*, *Clath*, and *Cha16* gene expression in different tissues of WCR third-instar larvae (gut, body, head and integument). (C) *silA*, *silC*, *Clath*, and *Vha16* gene expression in different tissues of WCR adults (gut, thorax, head and abdomen). Values shown are the means and standard errors (±SE) of three biological replicates each with two technical replicates. Different letters represent significant differences at P value < 0.05.

Supplementary data

Supplementary Table 1. Sequences and parameters of the primers used for dsRNA synthesis.

Primer name dsRNA	Sequence (5`-3`) for dsRNA synthesis	Product size (pb)
dsGFP-F	TAATACGACTCACTATAGGGGGTGATGCTACATACGGAAAG	370
dsGFP-R	TAATACGACTCACTATAGGGTTGTTGTCTGCCGTGAT	
dsLaccase2-F	TAATACGACTCACTATAGGGATGTGCAAGAGCTTGTAGGG	183
dsLaccase2-R	TAATACGACTCACTATAGGGATGCGATTGGCTGTTAGAAG	
dsvATPase-A-F	TAATACGACTCACTATAGGGTATTGTACAGGTG	258
dsvATPase-A-R	TAATACGACTCACTATAGGGCAATTTCCAAG	
dsSilA-F	TAATACGACTCACTATAGGGCAGAACCGTCATCCAGATA	382
dsSilA-R	TAATACGACTCACTATAGGGCCATCAATAACGCTAACAAGA	
dsSilC-F	TAATACGACTCACTATAGGGGAACTTTCGCACAAAGACGA	389
dsSilC-R	TAATACGACTCACTATAGGGACACTCCAGCATCCCATGTA	
dsChc-F	TTAATACGACTCACTATAGGGAGAGGAAGATTGGCTGATTTGGA	291
dsChc-R	TTAATACGACTCACTATAGGGAGACACGATGTGCATACCACACA	
dsVha16-F	TTAATACGACTCACTATAGGGAGATCATGAGGCCGGAACTTATC	327
dsVha16-R	TTAATACGACTCACTATAGGGAGAAGAGGTAAATGGCGACGATG	
dsAP50-F	TTAATACGACTCACTATAGGGAGA CTCCCGATGGAGAATTTGAA	316
dsAP50-R	TTAATACGACTCACTATAGGGAGA GCCATTCGCTTTATTTTCCA	
dsArf72A-F	TTAATACGACTCACTATAGGGAGA TTGGGATTAGACGGTGCAG	200
dsArf72A-R	TTAATACGACTCACTATAGGGAGAATGATTGCATCTGTATTGCTGTAGT	
dsRab7-F	TTAATACGACTCACTATAGGGAGA AATTCCTCATCCAAGCATCG	299
dsRab7-R	TTAATACGACTCACTATAGGGAGA ATCACCGTTGGTGTTGGTTT	

F: Forward primer; R: Reverse primer; Underlined sequence: T7 promoter

Supplementary Table 2. Sequences and parameters of the primers used for qPCR analysis.

Primer name qPCR	Sequence (5`-3`) for qPCR	Product size (pb)	Slope	R^2	Eff. (%)
qPCRLaccase2-F	GAGCAGCTTGCCAAGTATGT	109	-3.169	0.998	106.8
qpcrLaccase2-R	TTGTCCGTTTCTGCCAGAGA				
qPCRvATPase-A-F	GGAAGAAGATGATCTAGCCGAAATT	67	-3.361	0.993	98.4
qPCRvATPase-A-R	TTGTCCGTTTCTGCCAGAGA				
qPCRSilA-F	ACGCACTTAAACCTATACGGAAA	165	-3.29	0.99	101.3
qPCRSilA-R	CACAATGAATGACGCTGTTACC				
qPCRSilC-F	GAACTTTCGCACAAAGACGA	116	-3.457	0.996	94.6
qPCRSilC-R	TAACTTGCGCTCAAAACACC				
qPCRClath-F	GGCCAGAGAGAGCTACATCG	196	-3.437	0.998	95.4
qPCRClath-R	AGCTAGACGAGCGAAGTTGG				
qPCRVha16-F	TCCTCATTTTCGCCGAAGTA	124	-3.251	0.999	103
qPCRVha16-R	GCAACTTTTGGTACGCTGTG				
qPCRAP50-F	ATCGGAGAATGCCATTGTGT	112	-3.482	0.998	93.7
qPCRAP50-R	GCGAGTCCATTTCTTTTTGG				
qPCRArf72A-F	ATTTAGGGGGCCAGACAAGT	147	-3.493	0.99	93.3
qPCRArf72A-R	GCTCTTCTTCCCTTAACATTGG				
qPCRRab7-F	CCTCATTCAAATCCCTGGAC	109	-3.339	0.993	99.3
qPCRRab7-R	TCGAGGTCGACTTTGTTTCC				
qPCRβ-actin-F	TCCAGGCTGTACTCTCCTTG	134	-3.42	0.993	96.1
qPCRβ-actin-R	CAAGTCCAAACGAAGGATTG				

F: Forward primer; R: Reverse primer

R^2: Correlation coefficient, Eff. (%): percent primer efficiency

ARTIGO 2

Normas de formatação da Revista Biological Control (Versão Preliminar)

