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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação *Stricto Sensu* Biotecnologia Vegetal, para a obtenção do título de "Doutor".

Orientador
Dr. Fernando Hercos Valicente

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APROVADA em 25 de fevereiro de 2015.

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RESUMO

Variedades de milho transgênico que expressa a proteína de *Bacillus thuringiensis* tem sido desenvolvidas para o controle de pragas como *S. frugiperda*. No entanto, mecanismos de resistência têm sido documentados e pouco se sabe a respeito. RNAi (RNA de interferência) é um processo biológico que causa o silênciamento de moléculas de RNAm-alvo e tem sido utilizado como uma ferramenta poderosa para o estudo das funções dos genes e pode ser utilizado para compreender o mecanismo de resistência e o papel dos genes de Bt na toxicidade ao inseto. Estudos de RNAseq foram realizados para examinar a expressão diferencial de genes em neonatas de *S. frugiperda* resistentes e susceptíveis a proteína Cry1F. Para obter um transcriptoma abrangente, o RNAm foi sequenciado a partir do intestino médio de larvas do terceiro instar. O sequenciamento com Illumina gerou ~ 903.100.000 milhões de sequencias e depois da montagem com Trinity e remover sequencias duplicadas, um total de 88.378 *contigs* foram gerados. O tamanho médio dos *contigs* foi de 1.121 pb variando de 201 a 29.329 pb com N50 em 2.726. Um total de 18.749 *contigs* tiveram *hits* no NCBI e 52% dessas sequências tinham maiores similaridades com genes de *Bombyx mori*. Quase 72% das sequências para as quatro condições experimentais foram mapeados com o transcriptoma de referência e um total de 17.524 *contigs* foram utilizados para as análises estatísticas no programa R e DESeq, EdgeR e Limma foram analisados separadamente para identificar genes diferencialmente expressos (DEG). DESeq foi mais informativo e um total de 406 genes mostraram diferenças nas expressões para Cry1F entre suscetíveis e resistentes. Quando as larvas susceptíveis foram alimentadas com milho Cry1F, 770 genes foram diferencialmente expressos no DESeq e um baixo número (135) de genes para as larvas resistentes alimentadas com o milho Cry1F. Para avaliar se um gene especifico da caderina está envolvida no mecanismo de resistência à proteína Bt Cry1F. Este foi reduzido em 50% de expressão para larvas neonatas alimentadas com gotículas de dsRNA específico, na concentração de 2 µg/mL e 24 horas de exposição em temperatura ambiente, foi possível detectar uma supressão em 24 horas após as larvas serem transferidas para a dieta artificial. As larvas alimentadas com dsRNA da caderina não apresentaram diferença na mortalidade quando expostas à toxina Cry1F em comparação com os controles. Com um transcriptoma de referência do intestino médio da *S. frugiperda* foi possível selecionar genes candidatos para o RNAi que mostrou ser uma técnica eficiente para silenciar genes em *S. frugiperda*, e pode ser usado em estudos futuro podendo apresentar resultado com diferentes genes candidatos envolvidos no mecanismo de resistência em *S. frugiperda.*

Palavras-chave: *Spodoptera frugiperda*. Resistência. RNAi. Silenciamento gênico. Sequenciamento. Genes diferenciamente expressos. Caderina.

ABSTRACT

Transgenic maize varieties expressing protein of *Bacillus thuringiensis* has been developed for the control of pests such as *S. frugiperda*. However, resistance mechanisms have been documented and little is known about it. RNAi (RNA interference) is a biological process that causes silencing of molecules mRNA-target and has been used as a powerful tool for the study of gene functions and can be used to understand the mechanism of resistance and the role of genes Bt toxicity to insects. RNAseq studies were conducted to examine the differential expression of genes in neonate of *S. frugiperda* resistant and susceptible to Cry1F protein. To obtain a comprehensive transcriptome the mRNA was sequenced from the midgut of third instar larvae. The sequencing with Illumina generated ~ 903.100.000 million of sequences and after assembly with Trinity and remove duplicate sequences, a total of 88.378 contigs were generated. The average contigs size was 1.121 bp varied from 201 to 29.329 bp with N50 in 2.726. A total of 18.749 contigs had hits in the NCBI and 52% of these sequences were more similarities with Bombyx mori genes. Almost 72% of the sequences for the four experimental conditions were mapped with the reference transcriptome and a total of 17.524 contigs were used for statistical analysis in R and DESeq program, Edger and Limma were analyzed separately to identify differentially expressed genes (DEG). DESeq was more informative and a total of 406 genes showed differences in expressions to Cry1F between susceptible and resistant. When susceptible larvae were fed with corn Cry1F, 770 genes were differentially expressed in DESeq and a low number (135) of the genes to the resistant larvae fed Cry1F with corn. To assess whether a specific cadherin gene is involved in the mechanism of resistance to Bt protein Cry1F. This was reduced by 50% expression of the neonate larvae fed with droplets of specific dsRNA at a concentration of 2 µg / mL and 24 hours exposure at room temperature, it was possible to detect a suppression in 24 hours after the larvae were transferred to artificial diet. Larvae fed with cadherin dsRNA showed no difference in mortality when exposed to Cry1F toxin compared with controls. With the reference transcriptome of the midgut of *S. frugiperda* was possible to select candidate genes for RNAi which proved to be an efficient technique for silencing genes in *S. frugiperda*, and can be used in future studies may provide different results with candidate genes involved in mechanism of resistance in *S. frugiperda.*

Keywords: *Spodoptera frugiperda*. Resistance. RNAi. Gene silencing. Sequencing. Differentially expressed genes. Cadherin.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

As larvas de *Spodoptera frugiperda* causam danos econômicos, alimentando-se de folhas e partes reprodutivas de plantas de milho, soja, algodão entre outras (BARROS et al., 2010). A alimentação larval afeta indiretamente a produção de grãos; além disso, danos no tecido meristemático podem afetar a arquitetura da planta (BUNTIN, 1986; WISEMAN; DAVIS, 1979) e quando as larvas estão em instares mais avançado os danos causados por esse inseto podem ser ainda maiores (CAPINERA, 1999; FLANDERS, 2007). Há vários anos o homem vem buscando um sistema de controle de pragas menos tóxico para os seres humanos e o meio ambiente em geral, e que apresente maior eficiência e especificidade no controle de pragas agrícolas. A bactéria entomopatogênica *Bacillus thuringiensis* tem sido utilizada como alternativa dentro do controle biológico de pragas. *B. thuringiensis* é uma bactéria do solo que se desenvolve em condições aeróbicas em meios artificiais bastante simples. Essa bactéria entra em processo de esporulação durante a fase estacionária, acumulando assim proteínas tóxicas denominadas de proteínas Cry codificada por diversos genes *cry*. Essas toxinas têm sido utilizadas na produção de inseticidas biológicos e também para transformação de plantas, sendo assim conhecidas como plantas Bt (CAPINERA, 1999).

Plantas trangenicas expressando proteínas Bt têm sido amplamente utilizadas desde 1996 para controlar pragas-chave. No entando casos de resitencia a essas plantas têm sido relatados, como o caso das populações de campo de *S. frugiperda* em Porto Rico que desenvolveram altos níveis de resistência ao milho Bt evento de TC1507 expressando o gene da toxina Cry1F (STORER et al., 2010). Este caso de resistência ocorreu depois de quatro anos de comercialização, tornando-se o caso mais rápido documentado envolvendo

resistência à uma cultura de Bt a campo, e o primeiro caso de resistência levando à retirada da variedade de milho Bt do mercado (TABASHNIK; VAN RENSBURG; CARRIÈRE, 2009). Múltiplos fatores podem estar envolvidos na evolução da resistência à Cry1F em Porto Rico, no entanto, mais estudos são necessários para entender como a resistência evoluiu.

A RNAi também tem um enorme potencial para aplicação na entomologia (PRICE; GATEHOUSE, 2008; XU et al., 2012) e tem sido descrita e usada em estudos de função gênica em organismos modelo por mais de uma década, tendo vários estudos sido realizadas em insetos por microinjeção (AMDAM et al., 2003; BROWN et al., 2009; SUAZO; GORE; SCHAL, 2009), ingestão de dsRNA em dietas artificiais ou expressas em plantas transgênicas (BAUM et al., 2007; WHYARD; SINGH; WONG, 2009a). Outra forma de ingestão oral do RNA de dupla fita (dsRNA) é atravéz de gotícula da solução de dsRNA descrito por (TOPRAK et al., 2013). A supressão específica causada pela técnica de RNAi pode ser letal caso o RNAm alvo seja codificador de uma proteína com uma função essencial para o inseto e com isso surgi a possibilidade de desenvolvimento de uma nova tecnologia para o controle de pragas ou para estudos de específicos mecanismos (BAUM et al., 2007; HUVENNE; SMAGGHE, 2010; MAO et al., 2007; PRICE; GATEHOUSE, 2008). A técnica de RNA de interferência (RNAi) transformou a pesquisa com insetos porque permite ao pesquisador suprimir um gene de interesse e, assim, vincular um fenótipo com a função do gene. Para fins básicos de pesquisa, a RNAi oferece uma rota para a genética funcional em todos os insetos, incluindo àqueles para os quais os recursos dos transgênicos ainda não existem (BELLÉS, 2010). Entretanto, para desenvolver métodos de controle de pragas mediada por RNAi é importante encontrar genes-alvo adequados e avaliar o efeito destes para o organismo alvo. O ideal é

que os genes-alvo não só devem ter efeitos sobre as pragas-alvo, mas também devem ser seguros para organismos não alvo (LI et al., 2013).

Nos últimos anos, a contínua melhoria das técnicas e a diminuição do custos dos sequenciamentos de nova geração fez com que o sequenciamento do RNA (RNA-seq) se tornasse uma escolha popular para estudos de expressão gênica. Em tais métodos a base de sequências tem revolucionado estudos sobre o transcriptoma, permitindo uma ampla gama de novas aplicações, incluindo a deteção de isoformas, de *splicing* alternativo, construção de transcriptomas de referência, ou a estudos de expressões específicas (DILLIES et al., 2013) e ainda pode ser uma maneira eficaz de identificar gens candidatos para a RNAi em organismos com informação genómica insuficiente (WANG et al., 2011).

Juntamente ao crescimento da popularidade da tecnologia de RNA-seq, uma série de softwares computacionais vem sendo desenvolvidos para analisar o genes diferencialmente expressos, tais como EdgeR (ROBINSON; MCCARTHY; SMYTH, 2010), DESeq (ANDERS; HUBER, 2010) e Limma (SMYTH, 2004) entre outros. Entretanto, nenhum trabalho relata qual o melhor método de análise, sendo essa uma escolha de acordo com cada experimento ou objetivos de estudo (SEYEDNASROLLAH; LAIHO; ELO, 2013).

2 OBJETIVOS

2.1 Objetivo geral

Identificar genes candidatos potencialmente envolvidos na toxicidade de *Bacillus thuringiensis* e mecanismos de resistência à *Spodoptera frugiperda*, utilizando-se a técnica de RNA de interferência (RNAi).

2.2 Objetivos específicos

a) Sequenciar o RNAm do intestino médio de larvas de *S. frugiperda* para montagem de um transcriptoma de referência.

b) Selecionar genes canditados com base no transcriptoma de referência e prévio conhecimento do modo de ação de Bt.

c) Analisar genes diferencialmente expressos entre população suscetível e população resistente de *S. frugiperda.*

d) Identificar o melhor método de entrega do dsRNA (RNAi via oral) para *S. frugiperda*.

e) Entender como o gene candidato específico para *S. frugiperda* responde ao dsRNA.

f) Verificar o envolvimento do gene canditado com a proteína CRy1F avaliando bioensaios.

3 REFERENCIAL TEÓRICO

3.1 *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

Spodoptera frugiperda é um inseto endêmico do hemisfério ocidental, distribuído da América do Norte para a Argentina (CAPINERA, 1999). *S. frugiperda* é uma importante praga do milho e muitas outras culturas em todas as Américas. No Brasil, essa espécie é uma das pragas de insetos mais destrutivos e economicamente importantes no milho, e também provoca danos para outras culturas, incluindo a soja, algodão, arroz, sorgo e legumes, embora seja considerada como uma praga secundária destas culturas (CAPINERA, 1999; CARVALHO et al., 2013; STORER et al., 2012). As larvas jovens inicialmente consomem tecido da folha de um lado, deixando a camada epidérmica oposta intacta. Até o segundo ou terceiro instar, as larvas começam a fazer buracos nas folhas, e comer apartir da borda das folhas para dentro. Em estádios posteriores, densidades de larvas são normalmente reduzidas a 1-2 por planta, devido ao comportamento canibal (CAPINERA, 1999). No sexto instar as larvas podem comer mais do que todas as outras etapas combinadas e assim, causar dano em quase toda planta. Durante 4 e 5 instar com 2-3 dias de alimentação, as larvas consomem 80% do total da folha consumida durante todo o seu desenvolvimento (CAPINERA, 1999; NAGOSHI; MEAGHER, 2008). Danos extensos podem ocorrer frequentemente removendo todas as folhas e deixando apenas as nervuras e caules das plantas (CAPINERA, 1999; FLANDERS, 2007). As larvas também podem penetrar o ponto de crescimento da planta, destruindo seu potencial de crescimento (CAPINERA, 1999).

Diferentes estratégias têm sido utilizadas para gerenciar a lagarta do cartucho, incluindo práticas culturais, aprimoramento dos inimigos naturais, inseticidas Bt convencionais e culturas Bt (milho e algodão), além do uso de variedades de ciclo precoce, colheita precoce, plantio de variedades tolerantes,

rotação de culturas e inseticidas químicos (CAPINERA, 1999; NAGOSHI; MEAGHER, 2008). No entanto, as estratégias são inconsistentes e muitas vezes insatisfatórias para o controle da *S. frugiperda* na cultura do milho, devido ao seu movimento para a região espiral da planta de milho, onde as larvas são protegidas das pulverizações de inseticidas foliares (SIEBERT et al., 2008). Além disso, a população da lagarta do cartucho tem desenvolvido resistência a várias classes de inseticidas (ADAMCZYK JÚNIOR et al., 1997).

A mais recente estratégia para controlar a lagarta do cartucho tem sido o uso de milho e algodão transgênicos (Bt) (SIEBERT et al., 2008). As culturas transgênicas que produzem toxinas do Bt podem controlar pragas-chave, reduzindo assim a dependência de aplicações de inseticidas químicos. Toxinas Bt ingeridas podem matar insetos suscetíveis pela ligação nos receptores e rompimento das membranas intestinais. Milho expressando a proteína Cry1F tem sido utilizado para o controle da lagarta do cartucho (SIEBERT et al., 2008). Estudos demonstraram que híbridos de milho contendo Cry1F proporcionam um melhor controle da lagarta do cartucho do que híbridos de milho que produzem Cry1Ab sozinho (HARDKE et al., 2011; SIEBERT et al., 2008; WAQUIL; VILLELA; FOSTER, 2002). Dow AgroSciences (Indianapolis, IN) e Dupont Pioneer (Johnston, IA) desenvolveram híbridos de milho que expressam a proteína inseticida Cry1F de *B. thuringiensis* var. *aizawai* (STORER et al., 2010, 2012), que estão disponíveis comercialmente desde 2003 e comercializados como Herculex I proteção contra insetos (transformação evento TC1507).

3.2 Manejo de resistência às culturas expressando toxinas Bt

Os cultivos transgênicos que expressam toxinas de *B. thuringiensis* usado para controle de insetos-praga têm sido amplamente utilizados nos Estados Unidos e no mundo desde 1996 (SHELTON; ZHAO; ROUSH, 2002).

A estratégia de refúgio de altas doses tem sido amplamente adotada para gerenciar evolução da resistência (TABASHNIK et al., 2003). A parte de refúgio da estratégia requer que as pragas-alvo tenha um refúgio contra as toxinas para manter uma fonte de alelos de susceptibilidade e diminuir a seleção para resistência. A estratégia de refúgio e dose elevada, também envolve o uso de plantas que expressam uma alta dose de toxina Bt, que deve matar mais de 95% de heterozigotos portadores de um alelo resistente, impedindo assim a passagem de alelos resistentes para os heterozigotos da geração seguinte. Desse modo, baixa dose seria qualquer quantidade que não atenda à definição de alta dose. Assim, assumindo a expressão de alta dose da proteína em determinada planta Bt, a evolução de resistência pode ser retardada se: (1) os raros insetos homozigotos resistentes (RR) da cultura Bt acasalem com os insetos homozigotos suscetíveis (SS) da área de refúgio e (2) se o heterozigoto (RS) resultante do acasalamento for morto pela concentração da proteína inseticida expressa na planta Bt. Nessa situação, a dose é uma medida do custo adaptativo relativo do heterozigoto RS em relação à diferença entre os homozigotos RR e SS. Em outras palavras, em situações em que a herança da resistência é recessiva, o resultado final é a alta mortalidade dos indivíduos heterozigotos (RS), porque estes se comportariam fenotipicamente como homozigotos suscetíveis (SS) (GOULD, 1988; TABASHNIK et al., 2003; TABASHNIK; VAN RENSBURG; CARRIÈRE, 2009). A mortalidade dos indivíduos heterozigotos é um dos pontos fundamentais a serem considerados no manejo da resistência de insetos a proteínas de Bt, uma vez que no início do processo de seleção eles são os principais carreadores dos alelos de resistência (GOULD, 1988).

Uma estratégia adicional para retardar à resistência é a utilização de plantas Bt de segunda geração que produzem duas toxinas Bt distintas ativas contra a mesma praga. Nessa abordagem, que é chamado de "pirâmide", espera-

se retardar a resistência das pragas mais eficazmente quando a selecção para a resistência à uma das toxinas não provocar resistência cruzada a outras toxinas (ZHAO et al., 2005). A resistência contra as toxinas com diferentes modos de ação é rara em populações de campo (GOULD, 1988). A estratégia de alta dose combinada com o uso de refúgios de culturas não Bt e pirâmides de diferentes toxinas é considerada a melhor abordagem técnica para o manejo da resistência (GOULD, 1988).

3.2.1 Resistência a culturas de Bt

Mais de duas décadas após a comercialização inicial de culturas Bt, a maioria das populações pragas-alvo permanece susceptível. A resistência em campo foi documentada em alguns poucos casos, incluindo *S. frugiperda* ao milho Cry1F em Porto Rico (MATTEN; HEAD; QUEMADA, 2008a; STORER et al., 2010), *Busseola fusca* (Lepidoptera: Noctuidae) ao milho Cry1Ab na África do Sul (VAN RENSBURG, 2007), Helicoverpa *zea* ao algodão Cry1Ac e Cry1Aa no Sudeste dos Estados Unidos, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) para o algodão Cry1Ac na Índia (DHURUA; GUJAR, 2011), e *Diabrotica virgifera virgifera* LeConte (Coleoptera : Chrysomelidae) para milho Cry3Bb1 em Iowa, Estados Unidos (GASSMANN et al., 2011). Em todos os casos, a resistência de campo ocorreu quando um ou mais requisitos da estratégia de refúgio de alta dose não foram atendidas (HUANG; ANDOW; BUSCHMAN, 2011). Incorporar a compreensão dos padrões observados de resistência em campo nas futuras estratégias de manejo da resistência ajudará a minimizar problemas com a resistência e maximizará os benefícios das gerações atuais e futuras nos cultivos transgênicos (TABASHNIK; VAN RENSBURG; CARRIÈRE, 2009).

3.2.2 *S. frugiperda* resistência em Porto Rico

O milho TC1507 expressando Cry1F de *B. thuringiensis* var *aizawai* foi registrado nos Estados Unidos em 2001 para controlar pragas de Lepidópteros importantes, incluindo *O. nubilalis*, *Diatraea grandiosella* Dyar (Lepidoptera: Crambidae), *H. zea* (Lepidoptera:. Noctuidae) (*Helicoverpa zea*) e *S. frugiperda* (SIEBERT et al., 2008). O milho TC1507 está disponível comercialmente nos Estados Unidos desde 2003, mas em Porto Rico tem sido cultivada desde 1998 como parcelas experimentais, desenvolvimento e produção de sementes híbridas (STORER et al., 2012). Dano inesperado em híbridos de milho Cry1F foi relatado em 2006, em Porto Rico e elevados níveis de resistência ao Cry1F na lagarta do cartucho foram posteriormente relatados (MATTEN; HEAD; QUEMADA, 2008a; STORER et al., 2010). Resistência a campo envolvendo *S. frugiperda* ao milho Bt expressando a proteína Cry1F ocorreu após 4 anos de comercialização, tornando este o caso o mais rápido documentado da resistência desenvolvida em campo para uma cultura Bt e o primeiro caso de resistência que levou à retirada de uma cultura Bt do mercado (TABASHNIK; VAN RENSBURG; CARRIÈRE, 2009).

STORER et al. (2010) confirmaram que as falhas de controle do milho TC1507 em Porto Rico foram associadas com o alto nível de resistência da população de *S. frugiperda* com base em bioensaios em dieta. A maior concentração de Cry1F testada contra a população resistente não causou mortalidade significativa (10.000 ng Cry1F / cm^2), o que sugere uma relação de resistência em excesso de 1.000 vezes. Para avaliar a herança, foram realizados bioensaios com a progênie F_1 de cruzamentos recíprocos das populações suscetíveis e resistentes, e as estatísticas de dose-resposta foram comparadas. A mortalidade e a inibição do crescimento de dados da progênie suscetível, resistente e F_1 foram utilizadas para calcular o domínio da resistência. A resistência à Cry1F mostrou ser autossómica e altamente recessiva. A

sensibilidade das colônias resistentes e suscetíveis à Cry1Ab e Cry1Ac também foi avaliada, e nenhuma indicação de forte resistência cruzada a qualquer toxina foi encontrada (STORER et al., 2010, 2012).

Múltiplos fatores vêm sendo discutidos para tentar entender o que teria contribuído para a evolução da resistência à Cry1F em *S. frugiperda* em Porto Rico (STORER et al., 2012). Porto Rico é uma ilha que oferece um ecossistema isolado subdividido por terreno montanhoso. Isso restringe a migração e dispersão restrita e permite que as populações locais respondam à seleção. Além disso, o ambiente tropical de Porto Rico permite o cultivo durante o ano todo com várias gerações expostas à pressão de seleção em um único ano de crescimento. O uso de inseticidas Bt formuladas para gerenciar *S. frugiperda* em vegetais e sementes de milho, juntamente ao uso de outros eventos de milho Bt que produzem Cry1Ab também pode ter contribuído para a seleção (STORER ET al., 2012).

3.2.3 Modo de ação das toxinas Bt

Teoricamente qualquer alteração bioquímica e/ou na fisiologia do intestino do inseto que afetar um ou mais passos no modo de ação de toxinas de Bt poderia interferir com a toxicidade e conferir resistência. Modificações nas proteases intestinais que são criticamente envolvidas na solubilização e processamento proteolítico das proteínas Cry no intestino médio do inseto têm sido encontradas em várias estirpes de insetos resistentes ao Bt geradas pelas seleções de laboratório (LI et al., 2004; OPPERT et al., 1997a). A modificação dos sítios de ligação de toxinas Cry no intestino médio resultante na redução da ligação da toxina, foi classificada como o mecanismo responsável pelos mais altos níveis de resistência a toxinas Cry (ESTADA et al., 1994; FERR et al., 1991; FERRÉ; VAN RIE, 2002; TABASHNIK et al., 1994). Mecanismos de resistência alternativa têm sido propostos, incluindo a retenção da toxina Cry

pela matriz peritrófica do intestino médio (HAYAKAWA et al., 2004), a agregação de toxinas Cry por esterases intestinais (GUNNING et al., 2005) e a melanização elevada da atividade das células do intestino médio e hemolinfa (MA et al., 2005; RAHMAN et al., 2004).

Dados de estudos de resistência sugerem que a ligação de toxinas Cry aos receptores presentes na membrana do intestino médio é um passo crítico no modo de ação de toxinas Cry (FERRÉ; VAN RIE, 2002). Trabalhos relataram que as alterações nos receptores de toxinas estariam associadas com a resistência, incluindo proteínas caderina-*like* (FERRÉ; VAN RIE, 2002; GAHAN; GOULD; HECKEL, 2001; MORIN et al., 2003), aminopeptidase (APN) (TIEWSIRI; WANG, 2011) e fosfatase alcalina (ALP) (JURAT-FUENTES et al., 2011).