Selection and characterization of *Bacillus thuringiensis* strains active against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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ABSTRACT

Bacillus thuringiensis is widely used for insect pests control due the insecticidal activity of its proteins (δ-endotoxins and Vip). This study aimed to select and characterize *B. thuringiensis* strains toxic against *Helicoverpa armigera* (Hübner), *Anticarsia gemmatalis* (Hübner), *Diatraea saccharalis* (Fabricius), *Spodoptera cosmioide* (Walker) and *Pseudoplusia includes* (Walker), considered economically important pests of many cultivated crops. Among one hundred fifty *B. thuringiensis* strains evaluated, eight (426, 520B, 1636, 1641, 1644, 1648, 1657 and 1658) showed high toxicity against *H. armigera*. The strains 1641 and 1658 were the most active against *H. armigera* larvae with LC_{50} of 149.8 ng/cm^2 and 150.1 ng/cm^2, respectively. The strains with high insecticide activity against *H. armigera* were evaluated against *Anticarsia gemmatalis*, *Diatraea saccharalis*, *Spodoptera cosmioide* and *Pseudoplusia includes*. The bioassay results showed that most of the strains were also toxic to these insect pests. The protein analysis by SDS–PAGE of the *B. thuringiensis* strains demonstrated bands of approximately 140 kDa and 55 kDa and protein profile similar to standard HD-1 strain. PCR screening was performed to determine the *vip* and *cry* gene content of the strains and different profiles for these genes were found. The results showed on this study suggest some *B. thuringiensis* strains that have great potential to be used in bioinsecticide formulations which could be employed in the integrated pest management of *H. armigera* and other Lepidoptera species.

Keywords: *Helicoverpa armigera*, *Bacillus thuringiensis*, *cry* gene, *vip* gene, bioinsecticide.

1 INTRODUCTION

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is a pest of various economically important crops and widely distributed around the world. The first record of the occurrence of *H. armigera* in the American continent was made in Brazil in 2013 (Czepak et al 2013; Tay et al., 2013; Specht et al., 2013), increasing its geographical distribution that until the date was related in Europe, Asia, Africa and Australasia (Tay et al., 2013).

Since then, this pest has dispersed fastly for South and Central America, being found in Argentina (Murúa et al., 2014; Arneodo et al., 2015), Paraguay (SENAVE, 2013), Bolivia, Uruguay (Arnemann et al., 2016; Kriticos et al., 2015) and Puerto Rico (Smith, 2014). The strong migratory abilities of the *H. armigera* increase its potential invasion risk in North America (Kriticos et al. 2015), which has been supported by the occurrence register of some *H. armigera* individuals in Florida, USA (Hayden and Brambila, 2015; El-Lissy, 2015). In the 2012/2013 crop season, the Brazilian farmers had serious economic losses due high levels of *H. armigera* larvae infestations especially in the soybean, cotton and corn yields from several states, which resulted in demange to crops estimated about US$ 800 million (Bueno et al., 2014).

Maize, soybean and cotton are some of the main Brazil's agribusiness crops (MAPA, 2015), however other Lepidoteran insect pests beside the *H. armigera* as such *Anticarsia gemmatalis* (Hübner), *Spodoptera cosmioide* (Walker) *Pseudoplusia includes* (Walker) (Lepidoptera: Noctuidae) and *Diatraea saccharalis* (Fabricius) (Lepidoptera: Crambidae) have attacked these cultures compromising their productivity (Bueno et al., 2011; Palma et al., 2015; Farias et al., 2013; Bernardi et al., 2012). Alternative strategies that aid to effectively control *H. armigera* and also these other insect species, need to be exploited aiming to reduce the crop losses.

Bacillus thuringiensis (Bt) is an aerobic gram-positive bacterium able to produce insecticidal crystal proteins (ICPs) during the sporulation phase. The ICPs can be toxic to different orders of insect as such Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Blattaria and also nematodes (Schnepf et al., 1998; van Frankenhuyzen, 2009). The insecticidal proteins sintetized as crystalline parasporal inclusions comprise two families, Crystal (Cry) and Cytolytic (Cyt) proteins, classified by their amino acid sequence in 74 Cry classes and 3 Cyt classes. To date, more than 820 different genes responsive for the codification of these proteins have been identified (Crickmore et al., 2016).

In addition, *B. thuringiensis* can synthesize another type of insecticidal protein that has been designated as vegetative insecticidal protein (Vip), it is produced during the vegetative growth phase and subsequently secreted into the culture medium (Warren et al., 1996). The Vip proteins include the classes Vip1, Vip2, Vip3 and Vip4 that do not display sequence homology with the Cry or Cyt proteins. These toxins also have demonstrated activity against different insects (Estruch et al., 1996; Warren, 1997, Sattar and Maiti, 2011). The *B. thuringiensis* toxins present specificity against a restrict number of target insect species and they usually are not toxic to beneficial and non-target organisms including humans which allow their effective and safe use in the agriculture (Shelton et al., 2002; Raybould and Vlachos, 2010; Kock et al., 2015).