3.3 RNA de interferência (RNAi)

A descoberta do RNA de interferência em 1997 por Fire e Mello fez alterações no modo pelo qual estudos de genômica funcional em eucariotos são realizados. O RNAi permitiu a análise funcional de genes em organismos não modelos, sem o requisito de um mutante (BURAND; HUNTER, 2013). O RNAi, também conhecido como o silenciamento de genes mediado por dsRNA, é um fenômeno que ocorre naturalmente em eucariotos, em que um RNA de cadeia dupla diminui ou suprime a expressão de um gene-alvo (SCOTT et al., 2013). O RNAi como uma ferramenta de pesquisa entomológica ja foi utilizado para elucidar genes envolvidos em processos fisiológicos, embriogênese, reprodução e comportamento em insetos-modelo e não modelo (BELLÉS, 2010). Esse método é eficiente e muito variável, dependendo das espécies de insetos (BELLÉS, 2010; TERENIUS et al., 2011), o método de entrega de RNAi (SCOTT et al., 2013; TERENIUS et al., 2011) e o gene-alvo para o RNAi (BELLÉS, 2010). Ao ganhar um melhor entendimento da relação entre a entrega

do dsRNA e a eficácia do método de entrega, bem como identificar os genes que são sensíveis a RNAi poderemos entender melhor os mecanismos associados com RNAi nos sistemas de insetos (TERENIUS et al., 2011).

3.3.1 A descoberta do RNAi

Em uma tentativa de intensificar a cor púrpura de flores de petúnias, dois grupos de pesquisadores inseriram cópias extras de genes responsáveis pela pigmentação. Como resultado, ao invés de conseguirem flores de coloração mais intensa, obtiveram flores de cores variadas ou até completamente brancas (NAPOLI; LEMIEUX; JORGENSEN, 1990). Essas observações, provavelmente as primeiras manifestações de silenciamento por RNA foram chamadas na época de cossupressão, pois tanto as cópias extras quanto os genes endógenos foram silenciados. Cerca de 8 anos depois, estudando o nematoide *Caenorhabditis elegans*, um grupo de pesquisadores descobriu que o responsável pelo desencadeamento do processo de silenciamento era um dsRNA, e o termo RNAi foi proposto (FIRE et al., 1998). Tal descoberta rendeu em 2006, o premio Nobel de Fisiologia a dois pesquisadores, Andrew Z. Fire e Craig C. Mello (nobelprize.org).

O fenômeno de silenciamento gênico baseia-se na presença de moléculas de pequenos RNAs não codantes (ncRNA "*non-coding* RNA") geralmente produto da clivagem de moléculas maiores de dsRNA, com sequências homólogas a genes endógenos de um organismo. Está presente em fungos, quando é chamado de *quelling*; em plantas, chamado de PTGS (pos transcriptional gene *silence*) e em animais chamado de RNAi, indicando uma origem remota em um ancestral comum de fungos, plantas e animais (FAGARD et al., 2000).

O silenciamento de genes por introdução de dsRNA exógeno ficou conhecido como RNA de interferência, Kennerdell e Carthew (1998) descobriram que os efeitos do gene de supressão de RNAi semelhantes, observados em *C. elegans* podem ser traduzidos para os insetos. E ainda demonstraram *knockdown* por RNAi em embriões de *Drosophila.* Esse foi um dos primeiros estudos a demonstrar RNAi em um inseto, e encontrou semelhanças com RNAi no nematoide *C. elegans*, incluindo o dsRNA foi mais eficaz em causar o silenciamento do gene que qualquer senso ou antissenso RNAs (KENNERDELL; CARTHEW, 1998).

3.3.2 RNAi no controle de pragas

Depois de uma década utilizando RNAi como um método para o estudo da função dos genes, o potencial de RNAi em entomologia emergiu como uma nova ferramenta para o controle de pragas/insetos (BAUM et al., 2007). O RNAi pode causar a mortalidade dos insetos, silenciando genes que são essenciais para a sobrevivência do inseto e possui qualidades desejáveis para o controle de insetos, como um novo modo de ação e especificidade de espécie (MAO et al., 2007).

Um dos primeiros estudos de RNAi em inseto foi utilizado microinjeção de dsRNAs em *Drosophila* para induzir o silenciamento de genes mediados por RNAi (KENNERDELL; CARTHEW, 1998). A microinjeção de dsRNA é um método útil para a entrega de dsRNA para uma determinada célula ou região do corpo. No entanto, a microinjecção não é um método adequado de fornecimento para o propósito de controle de pragas/insetos. Portanto, foram avaliados o potencial de outros métodos de administração de dsRNA, como alimentação oral (WANG; GRANADOS, 2001) sugeriram que o intestino médio do inseto é como um sitío-alvo para o controle de insetos, pois é a única região

do intestino que não é protegida por uma cobertura de cutícula e é uma interface ativa com o meio ambiente.

Baum et al. (2007) demonstraram o potencial de controle da larva da raiz do milho ocidental por administração oral de RNAi. O milho transgênico expressando dsRNAs de um gene essencial da larva (vATPase) causou uma redução na alimentação do insetos (BAUM et al., 2007), e também indicou que o modo de ação de RNAi pode ser usado para complementar a estratégia atual de expressar a toxina (s) Bt em culturas para controle de pragas de insetos. Além de seu novo modo de ação, RNAi também é de interesse para o controle de insetos, uma vez que pode atuar como um insecticida específico da espécie. A especificidade do RNAi foi demonstrada dentro do gênero *Drosophila* ao alimentar com dsRNAs, isso causou seletiva mortalidade em uma espécie, sem afetar as outras três espécies estudadas (WHYARD; SINGH; WONG, 2009).

3.3.3 Vias e Mecanismo do RNAi

Foram encontradas três principais vias de RNAi caracterizadas por MicroRNAs não codificante. Essas vias são microRNA (miRNA), piwiRNA (piRNA) e pequena RNA interferente (siRNA). As vias de miRNA e siRNA funcionam como reguladoras negativas da expressão do gene, enquanto as função da via piRNA é para defesa contra elementos transponíveis (ARAVIN; HANNON; BRENNECKE, 2007). Todas as vias RNAi são evolutivamente conservadas, embora genes do núcleo da via siRNA são mais variáveis entre os insetos. Dois estudos (SHREVE et al., 2013; SWEVERS et al., 2013) examinaram a conservação das proteínas RNAi fundamentais entre diferentes espécies de insetos e *C. elegans* no miRNA e vias de siRNA. Núcleo RNAi genes implicados na via de miRNA (SHREVE et al., 2013; SWEVERS et al., 2013) e via piRNA (SWEVERS et al., 2013) são mais conservados entre espécies do que os genes do núcleo de RNAi da via de siRNA. A via de siRNA

é ativada por dsRNA exógeno, e esta via serve para defender o genoma contra a invasão de ácidos nucleicos. Experimentos com RNAi exploram o siRNA via entregando dsRNA para induzir *knockdown* do gene-alvo. Quando um dsRNA é introduzido em uma célula, é clivado em ~21bp RNAs interferentes (siRNAs) por uma enzima ribonuclease tipo III, Dicer-2. A etapa seguinte consiste na parte efetora da via de silenciamento, promovida pelo complexo RISC. Moléculas de siRNAs se ligam ao RISC que as direcionam para sequência complementar do RNA mensageiro-alvo (FIRE et al., 1998). Uma das subunidades desse complexo de 500 kDa é uma proteína da família Argonauta, AGO 2 (HAMMOND et al., 2001) composta estruturalmente por domínios funcionais denominados PAZ (em N-terminal) e PIWI (em C-terminal). A atividade catalítica do complexo RISC-siRNA é associada exclusivamente a AGO 2 (RAND et al., 2004) e seu domínio PIWI apresenta um enovelamento similar ao de uma RNAse H (ribonuclease envolvida na degradação das moléculas de RNA presentes em híbridos RNA/DNA) (SONG et al., 2003). Ou seja, a atividade catalítica responsável pela clivagem do RNA alvo no silenciamento gênico por RNA (denominado SLICER) é determinado pelo domínio PIWI presente em AGO 2, sendo essa proteína também responsável pela clivagem da fita não guia do siRNA associado à RISC (BURAND; HUNTER, 2013). O RNAm é clivado, e a proteína para qual o RNAm codifica não é expressa (BURAND; HUNTER, 2013). Os efeitos sistêmicos do RNAi são mediados através da produção de novos dsRNAs por RNA polimerase dependente de RNA (RdRp), que utiliza o RNA-alvo como molde e é produzido por cadeias de siRNA. Os dsRNAs secundários podem ser exportados das células e espalham o efeito de RNAi para outras células. As proteínas de transporte SID-1 e SID-2 foram identificadas em *Caenorhabditis elegans*, que possui a enzima RdRp. Mecanismos de transporte podem ser diferentes entre os diferentes organismos e ainda não foram bem elucidadas em insetos (PRICE; GATEHOUSE, 2008).

3.3.4 Métodos de administração do dsRNA para insetos

Um dos fatores que podem influenciar a eficiência do RNAi em insetos é a capacidade das células de absorver o dsRNA. A principal via de exposição do dsRNA é via ingestão oral, no entanto, a ingestão não é sinônimo de absorção; o dsRNA deve penetrar nas células do inseto-alvo para que tenha interação com a via de RNAi (TERENIUS et al., 2011). Podem ser empregados vários métodos de administração de dsRNA para insectos, dependendo da aplicação do RNAi, espécies de insetos, estágio de vida do inseto e a expressão do gene-alvo. Muitas experiências entomológicas de RNAi dependem de RNAi extracelular em que os dsRNAs são distribuídos na hemolinfa ou intestino, e as células devem captar esses dsRNAs (YU et al., 2013). O RNAi Extracelular é classificado como ambiental e/ou sistêmico (HUVENNE; SMAGGHE, 2010; WHANGBO; HUNTER, 2008). O RNAi Ambiental é o fenômeno em que dsRNAs são captados a partir do meio ambiente das células, e o efeito *knockdown* do gene está exposto nessas células. O RNAi sistêmico ocorre quando o efeito de silenciamento é passado a partir de uma célula para outra (HUVENNE; SMAGGHE, 2010; WHANGBO; HUNTER, 2008).

Os três métodos mais comuns de entrega de dsRNAs ao insetos incluem microinjeção, oral e imersão (SCOTT et al., 2013b; YU et al., 2013). Um dos primeiros métodos utilizados para a entrega de dsRNA foi microinjecção. Isso foi feito em ambos em um nematoide (*Caenorhabditis elegans*) (FIRE et al., 1998) e um inseto (*Drosophila melanogaster*) (KENNERDELL; CARTHEW, 1998). A Microinjeção é usada para adiministrar dsRNA diretamente na hemolinfa do inseto ou em um embrião de insetos. Inserir o dsRNAs nas células intestinais por meio de alimentação requer a absorção de dsRNAs pelas células. A entrega de dsRNAs é eficaz em insetos que apresentam uma resposta robusta ao RNAi. O método oral de alimentação pode ser utilizado para entregar dsRNA de uma forma de alto rendimento,

evitando ao mesmo tempo os danos mecânicos provocados por microinjecção (SCOTT et al., 2013). Além disso, a entrega do dsRNA através do método oral de alimentação é de particular interesse para controle de insetos (BAUM et al., 2007). Outra forma de entrega que tem sido publicada é através da via oral, porém os insetos são alimentados com gotas de soluções contendo dsRNA na concetração determinada para o específico experimento (TOPRAK et al., 2013).

Existe uma grande diversidade entre Lepidópteros e no que diz respeito à sua sensibilidade ao RNAi sistêmico podendo ocorrer alto ou nenhum silenciamento em diferentes concentrações de dsRNA (TERENIUS et al., 2011). Em algumas espécies, incluindo *H. Cecropia*, *Antheraea pernyi* e *M. sexta*, níveis elevados de silenciamento podem ser alcançados por aplicação de quantidades muito baixas de dsRNA (BETTENCOURT; TERENIUS; FAYE, 2002; HIRAI et al., 2004; TERENIUS et al., 2007). Em *H. Cecropia*, efeitos hereditários de RNAi sobre os embriões da próxima geração também foram relatados seguindo a microinjeção de dsRNA em pupas (BETTENCOURT; TERENIUS; FAYE, 2002). Da mesma forma, em estudos com *M. sexta*, concentrações de dsRNA aplicadas foram bastante baixas (menos de 10 ng/mg de tecido) e os genes selenciados estavam relacionados com a investigação da resposta imune, mas os efeitos ainda variaram muito (ZHUANG et al., 2008). Mais estudos são necessários para estabelecer uma relação dose/resposta entre a concentração clara de dsRNA e da eficiência do silenciamento, e com maior precisão determinar a sensibilidade de RNAi (TERENIUS et al., 2011).

Bellés (2010) sugere algumas causas potenciais a insensibilidade ao RNAi, incluindo razões específicas para cada espécie, o tecido ou o gene. Pode ocorrer a degradação do dsRNA no intestino do inseto, uma vez que insetos como lepdopteros apresentam intestino médio composto de RNAses e pH alcalino que poderiam facilitar a degradação do dsRNA, a deficiência da

absorção do dsRNA, e também possa existir uma baixa resposta do genes após tratamento com dsRNA (BELLÉS, 2010).

Com base na literatura é possível resumir cinco fatores importantes que podem influenciar o efeito de silenciamento e, portanto, a eficiência do RNAi como técnica de controle de inseto: (1) para cada gene-alvo e organismo uma ótima concentração tem de ser determinada para induzir silenciamento ideal (MEYERING-VOS; MÜLLER, 2007; SHAKESBY et al., 2009). (2) A especificidade da sequência utilizada, uma vez que determinará a diminução da expresão do gene-alvo no organismo visado pode também atingir outros insetos não alvo (ARAUJO et al., 2006). (3) O comprimento do fragmento dsRNA é um fator determinante da captação e silenciamento eficiente em organismos intactos (SALEH et al., 2006). Para experimento com alimentação oral as sequências variam entre 300 e 520 pb. No entanto, existe estudo utilizando apenas siRNA (KUMAR et al., 2014) para o silenciamento de genes. (4) O tempo de duração da supressão, como, por exemplo, o silencimanto de *A. Pisum* que persistiu durante 5 dias, em seguida foi reduzido (SHAKESBY et al., 2009) Ou seja, para cada inseto o tempo ideal precisa ser determinado. (5) Estágio de vida do organismo-alvo também é um fator importante. Embora as fases da vida mais tardia sejam mais fácies para manipulação, os estágios mais jovens muitas vezes mostram efeitos de silenciamento maiores. Por exemplo, nenhum efeito de silenciamento foi observado após o tratamento do quarto estágio de *R. Prolixus com n*itropin 2 dsRNA em comparação com 42% silenciamento ao usar o segundo instar (ARAUJO et al., 2006). Também no caso da lagarta do cartucho (*Spodoptera frugiperda*) um forte efeito de silenciamento foi observado em larvas do quinto instar em comparação com a fase adulta do inseto (GRIEBLER et al., 2008).

Para o desenvolvimento de métodos de controle de pragas mediada por RNAi, o ideal é que o gene-alvo não só deve ter efeitos sobre as pragas-alvo, mas também deve ser seguro para organismos não alvo (LI et al., 2013). Porém, muitos organismos de grande importância na economia e na saúde da população humana são organismos não modelo e, portanto, não dispõem de recursos genéticos eficientes. Contudo, com o advento do sequenciamento de nova geração (Next Generation Sequencing-NGS), e a diminuição dos custos do sequenciamento, é possível sequenciar os genomas de organismos em diferentes fases de desenvolvimento (LEGEAI et al., 2014).

3.4 Análise da expressão gênica

A análise da expressão gênica é feita, por exemplo, para identificar genes expressos diferencialmente entre tecidos ou entre diferentes condições experimentais, para discriminar as doenças heterogêneas, como o câncer, ou para elucidar a relação entre a expressão do gene e covariáveis, tais como a sobrevivência ou o grau do tumor (BARRY; NOBEL; WRIGHT, 2005). O transcriptoma é o conjunto completo de transcritos em uma célula e um resumo de todas as expressões de genes. É essencial construir e compreender o transcriptoma com precisão, a fim de interpretar os elementos funcionais do genoma, componentes moleculares das células, desenvolvimento de organismos e mecanismos de doenças (WANG; GERSTEIN; SNYDER, 2009).

Tecnologia de microarranjos é uma abordagem baseada em hibridização, o que requer conhecimento prévio da sequência do gene a ser anostrado. Também fornece uma compreensão da análise do transcriptoma uma vez que pode gerar os dados de expressão para milhares de genes simultaneamente (WANG; GERSTEIN; SNYDER, 2009). Limitações, no entanto, existem também nos microarranjos, por exemplo, a especificidade do arranjo para cada amostra, a saturação do fundo (*background*) e a qualidade e

densidade variáriaveis dos *spots;* fatores os quais têm dificultado as análises comparativas entre experimentos e, geralmente levadas à necessidade de métodos normalizadores complexos. Devido a isso, a utilização da tecnologia do RNA-seq surgiu como uma alternativa eficiente para solucionar esses problemas, bem como para suprir dados transcriptômicos independentemente da necessidade de uma sequência genômica de referência previamente descrita (WANG; GERSTEIN; SNYDER, 2009).

3.5 Sequenciadores de nova geração e RNA-Seq

Dois experimentos da década de 1970 sobre sequenciamento de DNA estabeleceram um marco na ciência que ditaria o rumo a ser seguido nos próximos anos nessa área do conhecimento. Tais experimentos possibilitaram o desenvolvimento de métodos de obtenção da sequência de fragmentos de DNA em laboratório (MAXAM; GILBERT, 1977; SANGER; COULSON, 1975). Um dos métodos, chamado de terminação da cadeia ou sequenciamento *dideoxi*, passaria a ser conhecido como método Sanger de sequenciamento. Na década de 80, com o desenvolvimento dos primeiros protótipos de sequenciadores semiautomáticos de DNA, esse método se tornaria o método mais comum de sequenciamento de DNA, utilizado em rotina até os dias de hoje. O concomitante desenvolvimento de argorítmos de montagem de genomas a partir de fragmentos sequenciados ao acaso tornaria o método Sanger a principal ferramenta do projeto de sequenciamento do genoma humano e de outros organismos nos anos seguintes (LANDER et al., 2001; VENTER et al., 2001).

Nos últimos anos, porém, o sequenciamento Sanger vem sendo substituído por tecnologia de sequenciamento de nova geração, ou NGS (Next Generation Sequencing). Essas metodologias proporcionam um aumento exponencial na quantidade de bases sequenciadas e, consequentemente, alteram significativamente a relação custo/benefício de obtenção de sequências de DNA,

oferecendo um preço final por base sequenciada mais atraente e em menor tempo (EKBLOM; GALINDO, 2011). As plataformas de sequenciamento de nova geração comercialmente disponíveis incluem o sistema Roche 454 (Roche Applied Science), GenomeAnalyser IIx (Illumina, Inc.), HiSeq (Illumina, Inc.) e SOLiD (Applied Biosystems).

Introduzido em 2006, o Illumina Genome Analyzer baseia-se no conceito de "sequenciamento por síntese" (SBS) o qual é semelhante ao sequenciamento de Sanger. Como acontece no 454 da Roche, o protocolo de sequenciamento do Illumina requer que as sequências a serem determinadas sejam convertidas em uma biblioteca de sequências especial, a qual permite a amplificação e imobilização das sequências para serem submetidas ao sequenciamento. Para esse propósito, dois adaptadores diferentes são adicionados às terminações 5' e 3' de todas as moléculas (KIRCHER; KELSO, 2010).

Essa biblioteca de cadeia dupla é desnaturada para obter DNAs de cadeia única. Essas cadeias simples são dispostas em concentrações muito baixas pelos canais de uma célula de fluxo. Essa "*flow cell*" possui na sua superfície com dois tipos de oligonucleotideos imobilizados e complementares aos dois adaptadores, utilizados para produzir a biblioteca de sequencimento. Esses oligonucleotideos hibridizam com as moléculas das cadeias das bibliotecas. Por síntese reversa, começando pela zona hibridizada, a nova molécula que está a ser criada encontra-se covalentemente ligada à "*flow cell*" (KIRCHER; KELSO, 2010; MARDIS, 2008). Essa nova molécula dobra-se e liga-se a outro oligonucleotídeo complementar ao segundo adaptador que não está ligado à placa, podendo ser usado para sintetizar uma segunda cadeia ligada também covalentemente à placa. Esse processo de dobra da molécula e de síntese reversa, chamada de amplificação em ponte, é repetida várias vezes e cria aglomerados de milhares de cópias da sequência original, muito próximos da

célula de fluxo (MARDIS, 2008). Esses aglomerados distribuídos aleatoriamente contêm cópias idênticas da mesma sequência. Desse modo as bibliotecas estão prontas para serem sequenciadas. No analisador de genoma, milhões de aglomerados são sequenciados simultaneamente, as moléculas de DNA são sequenciadas base a base em paralelo usando quatro nucleótidos marcados com fluorescência. As quatro bases competem umas com as outras para se ligar ao alvo, essa competição natural garante a alta precisão. Depois de cada síntese os fluorocromos são excitados por um laser, a cor obtida identifica a base que foi adicionada. Esse fluorocromo é depois retirado para que a próxima base se possa ligar ao template, é lida então cada base adicionada em cada ciclo (KIRCHER; KELSO, 2010).

Com as enormes quantidades de sequencias curtas produzidas por sequenciadores de última geração, programas de alinhamento tradicionais, por exemplo, BLAST (ALTSCHUL et al., 1990) são muito lentos e não são adequados para o mapeamento das sequências com os genomas de referência. Vários programas foram desenvolvidos para resolver o problema. Alguns exemplos incluem Bowtie (LANGMEAD et al., 2009), TopHat (TRAPNELL; PACHTER; SALZBERG, 2009), BWA (LI; DURBIN, 2009), e SOAP2 (LI et al., 2009).

Com essas ferramentas recentemente desenvolvidas, os sequenciamentos de nova geração são, agora, usados em muitos aspectos na pesquisa biológica, as descobertas de mutação, o sequenciamento de isolados, comparações com genoma de referência, permitindo metagenômica, definindo as interações DNA-proteína, descobrindo RNAs não codificadores, montagem, e até mesmo novas sequências genômicas e transcriptomas (MARDIS, 2008).

As plataformas Illumina e Solid são normalmente utilizadas para experimentos de RNA-Seq. Muitos tipos diferentes de análise como, por exemplo, identificação de SNPs, transcrição alternativa e de perfil da expressão

dos genes, podem ser aplicados sobre o resultado do alinhamento de sequências curtas. Em comparação com a tecnologia de microarranjo, RNA-Seq tem muitas vantagens, como por exemplo, alta resolução, baixo ruído de fundo, sem conhecimento prévio da sequência de referência exigido, e ser capaz de distinguir isoformas e expressão de alelos (WANG et al., 2011; WANG; GERSTEIN;SNYDER,2009).

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SEGUNDA PARTE

ARTIGO 1

Normas de formatação da Revista PlosOne (Versão Preliminar)

Transcriptomics of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae): Identifying candidate genes potentially involved in Bt toxicity and resistance mechanisms

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Abstract

Genomic information for *S. frugiperda* is still not completely available to assist current research in pest management. The lack of sufficient genomic information presents an impediment to development of RNAi techniques as well as an understanding of resistance evolution. Next Generation Sequencing technologies are routinely used to investigate gene expression and regulation as they relate to various environmental challenges. In this study, high-throughput RNAseq was conducted to examine differential gene expression in neonates of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) resistant and susceptible to Cry1F. To obtain a comprehensive transcriptome, we sequenced mRNA from midgut larvae of 3rd instar. Illumina sequencing generated ~ 903,100,000 RNA reads, after runned the Trinity and removed the isoforms a total of 88,378 contigs were generated. The average length of contig was 1,121 bp ranging 201-29,329 bp with N50 at 2,726. A total of 18.749 contigs had hits in NCBI and 52% of sequences had highest similarities with genes from *Bombyx mori*. Nearly, 72% of reads for all four experimental conditions were mapped to the reference transcriptome and a total of 17,524 contigs were used as an input in statistical analysis

program R while DESeq, EdgeR and Limma were applied separately to identify differentially expressed genes (DEG). DESeq was more informative and a total of 406 genes showed expression differences between susceptible and resistant Cry1F such as trypsin, crymotrypsin, ABCC2 transporter, serine proteases and P450 monoxigenases. When the susceptible larvae were fed with Cry1F corn a total of 770 genes differentially expressed were identified in DESEq and lower number of 135 genes for the resistant larvae fed with Cry1F corn. Current study provides a valuable resource for further molecular research in this and related pest species and provides insight on the role of host response to Cry1F toxin in *S. frugiperda* and also into the possible mechanisms of Bt resistance.

Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae), is an important pest of maize (*Zea mays*) and many other crops throughout the Americas [1]. In Brazil *S. frugiperda* is one of the most destructive and economically important insect pests on maize and also causes damage to other crops, including soybean, cotton, rice, sorghum and vegetables [1,4]. Transgenic maize (*Zea maize*)

expressing Cry1F protein from *Bacillus thuringiensis* has been released to control this insect pest since 2003, however, an unexpected damage to Cry1F maize hybrids was reported in 2006 in Puerto Rico and high levels of Cry1F resistance in field populations of *S. frugiperda* was subsequently documented in many areas [5,6]. Storer *et al.* (2010) confirmed the high-level of resistance to Cry1F as autosomal and recessive. *S. frugiperda* resistance represents one of four species which have been documented with field-evolved resistance to *Bt* crops. Multiple factors are thought to contribute to *S. frugiperda* resistance evolution [2], but the mechanism of resistance still remains unclear.