The mode of action of the Cry1 toxins involves several steps. After to be ingested by a susceptible insect larvae, the parasporal inclusions are solubilized and then the released Cry protoxins are proteolytically processed in the midgut lumen becoming active toxins. Sequential interactions of the toxins with insect midgut receptors facilitate the formation of a pre-pore oligomer structure and posteriorly its membrane insertion forming pores that cause the midgut cells death by osmotic shock (Bravo et al., 2011; Schnepf et al., 1998).

The most extensive method employed to control *H. armigera* is based on conventional synthetic inseticides but high levels of resistance have been developed for this pest with the heavy selection pressure (Avilla and González-Zamora, 2010; El-latif and Subrahmanyam, 2010; Torres-Vila et al., 2002). *B. thuringiensis* pesticides and transgenic plants expressing insecticidal proteins of *B. thuringiensis* are used successfully as alternative strategies in integrated pest management programs of many insect species thus contributing to a more sustainable crop protection (Sanahuja et al., 2011; Héma et al., 2009). However, resistance to *B. thuringiensis* toxins also has been related in some insects, including *H. armigera* (Janmaat and Myers, 2003; Liu et al., 2010; Storer et al., 2010; Tabashnik et al., 1994; Zhang et al., 2012, 2011; Welch et al., 2015; Devos et al., 2013; Alvi et al., 2012).

The identification of novel *B. thuringiensis* strains and genes more effective than those already known that can be employed in bioinsecticide and in genetically engineered crops contribute to wider the range of control options available to be used in insect pest management programs, especially when the traditional methods of control are not efficient because the evolution of resistance to *B. thuringiensis* proteins by the target insects. Then, with this propose many researches aiming the isolation, selection and characterization of new *B. thuringiensis* strains and also the identification of new genes have been performed (Baig et al., 2010; Hernández-Rodríguez et al., 2009; Valicente et al., 2010; Yu et al., 2011, Ye et al., 2012). The present study was undertaken to identify *B. thuringiensis* strains that could be employed in the development of appropriate strategies for the integrated pest management and integrated resistance management against *H. armigera, Anticarsia gemmatalis, Diatraea saccharalis, Spodoptera cosmioide* and *Pseudoplusia includes*.

2 MATERIAL AND METHODS

2.1 Insects

The larvae of *H. armigera* used in this study were provided by the Biological Control Laboratory from Embrapa Maize and Sorghum Research Center, Brazil. The insects were maintained in a climate-controlled room at 25 ± 2 °C, 70 ± 10% relative humidity and a photoperiod 12:12 (light:dark).

2.2 *Bacillus thuringiensis* strains

The one hundred fifty *B. thuringiensis* strains analyzed in this study were obtained from the *Bacillus thuringiensis* collection of Embrapa Maize and Sorghum (Sete Lagoas, Minas Gerais, Brazil). These strains were previously isolated from soil samples collected in different regions of Brazil and stored as a pellet in a freezer at -20 °C. The *B. thuringiensis* strain HD-1 subsp. *kurstaki* used as reference was provided by the United States Department of Agriculture (Columbus, Ohio, USA).

2.3 Selective Bioassays

Each *B. thuringiensis* strain was cultured in 40 ml of modified Luria–Bertani (LB) medium containing glucose (0.1% w/v), nutrient broth (0.8% w/v), $FeSO_4$ (0.002% w/v), $ZnSO_4$, (0.002% w/v), yeast extract (0.2% w/v), $MnSO_4$ (0.003% w/v), $MgSO_4$ (0.03% w/v), tryptone (1% w/v) and NaCl (0.5% w/v) with the pH adjusted to 7.5, in an orbital shaker set at 200 rpm and 28 ± 2 °C during 72 h. Bioassays were carried out by spreading 150 µl of the bacterial culture on the surface of artificial diet piece with 1 cm^3 placed into 50 ml plastic

cup. The treated diet was allowed to air dry at room temperature and then first-instar larvae were placed individually into each cup. The *B. thuringiensis* strain HD-1 and autoclaved water were used as positive and negative controls, respectively. The plastic cups were kept in a climate-controlled room at 25 ± 2 °C, 70% relative humidity, photoperiod of 12:12 (light:dark) and larval mortality was recorded after 7 days. Each bioassay was performed in triplicate with 24 larvae per replicate. The *B. thuringiensis* strains that displayed high level of mortality (above 75%) against *H. armigera* larvae were also evaluated against *A. gemmatalis*, *P. includens*, *D. saccharalis* and *S. cosmioide* larvae by selective bioassays as described for *H. armigera*.

The diet used in the bioassays with *H. armigera*, *A. gemmatalis* and *P. includens*, contained a mixture of 73.57 g of cooked bean, 58.57 g of wheat germ, 22.14 g of powdered milk, 9.17 g of brewer's yeast, 3.78 g of soybean bran, 3.5 g of ascorbic acid, 1.78 g of sorbic acid, 3.92 g of Nipagin®, 5.71 ml of formaldehyde, 1.78 ml of inhibition solution (4.2% of phosphoric acid and 41.8% of propionic acid), 5.71 ml of vitaminic solution, 12.5 g of agar and 1000 ml of water. The diet for *D. saccharalis* was prepared with 102.56 g of soybean bran, 69.2 g of sugar, 23.07 g of wheat germ, 0.51 g of choline chloride, 1.02 g of ascorbic acid, 5.89 g of Nipagin®, 1.28 ml of formaldehyde, 1.28 ml of inhibition solution, 0.51 ml of Vita Gold®, 17.94 g of agar and 1000 ml of water. For *S. cosmioide* was used the diet made with 118.92 g of cooked bean, 56.57 g of wheat germ, 36.21 g of brewer's yeast, 3.64 g of ascorbic acid, 1.17 g of sorbic acid, 2.25 g of Nipagin®, 2.96 ml of formaldehyde, 2.96 ml of inhibition solution, 14.28 g of agar and 1000 ml of water.