B. thuringiensis is a Gram-positive, spore-forming soil bacterium that produces insecticidal *Cry* proteins in insoluble inclusion bodies during the sporulation phase of growth [7]. Individual *Cry* proteins show toxicity toward a subset of arthropod or nematode species, and hence have been deemed safe for mammalian consumption. The general mode of action for Bt starts with toxin ingestion and culminates in the death of these insects following disruption of midgut epithelial cells [8,9]. Two models have been proposed regarding the mechanism by which cell disruption occurs in susceptible Lepidoptera as a result of Bt toxin

exposure: 1) the pore formation model, and 2) the signal transduction model. According to the pore formation model, toxin monomers bind to receptors on the luminal surface of midgut epithelial cells, which leads to toxin oligomerization and insertion into the cell membrane. Embedded toxins are believed to form a pore which affects ionic balance across the cell membrane and finally leads to cell death due to osmotic lysis [7]. In contrast, the signal transduction model proposes that the binding of the Bt toxin to specific receptors stimulate the G-protein coupled signaling pathway leading to activation of protein kinase A and apoptosis [10].

Understanding how a susceptible and resistant insect responds to sublethal exposure to Cry1F toxin may potentially identify genes associated with the mode of action of this toxin. Moreover, comparing the changes in midgut transcriptomes of Cry1F resistant and susceptible insect strains could detect the differential expression of genes in biochemical pathways associated with Cry1F resistance, and provide novel insights into resistance mechanisms in this species. However, lack of adequate genomic resources has hindered molecular-level studies among lepidopteran pest insects. Transcriptome profiling through massively parallel RNA-Sequencing (RNA-Seq) has transformed research

in non-model organisms without prior genomic resources[11–13] and provides a simple and potentially more comprehensive approach to measure changes in transcriptome expression in response to insecticid [14] and Bt protein exposures [15–17] challenges. In this study, using the RNA-seq technology, we examined the transcriptional differences between a susceptible and resistant *S. frugiperda* strain. A reference transcriptome was generated by assembly of Illumina cDNA sequencing data from third instar *S. frugiperda* midgut. In order to understand the molecular mechanisms underpinning the Cry1F toxin response in FAW, we compared transcriptional changes occurring in susceptible and resistant third instars of *S. frugiperda* when exposed to Cry1F protoxin. Identifying and comparing transcriptional changes in response to Cry1F protoxin in susceptible and resistant larvae will help us to understand Cry1F mode of action and the differences between the two strains. Further, we compared the midgut transcriptional repertoire of third instars of *S. frugiperda* Cry1F susceptible and resistant strains. Analysis of constitutive transcriptional differences between the susceptible and resistant *S. frugiperda* strains provides a global perspective of the evolution of resistance to Bt toxins and may further assist in identifying

toxins that are unaffected by resistance. Taken together, this approach enhances our knowledge regarding pest response to Cry toxin exposure and will help further our understanding of the evolution of resistance in other pest species.

Material and methods

Insect strains

The susceptible (SS) strain was purchased from BioServ (Frenchtown, New Jersey), and has been in continuous culture since November 1997 with regular screenings to monitor for any changes in insecticide susceptibility (VÉLEZ et al., 2013).

The resistant (rr) strain was generated by Dupont Pioneer (Johnston, Iowa) and originated from several hundred field collected fall armyworm egg masses from Puerto Rico maize fields during October 2008 and January 2009. Egg masses were brought into the laboratory in Johnston, Iowa, where 826 neonates were selected by exposing them to TC1507 leaf discs. Only larvae that survived a 4-day exposure (785 larvae) were maintained [18]. Then this collection was selected for one generation on maize leaf tissue expression Cry1F (TC1507). This rr strain

was crossed with SS strain first, then mass crossed themselves, which were further bioassayed with Cry1F (full length) at 200 ng/cm_2 for rr individuals. Finally there is 75% similarity in genetic background between SS and rr strain used in this experiments. Both population were reared on general Lepidoptera artificial diet from BioServ [18] at 27°C and 80% RH under a 16 h:8 h light:dark.

Larvae exposure to Cry1F

Cry1F maize leaf (Herculex 1, TC1507, Pioneer and DAS) was used for the treatment. Briefly, 30 larvae from 3rd instar of both SS and rr strain were transferred into no Bt (isoline) maize leaf for 24 hrs first at room temperature (RT), then 15 larvae of 3rd instar from either SS or rr strain were transferred into Bt or no Bt leave discs on 1% of agar in well for 24 hrs at RT, one larva per disc of leaf/well. The midgut of 3rd instar larva was then dissected on ice on next day, ~35mg pooled midgut tissues (3 midguts) were snap frozen in liquid nitrogen and saved in -80 ^{0}C until use. A total of 4 treatments (SS, SSBt, rr, and rrBt) with 3 replicates in each treatment.

RNA preparation

For single RNA preparation, the total RNAs were extracted from pooled samples with RNeasy Mini Kit (Qiagen, Cat. 74104) and treated with RNase-Free Dnase (Qiagen, Cat. 79254) to eliminate the DNA contamination according to the manufacturer's instructions. The quality and quantity of RNA samples were evaluated on 1% agarose gels and NanDrop-1000 (Thermo Fisher Scientific) respectively before submitted for RNAseq analysis.

Next generation sequencing-RNAseq

RNA samples were submitted for Next Generation Sequence (NGS) Core Facility of the University of Nebraska Medical Center for RNAseq analysis on HiSeq 2000 sequence analyzer (http://www.unmc.edu/genetics/ngs.htm) using the 100 bp paired end read method.

Reads mapping and differential expression analysis

For quality control of raw reads data, we applied a stringent quality filtering process by removing reads that do not have a minimum Phred quality score (Q64) of 20 per base corresponding to a 1% expected error rate across the whole sequence using Sickle/1.2

(https://github.com/najoshi/sickle) and any unknown sequence ‘N’ were removed using Prinseq (http://edwards.sdsu.edu/cgi-bin/prinseq/prinseq.cgi) according to the manual instructions, respectively. The raw sequence data past filtering was then used in *de novo* assembling with Trinity assembler (http://trinityrnaseq.sourceforge.net/). In order to reduce the redundancy of isoforms, the resulting assemblies were first processed by CD-HIT-EST with 95% similarity and then followed by CAP3.

To map the quality reads back to a reference transcriptome [19], the Bowtie aligner (http://bowtie-bio.sourceforge.net/index.shtml) was used. The records from the aligner in BAM format were further transformed by using Samtools (http://samtools.sourceforge.net/) for read count information for all treatments and control. All data analyses mentioned above were performed at the University of Nebraska Holland Computing Center (HCC) (http://hcc.unl.edu/main/index.php).

To identify differentially expressed genes among the different treatments, three statistical methods (DESeq, EdgeR and Limma) commonly used for detecting differential expression in RNA-seq studies [20] were employed and compared further for accuracy. The EdgeR

package was initially used to remove low read counts from all data sets at a threshold of cpm (count per million) <1. For EdgeR and Limma, TMM normalization was used to identify differentially expressed genes at adjusted P value (FDR) <0.05. For DESeq, an adjusted p value<0.05 was used along with size factor normalization with default settings.

Homology searches and gene ontology analysis

The resulting contig fasta files were annotated by using the BLASTx algorithms against nonredundant database (nr) in NCBI using an E-value cut-off of 10^{-25} on HCC (Holland Computing Center at http://hcc.unl.edu/main/index.php). For gene ontology analysis, BLAST2GO (http://www.blast2go.org) was employed for further analysis at default setting.

Results

Using the Illumina HiSeq2000 sequence analyzer, our run yielded a total of ~ 903,100,000 raw reads, each read a uniform length of 100 bp after removing adapter sequences. After filtering at Phred>20, the remaining ~876,007,000 reads were applied in de novo assembly with Trinity. With Trinity, a total of 113,000 contigs were generated. In

addition, the total contigs were reduced to 88,378 contigs by removing the redundant isoforms using CAP3 and CD-hit-EST respectively. The average length of contig was 1,121 bp ranging 201-29,329 bp with N50 at 2,726.

Annotation of reference transcriptome

Blast2GO was applied to the 88,378 contigs and a total of 18,749 contigs had hits in NCBI (Fig 1). FAW transcripts had highest similarities with genes from *Bombyx mori*, (9,797; 52%) *Danaus plexippus* (5,532; 29%) and also a less number of sequences with similarity to genes from *Helicoverpa armigera* (333), *Papilio xuthus* (330), *Tribolium castaneum* (276), *Spodoptera littorais* (155) and even *Spodoptera frugiperda* (237) (Fig 2).

Figures 3, 4 and 5 shows the distribution of different GO terms associated with the proteins coded by FAW 18,749 transcripts. They are broadly categorized into biological process, cellular component and molecular function. In the category of biological processes, genes were involved in cellular process (26%), metabolic process (29%) and single-organism complex (14%). Genes involved in binding (42%) and catalytic

activity (42%) were highly represented in the molecular function category. For cellular component the highest number of genes expressed were involved in cell (33%), membrane (24%) and macromolecular complex (13%). Searching for candidate genes previously described in the literature as involved in Bt toxicity, 55 contigs were found for cadherin, (13) aminopeptidases, (11) ABC transporters and 10 contigs for alkaline phosphatases.

Mapping and differential expression gene analysis

Nearly, 72% of reads for all four experimental conditions were mapped to the reference transcriptome among the replicates with at least one reported alignment at tolerance of 2-bp mismatches.

After removed the low read counts from all read counts data sets using cpm (count per million) <1, a total of 17,524 contigs were used as an input in statistical analysis program R and three different statistical methods, DESeq, EdgeR and Limma were applied separately to identify differentially expressed genes (DEG) from RNAseq data. The number of DEG's identified varies greatly with the treatment and to some extent based on the analysis method (Table 1). In total, 406, 252 and 2 DGEs

were identified with DESeq, EdgeR and Limma when resistant larvae were compared with the susceptible larvae, respectively. When compared the three methods of analysis, only two genes were shared between DESeq, EdgeR and Limma (Fig 6).

The resistant larvae unexposed compared to the larvae exposed to Bt corn (rr vs rrBt), indicate 135 and 15 genes that were classified as DEGs by DESeq and EdgeR respectively. But Limma did not identify any DEG for this treatment. The largest number of DEGs identified by Limma was in susceptible larvae fed with Bt corn (SS vs SSBt) 34 genes total. EdgeR classified 685 for this treatment and DESeq 770 DEGs and compared a total of 33 genes were commonly to the three methods (Fig 6). Resistant and susceptible larvae both exposed to Bt maize were compared (rrBt vs SSBt) 927, 913 and 14 DEGS were identified by DESeq EdgeR and Limma respectively, with 14 genes shared between the three methods (Fig 6). The results were not very informative when the three methods were compared for each treatment, so the DEGs from DESeq were used for homology searches and gene ontology analysis.

Annotation of DEGs from DESeq

We examined the genes that showed constitutive differences between the Cry1F resistant and susceptible larvae. Even though we backcrossed the resistant and susceptible strains in order to reduce variance in genetic background, a large number of DEGs by DESEq were detected as reported above. Fourteen transcripts annotated as cytochrome P450 monooxygenases and one trypsin was highly expressed in the resistant larvae. Serine proteases, chymotrypsin and *ABCC2* transporter were up- regulated in susceptible larvae. Nine transcripts coding for trypsin and seven for serine protease was up-regulated when the susceptible larvae were exposure to Bt Cry1F (SS vs SSBt), and a total of ten cytochrome P450 monooxygenases, seven chymotrypsin and five alkaline phosphatase (ALP) were identified as down-regulated genes for this condition. The lowest numbers of DEGs identified by DESeq larvae were in the treatment when the resistant larvae were exposed to Bt corn Cry1F. Trypsin was found to be down- regulated hovewer cathepsin and chymotrypsins were up regulated in rrBt. Resistant and susceptible larvae fed with Bt corn (rrBt vs SSBt), and it was the treatment with largest number of DEGs, from a total of 927, 396 transcripts were well annotated such as cytochrome P450 monooxygenases with 34 contigs down-

regulated and 9 trypsin, 12 serine protease and one defense protein highly expressed in the SSBt. Our data shows that trypsins were highly expressed when the susceptible larvae were exposed to Bt Cry1F and also expressed more alkaline phosphatase, serine proteases and a defense protein. However the resistant larvae had higher expression of cytochrome p450s when compared to susceptible larvae (Fig 7).

Discussion

Second generation sequencing technology provides a powerful tool for analyzing transcriptome of both model and non-model species. However without a preliminary genome sequence, the *de novo* assembly of a transcriptome is still difficult (Schuster, 2008). BLAST-based annotation allows the researchers to transfer knowledge from model organisms to non-model species. Among the genes with BLAST hits, most of them had homologs in the lepidopteran Bombyx *mori* as well as being a traditional model insect [21].

Some research groups have been working with different methods for the analysis of differentially expressed genes [20,22,23] but no one saying each method is the best one for such analyzes. Seyednasrollah et

al., 2013 comparing various methods concluded that the most similar ranking results were obtained with DESeq and edgeR, which share also the same underlying statistical model. Also Limma and DESeq seemed to be among the safest choices when the experiment has small numbers of replicates. Hovewer in this study Limma was highly conservative compared to DESeq and EdgeR, which provided more DEG information for Blat2go analysis.

For DEGs results indicate that the resistant FAW larvae generally do not exhibit a strong transcriptional response to the Cry1F protoxin exposure. In contrast, the susceptible larvae change the midgut transcriptome in response to this toxin. Chymotrypsin-like serine proteases and alkaline phosphatase, which are down regulated in susceptible larvae after toxin exposure, include genes putatively involved in Bt toxin mode of action. Such responses could be considered the first line of host defense against the toxin to prevent further tissue damage. Trypsin-like protease was up-regulate when the susceptible larvae of FAW were exposure to the Cry toxin. Rodriguez-Cabrera et. al., 2010 using RNAi could knockdown a trypsin-like proteinase and they also reported this proteinase is specifically involved in the Cry1Ca1 toxic

pathway in *S. frugiperda*. Changes to Cry toxin binding sites on insect midgut membranes have been the most commonly reported type of Bt resistance mechanism [25–27], but changes in the pattern of trypsin and chymotrypsin proteinases have also been found in some Bt-resistant insect strains suggesting a major role in resistance via the alteration of Cry protoxins activation [28–32]

We also identified several cytochrome P450 transcripts that were differentially expressed between resistant and susceptible larvae and also when both were exposure to Bt Corn, which identified more down regulated cytochrome P450 transcripts. These monooxygeneases play an important role in the degradation of insecticides [33] and were also observed to respond to Cry1Ab protoxin in Lepidoptera [34]. The precise role of cytochrome P450 in Cry protoxin processing is uncertain as these enzymes are generally thought to be involved in biotransformation of *Lipophilic xenobiotics* [35], but may indicate general response to environmental stress (Cry1F toxin exposure). Gene expression in the midgut of Cry1F resistant larvae changes very little in response to Cry1F toxin. These results might suggest that the toxin fails to engage in the

typical mode of action or is not capable of inflicting cellular damage in the midgut of resistant larvae.

In this study, we report a midgut transcriptome for *S. frugiperda*, an economically important pest of corn in the Brazil and United State. Since identified the specific sequence of candidate genes for *S. frugiperda,* from a referencial transcriptome or from the DEGs, that will allow use it in future design of RNAi experiments to try to identify their specific role in the Cry1F resistance mechanism.

Competing interests

The authors declare that they have no competing interest.

Acknowledgments

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ANEXOS

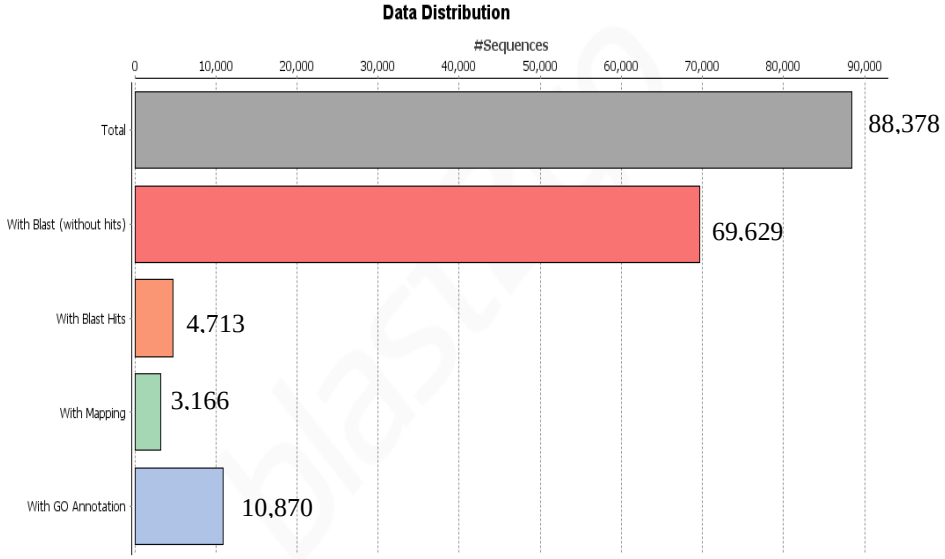

Figure 1. Annotation process with Blast2GO. Sequences without significant sequence alignments (red). Sequences without hits with functional information (orange). Sequences with information that did not surpass the annotation threshold (green). The total amount of annotated sequences (blue).

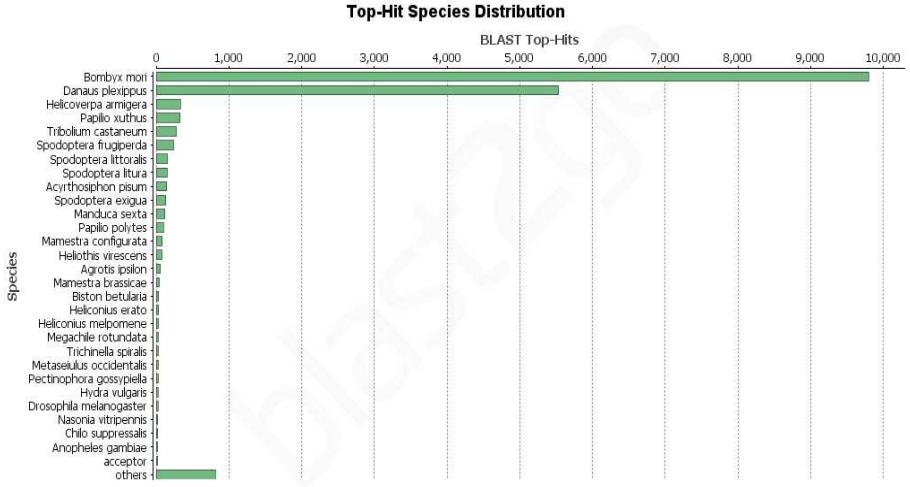

Figure 2. Species distribution of BLAST top-hits of *S. frugiperda* transcriptome assembly.

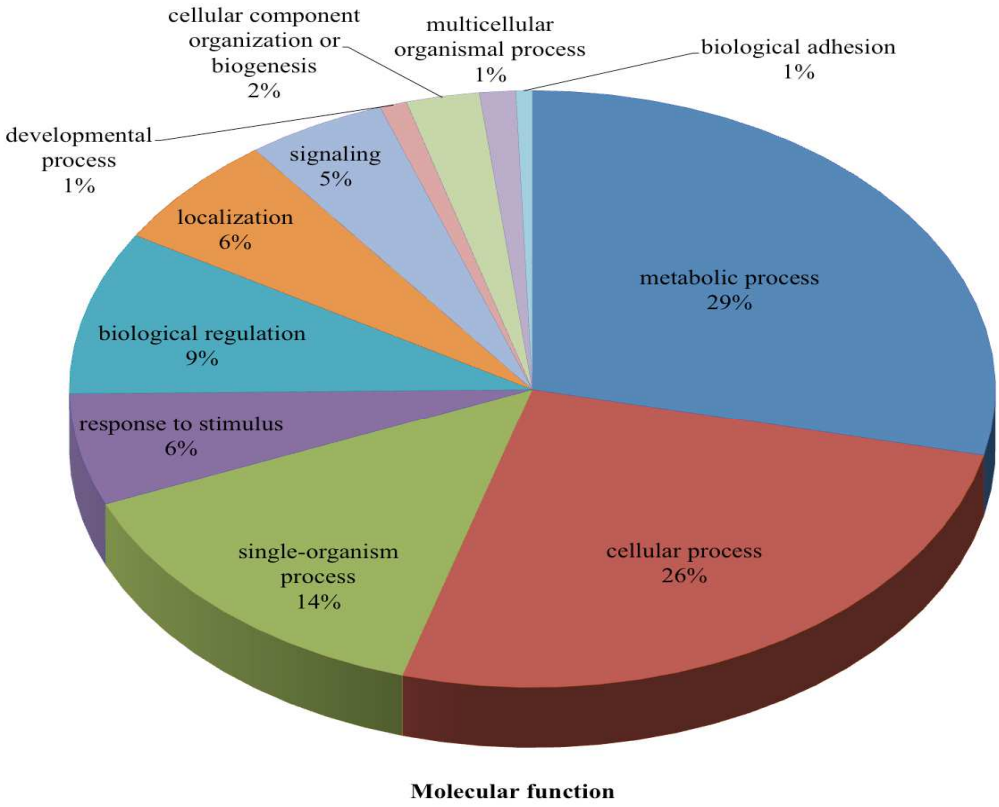

Figure 3. Gene ontology (GO) assignments for the *S. frugiperda* transcriptome. GO assignments (level 2) as predicted for their involvement in molecular Function.

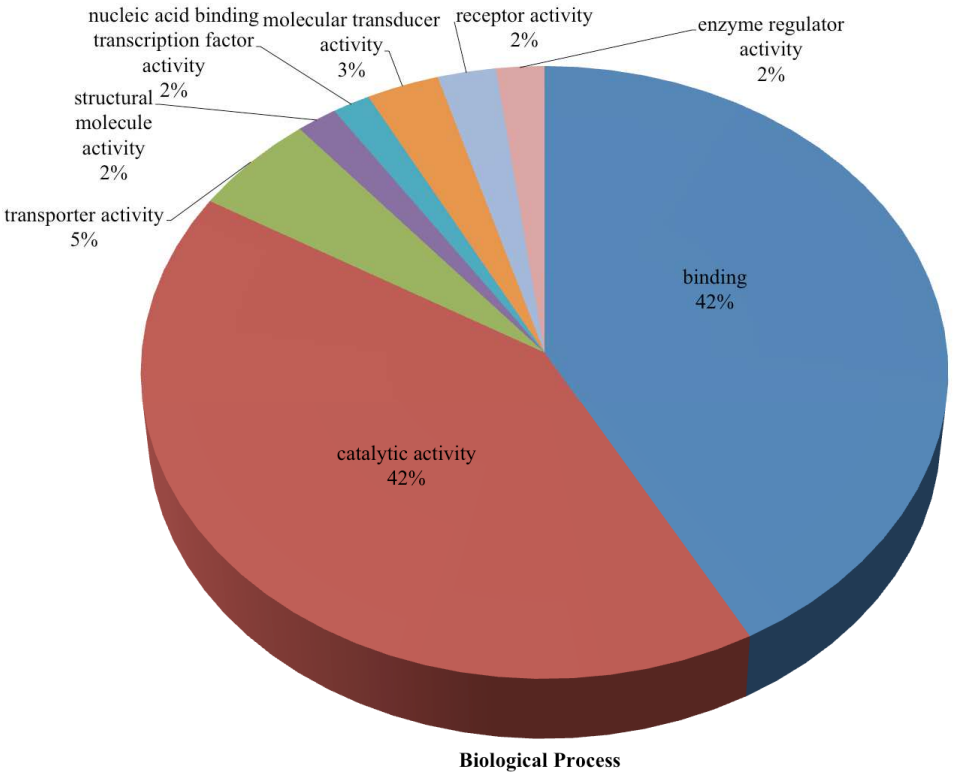

Figure 4. Gene ontology (GO) assignments for the *S. frugiperda* transcriptome. GO assignments (level 2) as predicted for their involvement in biological processes and (C) Cellular components.