2.4 Dose-mortality bioassays

The insecticidal activity of the selected *B. thuringiensis* strains against *H. armigera* larvae was evaluated by dose-mortality bioassays. The *B. thuringiensis* strains were grown on modified LB medium prepared according to the method described above including agar 1.2% (w/v) at 28 ± 2 °C during 96 h. Posteriorly, the bacterial cells were harvested, frozen overnight, lyophilized (LS 3000, Terroni, Brazil) for 18 h and used to prepare ten dilutions containing spore-crystal mixtures (from 20 to 2000 ng/cm^2). A volume of 35 µl of the dilution was applied uniformly over the diet previously dispensed in the wells of plastic bioassay trays (Bio-BA 128©; BioServ, USA) and air dried at room temperature. One first instar larva was placed into each well and the bioassay trays were kept under the same conditions of the selective bioassays. The *B. thuringiensis* strain HD-1 was used as positive control at the same concentrations of the selected *B. thuringiensis* strains and for the negative control was used autoclaved water. Larval mortality was recorded after 7 days. Three replicates with 24 larvae were used for each of ten dilutions. The lethal concentration (LC_{50}) values were calculated by probit analysis (Finney, 1971) using PoloPlus software.

2.5 Characterization of crystal protein (SDS–PAGE)

The *B. thuringiensis* strains were grown in 5 ml of modified LB medium using 50 ml polypropylene tubes at 200 rpm, 28 ± 2 °C for 96 h and then the proteins were extracted as described by Lecated et al. (1991) from 1.5 ml of grown culture. The samples (15 µl) were mixed with Bolt™ LDS Sample Buffer 4x (Life Technologies) (5 µl), boiled for 5 min and separated in a Bolt® 4–12% Bis-Tris Plus gel using Bolt™ MES SDS Running Buffer (Life Technologies),

following manufacturer's instructions. The molecular mass of the proteins was determined by using SeeBlue® Plus2 Pre-Stained Standard (Life Technologies).

2.6 Screening of *cry* and *vip* genes

The *B. thuringiensis* strains were characterized according to the presence of *cry* and *vip* genes. The PCRs were performed using specific primers designed to amplify the *cry1Ac*, *cry1B*, *cry1C*, *cry1D*, *cry1Ea/Eb*, *cry1Fa/Fb*, *cry1G*, *cry2Aa1*, *cry2Ab2*, *cry2Ac*, *vip1*, *vip2* and *vip3* genes (Ceron et al., 1994; Hernández-Rodríguez et al., 2009; Ben-Dov et al., 1997) (Table 1). The DNA extraction was performed as described by Shuhaimi et al. (2001) with some adaptations. From each sample, the PCR mixture included 3 µl of genomic DNA (90 ng), 0.25 µl of each primer at 10 µM, 0.5 µl of 10 mM dNTP mix, 0.4 µl of 25 mM $MgCl_2$, 1 µl of 10x reaction buffer and 1 U of Taq DNA polymerases (Kapa Biosystems) and water to a final volume of 10 µl.

Amplifications were carried out in an Eppendorf Mastercycler thermal cycler using the following cycling conditions: 2 min of initial denaturation at 95 °C, 30 amplification cycles of 1 min denaturation at 95 °C, 1 min annealing at 50-57 °C (according with the primer) and 1 min extension at 72 °C with a final extension step of 10 min at 72 °C. The amplifications using the vip and cry2 primers were carried out following conditions described by Hernández-Rodríguez et al. (2009) and Juárez-Pérez et al. (1997), respectively. The PCR products were visualized in 1-2 % agarose gels using the L-PIX Molecular Image transilluminator (Loccus Biotecnologia, Brazil).

2.7 PCR amplification and sequencing of *gyrB* fragments

The PCR reactions for amplification of the *gyrB* fragments of the *B. thuringiensis* strains were performed as reported above using the primers described by Manzano et al. (2003) and cycling conditions described by Awad et al. (2007). The PCR products were purified with ExoSAP-IT PCR product cleanup (USB Products Affymetrix) according to the manufacturer's instructions and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit v.3.1 (Life Technologies) and the same primers used in the PCR on an ABI PRISM 3500xL automatic sequencer (Applied Biosystems).

2.8 *gyrB* fragments alignment and phylogenetic tree construction

The alignment between the *gyrB* sequences of the *B. thuringiensis* strains including the sequences of the reference strains (Soufiane and Côté, 2009) was realized using MUSCLE version 3.8 (Edgar, 2004). Phylogenetic tree was constructed using the MEGA version 7 software (Kumar, 2016) and Neighbor-joining method (Saitou and Nei, 1987). The resultant tree topologie was evaluated by bootstrap analysis based on 1,000 resamplings to estimate the confidence.