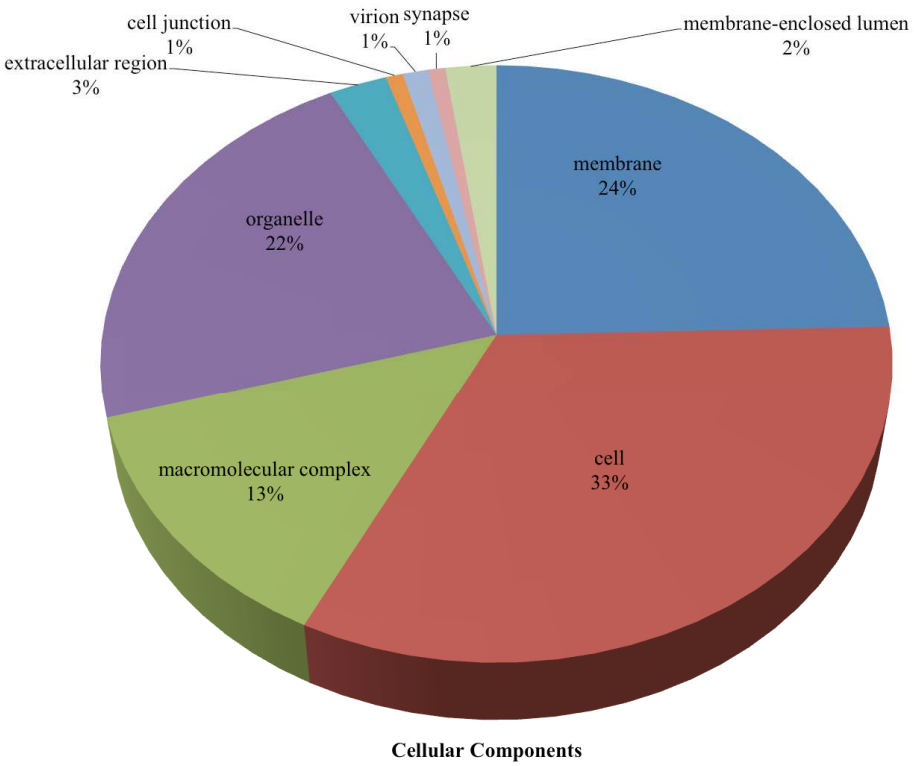

Figure 5. Gene ontology (GO) assignments for the *S. frugiperda* transcriptome. GO assignments (level 2) as predicted for their involvement in Cellular components

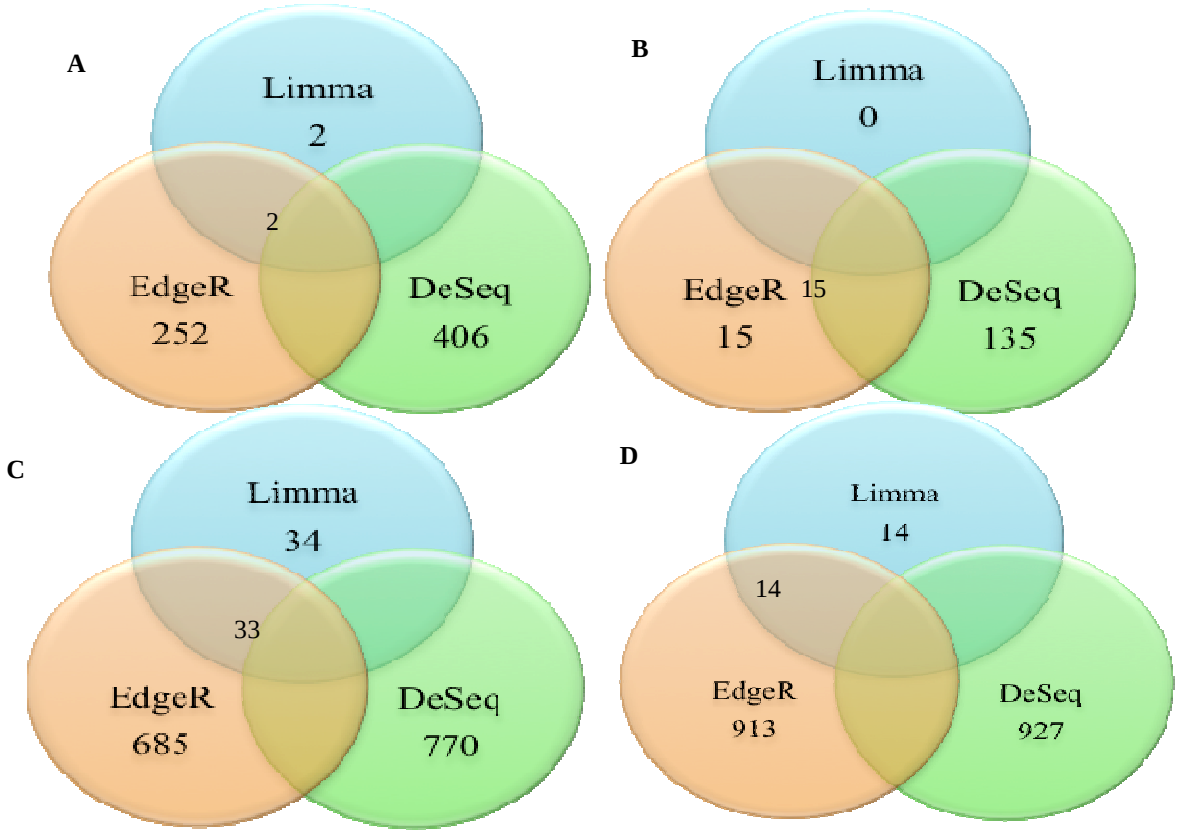

Figure 6. Venn diagrams representing the Comparisons of differentially expressed genes for each method of RNA-seq Limma, EdgeR and DESeq and shared contigs between those. **A**: rr vs SS; **B**: rr vs rrBt; **C**: SS vs SSBt; **D**: SSBt vs rrBt.

Table 1. Number of contigs identified as differentially expressed in comparisons (DESeq and Limma at padjust<0.05 and EdgeR at FDR<0.05)

Analysis method	rr vs SS			sr vs rrBt			SS vs SSBt			rrBt vs SSBt		
	up	down	total	up	down	total	up	down	total	up	down	total
DESeq	168	238	406	76	59	135	333	437	770	488	439	927
EdgeR	69	183	252	6	9	15	332	353	685	503	410	913
Limma	1	1	2	0	0	0	13	21	34	10	4	14

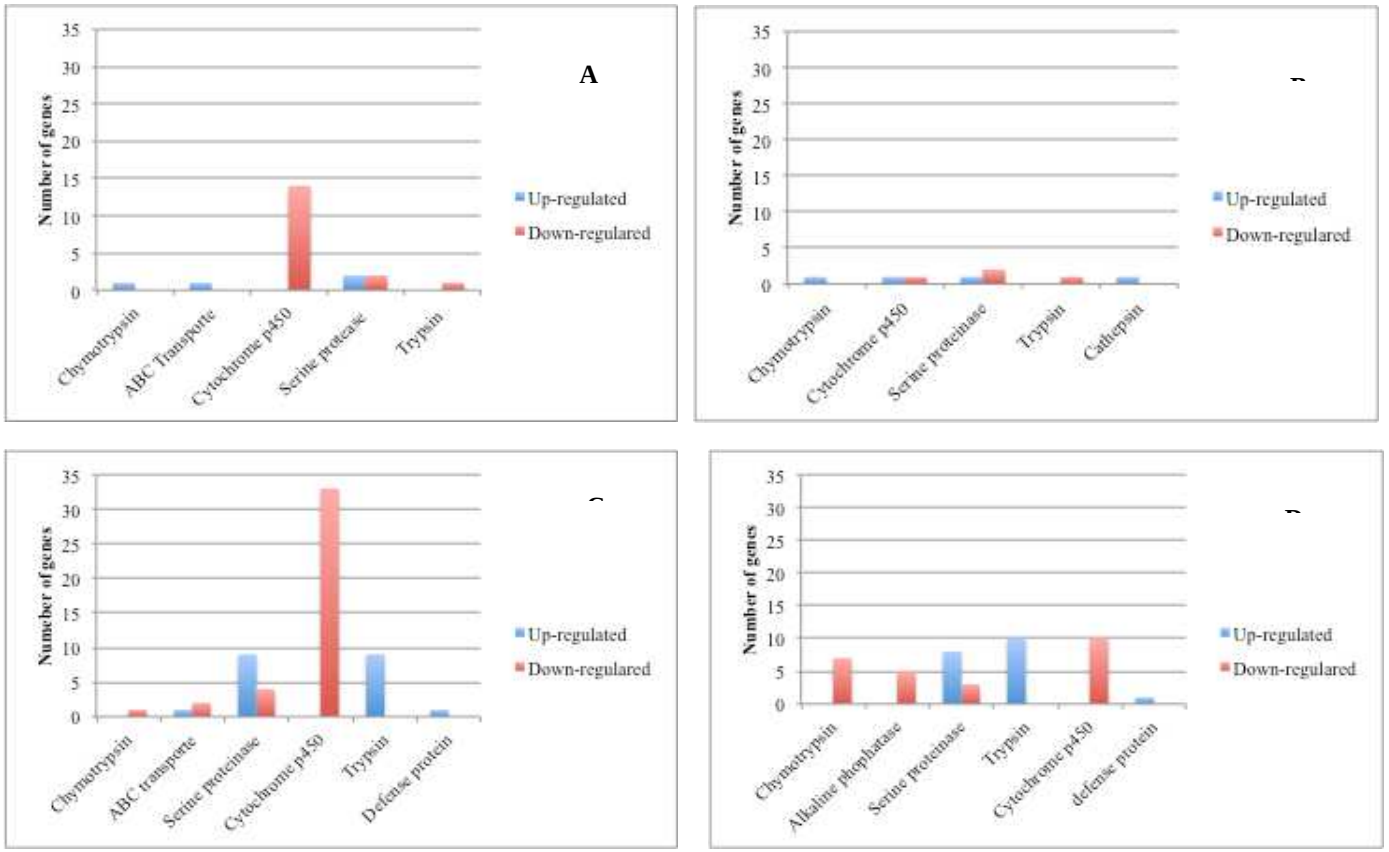

Figure 7. Genes that showed significant values in highly expressed or low expressed in each treatment using DESeq analisys. A (rr vs SS); B (rr vs rrBt); C (rrBt vs SSBt) and D (SS vs SSBt).

ARTIGO 2

Normas de formatação da Revista Environmental Microbiology

(Versão Preliminar)

Efficiency of RNA interference in *Spodoptera frugiperda* (Lepidoptera, Noctuidae) using dsRNA of cadherin-like protein as a candidate gene

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SUMMARY

Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) is an important pest of maize (*Zea mays* L.) and many other crops throughout the Americas. Transgenic maize expressing Cry1F protein from *Bacillus thuringiensis* has been in the market to control this insect pest since 2003. However, resistance to Cry1F was reported in Puerto Rico in 2006 and the mechanism resistance still remains unclear. This study evaluated the RNAi efficiency in fall armyworm larvae using specific dsRNA to silence a cadherin gene. In addition, bioassays were performed to evaluated if cadherin is involved in the mechanism of resistance to Cry1F. Cadherin gene expression was effectively reduced by 50% in neonate larvae fed with droplets of dsRNA (2µg/µL) after 24 hours of exposure. Larvae were transferred to artificial diet treated with Cry1F after 24 hours of exposure with dsRNA. No differences were found between larvae treated with Cry1F and controls. Indicating that this cadherin in *S. frugiperda* might not be involved in the toxicity of Cry1F. The RNAi to silence genes in midgut of *S. frugiperda* still needs to be further studied and other potential candidates genes involved in resistance mechanism can be used to try to identify they specific role for this insect.

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an important pest of maize (*Zea mays* L.) and many other crops throughout the Americas, including soybean, cotton, rice, sorghum and vegetables (CARVALHO et al., 2013; DA SILVA; VALICENTE, 2013; STORER et al., 2012). Transgenic maize expressing the Cry1F protein from *Bacillus thuringiensis* Berliner has been released to control this insect pest since 2003. However, resistance was documented in Puerto Rico in 2006 (STORER et al., 2010). The resistance mechanism remains unclear and our understanding is limited by a lack of genomic information on this species.

B. thuringiensis (Bt) Cry toxins exert their lethal actions by binding to receptors in insect midguts. Four midgut Cry protein receptors have been described, cadherin-like proteins (CADs), aminopeptidase N (APN), alkaline phosphatase (ALP) and glycolipids (BRAVO; GILL; SOBERÓN, 2007; BRAVO et al., 2011; GRIFFITTS; AROIAN, 2005). Two models outline the Cry toxin mode of action and associated mechanisms for resistance, the pore-formation and the signal transduction models. The initial steps for both models share similar initial steps

including toxin solubilization, activation by midgut proteases, and binding to the primary receptor Cadherin (GRIFFITTS; AROIAN, 2005; WANG et al., 2013).