3 RESULTS AND DISCUSSION

3.1 Insect bioassays

In order to select *B. thuringiensis* strains with high insecticidal activity against *H. armigera* larvae we performed bioassays to evaluate the toxicity of the strains. Among the one hundred fifty strains of *B. thuringiensis* examined

through the selective bioassays, only eight showed larval mortality greater than 75%, corresponding to 5.33% of the strains (Table 2). These strains (426, 520B, 1636, 1641, 1644, 1648, 1657 and 1658) besides the standard strain HD-1 that exhibited mortality of 100% were then selected for the dose-mortality bioassays. The most of the strains (48.66%) presented very low toxicity (0-25% mortality) against *H. armigera*, 42.66% of the strains exhibited low toxicity causing mortality from 25.1 to 50%, and 3.33% of the strains were moderately toxic (50.1-75% mortality) (Table 2). Some studies have demonstrated that the proportion of strains with high toxicity to different species of Lepidoptera is usully low as reported in our study (Apaydin et al., 2008; Azzouz et al., 2015; Bernhard et al., 1997; dos Santos et al., 2009; Valicente and Barreto, 2003).

Dose-mortality bioassays were performed to evaluate the LC_{50} value of each selected *B. thuringiensis* strain. The determined LC_{50} values of the strains varied between 149.8 ng/cm^2 to 1543.3 ng/cm^2 (Table 3). Comparatively, the *H. armigera* larvae were 10-fold less susceptible to the strain 426 that presented the highest LC_{50} value than to the strain 1641 that had the lowest LC_{50} value. The strains 1641 and 1658 were the most effective against *H. armigera* larvae and their LC_{50} values were similar to the standard HD-1 strain that showed LC_{50} of 158.5 ng/cm^2. The LC_{50} of the strains 1644 and 520B was not determined since the highest concentration tested (2000 ng/cm^2) was not enouth to kill 50% of the insects. Though the strains 1644 and 520B have had relatively weak larvicidal activity against *H. armigera* compared to the other *B. thuringiensis* strains, even at high concentration, they are potential source of genes to be employed in the development of insect resistant transgenic plants.

The insecticidal activity of the *B. thuringiensis* strains selected as highly toxic to *H. armigera* was assayed against *A. gemmatalis*, *D. saccharalis*, *S. cosmioide* and *P. includens*. The results demonstrated that the most of the strains also are active against the other Lepidoptera species tested, exhibiting mortality

superior to 75% (Table 4). However, the strain 1648 did not show insecticidal activity against *A. gemmatalis*, *D. saccharalis*, *S. cosmioide* and *P. includens*. In addition, the strains 426 and 520B were not toxic to *S. cosmioide* and *D. saccharalis*. The differencial susceptibility of the Lepidoptera species to each *B. thuringiensis* strain may be due the difference among the receptors present in their midgut since the existence of specific receptor for the Cry toxins is essential for the toxin activity (Pardo-López et al., 2013). The pH, protease composition and activity of the larval midgut affect the solubilization or proteolytic processing of the Cry toxins and may also lead to variations in the susceptibity (Talaei-Hassanloui et al., 2013; Fortier et al., 2007).

These results suggest that the selected *B. thuringiensis* strains present great potential to be used in the control of Lepidopera species considered important insect pest of different agriculture cultures. Further bioassays will be required to estimate the LC_{50} values of the strains that caused high mortality to the *A. gemmatalis*, *D. saccharalis*, *S. cosmioide* and *P. includens*.

3.2 Molecular characterization

The PCR amplifications using specific primers revealed that the *cry* and *vip* content of the *B. thuringiensis* strains were variable. The *vip2*, *cry1Fa/1Fb* and *cry2Ac* genes were the less frequent occurring in two strains or three strains while the *cry1B* gene presented the highest frequency (in eight strains), followed by *vip3* and *cry2Ab2* that were found in six and seven strains, respectively (Table 5). The 520B and 1657 strains displayed amplicons for both *vip1* and *vip2* genes. However, the 426, 1644 and 1658 strains showed amplification only for *vip1* gene, this gene profile indicates that these strains might contain extremely different, deleted or truncated *vip2* gene variants (Palma et al., 2013).

In agreement with our results indicating that the strains harbor the gene coding for at least one Cry1, Cry2 or Vip3 protein, some studies with *H. armigera* have evaluated the activity of different proteins belong to these classes and demonstrated high levels of toxicity (Avilla et al., 2005; Bird and Akhurst, 2007; Chakrabarti et al., 1998; Li and Bouwer, 2012; Liao et al., 2002; Sebastião et al., 2015; Chandrashekar et al., 2005; Ruiz de Escudero et al., 2014). In addition, the combinations of Cry and/or Vip proteins that can have synergistic or antagonistic interactions and the expression level of the proteins are factores that may influence the final toxicity of the *B. thuringiensis* strains (Chen et al., 2014; Ibargutxi et al., 2008; Liao et al., 2002; Xue et al., 2005; Li and Brower, 2014). The Cry and Vip proteins are usually order-specific and even species-specific (Estruch et al., 1996; Frankenhuyzen, 2009; Warren et al., 1997), this way, the strains that present diversity of genes can have a wider insect pest spectrum or increased activity. This characteristic is pretty interesting for the development of Bt-based biopesticides since a greater range of insect species could be controlled through of their use. Furthermore, the presence of several *cry* and *vip* genes in the *B. thuringiensis* strains that express proteins with different action mode is important to delay the development of resistance in the target insect. The selected *B. thuringiensis* strains harbor genes coding for proteins known to present insecticidal spectrum against other Lepidoptera species and also Coleoptera species, implaying that the strains might be also effective against them. However, bioassays should be performed in order to determine the toxic activity of these strains against other insect species since the correlation among gene content and the insecticidal activity of the *B. thuringiensis* strains sometimes is not verified (Apaydin et al., 2008; Martínez et al., 2005).

Previous studies have suggested that *cry1C* and *cry1D* are genetically linked at least in some *B. thuringiensis* strains (Ben-Dov et al., 1997; Ferrandis

et al., 1999; Martínez and Caballero, 2002). Our results are in aggrement with this hypothesis once these genes usually occurred together. It is interesting to note that the *cry1B* gene occurred in combination with *cry1C* or *cry1D* or both genes in all strains except in the 1658 and 1636 strain implaing that they may be linked on the cromossome.

The global area of Bt crops has increased tremendously over the past two decades occupying more than 84 million hectares in 2015 (James, 2015), due their substancial environmental and economic benefits that lead to a great acceptance and adoption by the producers (Wu et al., 2008; Carpenter, 2010; Klümper and Qaim, 2014). However with the problem of resistance evolution to *B. thuringiensis* toxins there is a continue necessity to find novel *B. thuringiensis* strains highly active against the target insects that can be useful for their control and resistance management.

Since the entomopathogenic propriety of the *B. thuringiensis* was discovered several research programmes have isolated *B. thuringiensis* strains from diverse habitats around the world and tested their insecticidal activity against a great variety of insect species. Furthermore, some studies have dedicated to screen insecticidal genes already characterized and to find new genes (Bravo et al., 1998; Elleuch et al., 2015; Hernández-Fernández et al., 2011; Ibarra et al., 2003; Palma et al., 2013).

As reported by Tay et al. (2013), the cultive of large areas of Bt crops (maize, cotton and soybean) expressing Cry1 toxin in Brazil, represents a high selection pressure to resistant *H. armigera* insects, especially when these cultivations are done without the appropriate strategies for resistance management. Additionally, this new scenario of the *H. armigera* distribution increases the potential of development of resistance also to Vip3Aa since wide areas of corn expressing this toxin are cultivated in American continent (Chakroun et al., 2016).

These factors may compromise the long-term effectiveness of the actual transgenic events available for the combating of *H. armigera*. The increase in the frequency of resistance to Cry1Ac in *H. armigera* field populations has been verified recently in China. In addition, it is suggested that when the percentage of resistant individuals in the population surpasses the level of 5%, there is a trend that the resistance increases rapidly (Jin et al., 2015). This way, if the farmers do not adopt management resistance tactics as the refuge area it is expected in the near future evolution of resistance to Cry and Vip toxins by the populations of *H. armigera* present in Brazil becomes the Bt crops inefficient and new transgenic events will be necessary.

The *B. thuringiensis* strains selected and characterized in this study could be employed in the formulation of bioinsecticides for the management of *H. armigera, A. gemmatalis, D. saccharalis, S. cosmioide* and *P. includens*. Furthermore, these strains are candidates for harboring new insecticidal genes or genes that synthetize proteins with higher activity than those already studied which could be isolated and used in the development of transgenic plants resistant to these insect pests.

3.3 SDS-PAGE protein profile

The analysis by SDS-PAGE of the spore-crystal mixtures extracted from the *B. thuringiensis* strains showed that all the strains had a similar protein profile to the HD-1 strain with the presence of two principal proteins of about 140 and 55 kDa. These molecular masses are related to the Cry1, Cry9 (130-140 kDa) and Cry2 (50-75 kDa) protein classes which can reflect in the insecticidal activity of the strains since these classes of Cry proteins usually have toxicity to Lepidoptera species (Bravo et al., 1998; Höfte and Whiteley, 1989). These

results are in agreement with those from PCR analysis suggesting that the strains indeed contain *cry1* and *cry2* genes and they are expressed.

3.4 *gyrB* fragments alignment and phylogenetic tree construction

The fragments of the *gyrB* gene of the *B. thuringiensis* strains, which encodes the protein subunit B of the DNA gyrase, were sequenced and their phylogenetic relationship with reference *B. thuringiensis* strains (Soufiane and Côte, 2009) was analyzed. As expected, Blast search for similar sequences on NCBI database demonstrated that the *gyrB* sequences were related to *B. thuringiensis* species. Three clusters at the 95% nucleotide sequence identities were reveled (Figure 2). The eight strains studied were grouped together in cluster I with the *B. thuringiensis higo, B. thuringiensis thompsoni, B. thuringiensis entomocidus, B. thuringiensis zhaodongensis, B.thuringiensis tolworthi, B. thuringiensis kenyae, B. thuringiensis oswaldocruzi, B. thuringiensis pulsiensis, B. thuringiensis poloniensis, B. thuringiensis finitimus* and *B. thuringiensis bolivia*, and they appear to be relatively homogeneous at the *gyrB* gene sequence level however the strains 426 and 520B apper to be more distinct from the strains 1636, 1641, 1644, 1648, 1657 and 1658.

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Figures and tables

Table 1 - Characteristics of general and specific primers for the detection of *cry* and *vip* genes in *B.thuringiensis* strains. (Continua).

	Gene (s) recognized	Sequence (5`-3`)	Tm (°C)	Size (pb)	Reference
CJ16	*cry1Ac*	F-GTTAGATTAAATAGTAGTGG	53	180	Cerón et al., 1994
CJ17		R-TGTAGCTGGTACTGTATTG			
CJ18	*cry1B*	F-CTTCATCACGATGGAGTAA	55	367	Cerón et al., 1994
CJ19		R-CATAATTTGGTCGTTCTGTT			
CJ10	*cry1C*	F-AAAGATCTGGAACACCTTT	55	130	Cerón et al., 1994
CJ11		R-CAAACTCTAAATCCTTTCAC			
CJ12	*cry1D*	F-CTGCAGCAAGCTATCCAA	55	290	Cerón et al., 1994
CJ13		R-ATTTGAATTGTCAAGGCCTG			
CJ14	*cry1Ea/cry1Eb*	F-GGAACCAAGACGAACTATTGC	57	147	Cerón et al., 1994
CJ15		R-GGTTGAATGAACCCTACTCCC			
CJ16	*cry1Fa/cry1Fb*	F-TGAGGATTCTCCAGTTTCTGC	57	177	Cerón et al., 1994
CJ17		R-CGGTTACCAGCCGTATTTCG			
CJ18	*cry1G*	F-ATATGGAGTGAATAGGGCG	50	235	Cerón et al., 1994
CJ19		R-TGAACGGCGATTACATGC			
spe-cry2Aa	*cry2Aa1*	F-GTTATTCTTAATGCAGATGAATGGG	45	498	Ben-Dov et al., 1997
spe-cry2Aa		R-GAGATTAGTCGCCCCTATGAG			
spe-cry2Ab	*cry2Ab2*	F-GTTATTCTTAATGCAGATGAATGGG	45	546	Ben-Dov et al., 1997
spe-cry2Ab		R-TGGCGTTAACAATGGGGGGAGAAAT			
spe-cry2Ac	*cry2Ac*	F-GTTATTCTTAATGCAGATGAATGGG	45	725	Ben-Dov et al., 1997
spe-cry2Ac		R-GCGTTGCTAATAGTCCCAACAACA			
vip1	*vip1*	F-TTATTAGATAAACAACAACAAGAATA TCAATCTATTMGNTGGATHGG	50	585	Hernández-Rodríguez et al., 2009
		R-GATCTATATCTCTAGCTGCTTTTTCAT AATCTSARTANGGRTC			

Table 1 - Characteristics of general and specific primers for the detection of *cry* and *vip* genes in *B.thuringiensis* strains. (Conclusão).

	Gene (s) recognized	Sequence (5`-3`)	Tm (°C)	Size (pb)	Reference
vip2	*vip2*	F-GATAAAGAAAAAGCAAAAGAATGGG RNAARRA R-CCACACCATCTATATACAGTAATATTT TCTGGDATNGG	50	845	Hernández-Rodríguez et al., 2009
vip3	*vip3*	F-TGCCACTGGTATCAARGA R-TCCTCCTGTATGATCTACATATGCATT YTTRTTRTT	47	1621	Hernández-Rodríguez et al., 2009

(F) forward; (R) reverse.

Table 2 - Groups of *B. thuringiensis* strains based on their toxicity against *H. armigera*.

Group	Toxicity against *H. armigera* in % motality	Number of strains	% of strains
I	0-25	73	48.66
II	25.1-50	64	42.66
III	50.1-75	5	3.33
IV	75.1-100	8	5.33

Table 3 - LC_{50} of the *B. thuringiensis* strains against first instar larvae of *H. armigera* after 7 days of bioassay.

Strain	LC_{50} (ng/cm^2)	Fiducial limit (95%)
HD-1	158.5	128.8 - 193.8
426	1543.3	985.9 - 2870.0
520B	N.D.	-
1636	185.0	139.2 - 250.2
1641	149.8	114.7 - 198.6
1644	N.D.	-
1648	725.6	489.7 - 1200.5
1657	1500.1	808.4 – 4339
1658	150.1	114 - 194.4

N.D. Not determined. Less than 50% mortality was obtained even at concentrations as high as 2000 ng/cm^2.

Table 4 - *Cry* and *vip* genes present in *B. thuringiensis* strains and mortality.

Strains	Gene profile	% mortality
HD-1	*cry1Ac, cry2Aa1, cry2Ab2, cry2Ac, vip1, vip3*	100±0.00
426	*cry1Ac, cry1B, cry1C, cry1D, cry1EA/1Eb, cry1Fa/1Fb, cry2Aa1, vip1*	100±0.00
520B	*cry1B, cry1C, cry1D, cry1Ea/1Eb, cry2Aa1, cry2Ab2, vip1, vip2, vip3*	98.33±1.67
1636	*cry1Ac, cry1B, cry1G, cry2Aa, cry2Ab, vip2, vip3*	98.55±1.45
1641	*cry1Ac, cry1B, cry1D, cry1Ea/1Eb, cry1G, cry2Aa1, cry2Ab2, vip1, vip3*	98.55±1.45
1644	*cry1B, cry1C, cry1D, cry2Ab2, cry2Ac, vip1, vip3*	100±0.00
1648	*cry1B, cry1C, cry1D, cry2Aa1, cry2Ab2, vip3*	98.06±1.94
1657	*cry1B, cry1D, cry1Ea/1Eb, cry1Fa/1Fb, cry1G, cry2Ab2, cry2Ac, vip1, vip2, vip3*	78.46±3.92
1658	*cry1Ac, cry1B, cry1Fa/1Fb, cry1G, cry2Aa1, cry2Ab2, vip1, vip3*	98.55±1.45

Percentage of mortality values are expressed as means ± standard deviation.

Table 5 - Toxicity of the *B. thuringiensis* strains against different Lepidoptera species.

Strain	*Anticarsia gemmatalis*	*Spodoptera cosmioide*	*Pseudoplusia includens*	*Diatraea saccharalis*
HD-1	100±0.00	100±0.00	100±0.00	96.79±1.62
426	100±0.00	27.29±8.22	100±0.00	43.91±14.50
520B	100±0.00	13.13±5.37	85.55±1.12	12.50±6.49
1636	98.55±1.45	100±0.00	100±0.00	100±0.00
1641	98.24±1.75	76.99±8.18	86.75±4.55	95.65±4.35
1644	100±0.00	95.65±4.35	96.96±3.03	100±0.00
1648	23.33±13.02	8.33±4.81	7.19±3.73	40.68±6.34
1657	100±0.00	91.30±2.51	97.10±2.90	100±0.00
1658	97.91±2.08	100±0.00	100±0.00	97.09±1.46

Percentage of mortality values are expressed as means ± standard deviation.

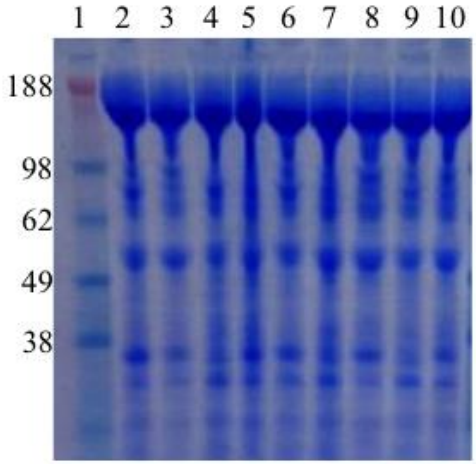

Figure 1 - Protein profile of *B. thuringiensis* strains toxic against *H. armigera* analysed by SDS-PAGE. 1: SeeBlue Plus2 Pre-stained Protein Standard (Life Technologies), 2: 426, 3: 528B, 4: 1636, 5: 1641, 6:1644, 7: 1648, 8: 1657, 9: 1658, 10: HD-1

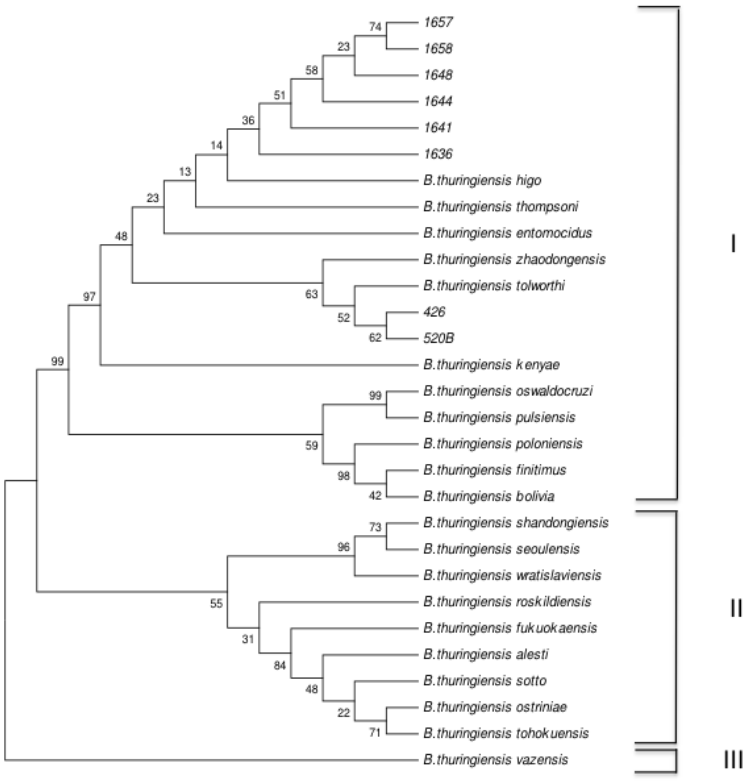

Figure 2 - Phylogenetic tree based on *gyrB* sequences of the *B. thuringiensis* strains constructed using MEGA program relying on the Neighbor-Joining method.