RNA interference (RNAi) has emerged as a powerful tool for the rapid analysis of gene function in a variety of organisms (AGRAWAL; MALHOTRA; BHATNAGAR, 2004). Transgenic crops have been developed to express insecticidal dsRNAs and offer a new approach for agricultural pest control (BAUM et al., 2007; MAO et al., 2007). For transgenic crops expressing an insecticidal dsRNA, lack of direct or indirect exposure provides an additional barrier for toxicity (BACHMAN et al., 2013). Lepidoptera have demonstrated variable susceptibility to ingested dsRNA and high concentrations are required to elicit a response in this Order relative to Coleopterans (HUVENNE; SMAGGHE, 2010; TERENIUS et al., 2011). Additionally, rapid degradation of dsRNA in the hemolymph of *Manduca sexta* has been reported and attributed to nuclease activity, indicating that sensitivity to RNAi may be influenced by the instability of dsRNA within the insect (GARBUTT et al., 2013). Cadherin genes in Lepidoptera already have been silenced in other species including, *Mythimna separate* (GRIFFITTS; AROIAN, 2005;

WANG et al., 2013) and *Spodoptera exigua* (PARK; KIM, 2013; REN et al., 2013). Other case of silencing has been reported in Lepidoptera such as aminipeptidase and alkaline phosphatase in *Manduca sexta* (FLORES-ESCOBAR et al., 2013), and trypsin in *Spodoptera frugiperda* (RODRÍGUEZ-CABRERA et al., 2010). Direct microinjection is the most commonly used procedure for delivery of double-stranded RNA (dsRNA) into organisms. However, Simple more convenient means of dsRNA delivery have been explored, including soaking (ORII; MOCHII; WATANABE, 2003), oral feeding (RODRÍGUEZ-CABRERA et al., 2010), and transgenic plant expression (BAUM et al., 2007; MAO et al., 2007).

Following the revolution in sequencing technologies in the last decade and the resulting availability of large amounts of sequence data, effective methods for assigning function to a gene, such as RNAi, are of paramount importance. Unfortunately, not all species are equally susceptible to RNAi. Despite recognition in the research community that species differ in their sensitivity to RNAi few explanations for these differences have been proposed and experiments designed to explain

observed differences in susceptibility are rare (BELLÉS, 2010; GARBUTT; REYNOLDS, 2012; TERENIUS et al., 2011).

This study evaluated the efficiency of RNAi in *S. frugiperda* and the role of cadherin in Cry1F resistance. To address the association of cadherin in Cry1F toxicity, cadherin gene expression was suppressed using RNAi followed by exposure to Cry1F. This study demonstrated when the expression of this specific cadherin was suppressed in neonates of *S. frugiperda* did not affected in the toxicity of Cry1F after 24 hours of exposure to the toxin

MATERIALS AND METHODS

Insect rearing. A Cry1F susceptible *S. frugiperda* strain was used to perform the experiments. This population was purchased from BioServ (Frenchtown, New Jersey), and has been in continuous culture since November 1997 with regular screenings to monitor for any changes in insecticide susceptibility. Larvae were reared at the University of Nebraska-Lincoln using the protocol described in (VÉLEZ et al., 2013) on artificial general Lepidoptera diet (BioServ, Frenchtown, New Jersey) at 27°C and 80% RH under a 16 h:8 h light:dark.

cDNA and dsRNA synthesis. Total RNA was extracted from 10 neonate larvae with RNeasy minikit (Qiagen). The quantity and quality of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Franklin, MA). cDNA was synthesized from 1 μg of total RNA using a Quantitech reverse transcription kit (Qiagen) following manufacturer's instructions. Primers were designed using Primer3Plus (ROZEN; SKALETSKY, 2000) and T_7 promoter sequences were placed in front of both forward and reverse primers (Table 1). The 348 bp cadherin fragment originated from a reference transcriptome database of *S. frugiperda* larval midgut (Silva et al., 2015 unpublished). For a negative control a 375 bp fragment of a non-specific GFP (green fluorescent protein) gene was amplified from the plZT/V5-his expression vector (Invitrogen) using the gene-specific primers given in Table 1. After sequence confirmation the double-stranded RNA (dsRNA) was synthesized using the MEGAScript RNAi kit following manufacturer's instruction (Ambion, Austin, TX, USA). The reaction mix was incubated for 16 h at 37° C, followed by 15 min of DNase treatment. dsRNA was purified using the RNAeasy Mini kit (RNA cleanup, Qiagen) to the manufacturer's protocol and minikit (Qiagen, Valencia, CA). The quantity

and quality of dsRNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Franklin, MA) and analyzed by electrophoresis (1% agarose gel) to determine purity. Purified dsRNA was stored at -20°C until use.

Cadherin knockdown using RNAi. Approximately 200 neonate larvae were fed with 3 µL droplets of a 1% sucrose solution with 5% blue food coloring in nuclease-free water. Each dropplet contained approximately 2 µg/µL of dsRNA (Cadherin or GFP). Larvae were allowed to feed on dsRNA droplets for 16, 24, 36 and 48 hours at room temperature. Droplets were renewed every 12 hours. Check treatments were fed with 1% sucrose solution with 5% blue food coloring in nuclease-free water. After exposure in each time 10-pooled larvae per replication were collected from each treatment. Four replicates per treatment were performed. Only individuals with observable blue coloring integument were included for RNA extractions with RNeasy minikit (Qiagen).

Quantitative real time PCR (qRT-PCR) and analysis of gene expression. qRT-PCR was performed using the 7500 Fast System real-time PCR detection system (Applied Biosystems, Foster City, CA, USA)

and SYBR® Green Realtime PCR master mix (Toyobo, Osaka, Japan) following manufacturer's instructions. Gene-specific primers were designed using Primer3Plus (Table 2). Template cDNAs were used as a template and were synthesized as previously described. The 7500 Fast System SDS v2.0.6 Software (Applied Biosystems) was used to determine the slope, correlation coefficients, and efficiencies. The efficiencies of the primers were evaluated using 5 fold serial dilutions (1; 1/5; 1/25; 1/125; 1/625) in triplicate. Amplification efficiencies were higher than 93% for all the qRT-PCR primer pairs used in this study. All primer combinations showed a linear correlation between the amount of cDNA template and the amount of PCR product (Table 2). qRT-PCR cycling parameters included, a start at 94 °C for 10 min followed by 40 cycles of 1 min at 94 °C, 30 s at 60 °C, and 40 s at 72 °C. At the end of each PCR reaction, a melt curve was generated to confirm single peak and rule out the possibility of primer-dimer and non-specific product formation. β-actin was used as the housekeeping gene (Table 2). Three biological and three technical replicate were used. Relative quantification of cadherin expression was calculated using the comparative C_T ($\Delta\Delta C_T$) method (LIVAK; SCHMITTGEN, 2001).

Cry1F exposure Bioassay. Diet bioassays were conducted in 24-well titer plates, each well contained 1 ml of general Lepidoptera diet (BioServ, Frenchtown, New Jersey). Each well was treated with *S. frugiperda* Cry1F LC_{99} (VÉLEZ et al., 2013). Purified Cry1F was diluted in 0.1% Triton-X 100 non-ionic detergent to obtain uniform spreading on the diet surface. Each well was surface treated by applying 30 μL per well and dried under room temperature laminar flow. The negative control consisted of well treated with 30 μL of 0.1% Triton-X 100. Control larvae and dsRNA feed treatments were randomly divided into two groups. One group was placed on a negative control diet (0.1% Triton-X 100), and the other group was placed on a diet containing the toxin. Individual larvae with blue integument were transferred using a fine paintbrush into each well. Wells were covered with vented lids (Bioserv, Fenchtown, New Jersey), and trays were held in an incubator ar 27 °C and 80% RH. Bioassays were replicated three times for each treatment with 16 larvae per treatment (total of at least 48 larvae per treatment).

The one-way analysis of variance (ANOVA) was used for statistical analysis and Tukey test (at $P<0.05$) for statistical significance with Sigma Plot Program (version 12.0).

RESULTS

The 348 bp cadherin sequence from *S. frugiperda* larvae was sequenced and confirmed using BLASTX, showing high 90% similarity with *Spodoptera litura* (Fig. 1). GFP and cadherin dsRNA quality was evaluated on agarose gel (Fig. 2). Similarly qRT-PCR amplification was confirmed by the presence of a single peak in melting curve analyses and a specific band with expected size based on agarose electrophoresis. The primer efficiency value of cadherin primer was 95.3 % with a correlation coefficient (R^2) of 0.997 and for actin primer the value was 93 % of efficiency and 0.999 of correlation coefficient (R^2) (Table 2).

Cadherin and GFP dsRNA in sucrose solution were delivered to neonates by drop feeding. Expression of the target gene was monitored by qRT-PCR after 16, 24, 36 and 48 hours of exposure. After a few minutes the larvae started to walk in direction to the droplets and 12 hours later most the larvae were with the gut completely blue (Fig. 3). Only larvae with blue integument were selected to qRT-PCR and for bioassay analysis. Cadherin expression was reduced to an average of 50 % relative to water and GFP controls ($p<0.05$) (Fig. 4) after 24 hours of exposure with dsRNA. However, there were no significant differences of

expression after 16, 36 and 48 hours of exposure (Fig. 5). Different dsRNA concentrations were tested (data not shown) and 2µg/µL after 24 hours of exposure were showed the best results.

Preliminary experiments were performed the longevity of cadherin gene suppression after dsRNA treatment. After 24 hours of dsRNA feeding larvae were transferred individually to artificial diet without treatment to avoid cannibalism. After 24 and 48 hours larvae were collected to evaluate gene expression by qRT-PCR. Results indicated that cadherin was reduced around 50% compared to water and GFP controls after 24 hours. However, after 48 hours larvae showed higher cadherin expression and there were no significant differences between treatments (Fig 6).

Cry1F Bioassays. After 24 hours of feeding with dsRNA larvae with blue gut from each treatment were transfered to the artificial diet treated with pure Cry1F toxin (200 µg/cm^2). The following day the majority of larvae treated with Cry1F were dead in all treatment compared to the negative control (Fig 7).

DISCUSSION

RNAi has been used for investigating the function of several genes in different Lepidopteran species (BELLÉS, 2010). RNAi silencing in *S. frugiperda*, has been previously used to investigate the role of neuropeptide genes in the regulation of hemolymph juvenile hormone during different insect life stages (GRIEBLER et al., 2008; MEYERING-VOS et al., 2006). In addition of genes involved with *B. thuringiensis* toxicity (RODRÍGUEZ-CABRERA et al., 2010) as reported in this study.

Several dsRNA delivery methods were tested including microinjection and surface treating artificial diet (data not shown). With microinjection the mortality was too high, the possibility of damage and loss of body turgor pressure in larvae due to injection could be a limiting factor in the application of this method. The experiments with dsRNA in artificial diet did not show constant results, varying it in each repetition. While the droplets method provided a significant decrease (50%) on cadherin expression and low expression (50%) levels were detected after two days of exposure, the results were constant in each repetition. Similar results were obtained in a different study, when neonate larvae were fed

with a single dose of dsRNA at a concentration of 6 μg/μL and 1 hour of exposure then moved to leaf disks (TOPRAK et al., 2013).

When dsRNA delivery method was established, various concentrations of dsRNA were tested but 2 μg/μL was the best result in silencing. Several paper report that large amounts of dsRNA (>3 μg/μL) are necessary to reduce gene expression in Lepidoptera (TERENIUS et al., 2011), RAJAGOPAL et al., 2002 and SIVAKUMAR et al., 2007 showed silencing of a midgut aminopeptidase gene required 4 and 6 μg/μL of dsRNA/larva injected to fifth instar *S. litura* and *Helicoverpa armigera* respectively. However, results with lower concentrations reported effective silencing of several immune-related genes in *Manduca sexta* by injection of 100 ng of dsRNA in fifth instar larvae (ELEFTHERIANOS et al., 2006). Similarly, (TURNER et al., 2006; YANG et al., 2010) reported approximately 25 % - 50 % reduction in the expression of gut carboxylesterase and aminopeptidase genes two days after feeding dsRNA to thirst instar *Epiphyas postvittana* (1 μg) and *Diatraea saccharalis* (250 ng) larvae, respectively. This study supports the idea that RNAi requirements may vary according to the method of delivery, the efficiency in up take of dsRNA by the cells, the target gene,

the insect species and the developmental stage (HUVENNE; SMAGGHE, 2010; TERENIUS et al., 2011). Also genes expressed at lower levels may be targeted more efficiently than genes expressed at higher levels by the same doses of dsRNA; thus, it may be necessary to optimize the amount of dsRNA for each gene (TOMOYASU; DENELL, 2004).

The low expression of cadherin after 24 hours of dsRNA exposure could indicate that dsRNA is degraded after 24 hours. The midgut of most Lepidoptera larvae is a hostile environment for RNA because of its alkaline pH and the presence of high RNase activity (TERRA; FERREIRA, 1994). In *Bombix mori* larvae, a midgut dsRNAse has been implicated in interfering with RNAi. *B. mori* larval midgut juices were mixed with dsRNA and complete degradation of dsRNA was observed after 24 hours (LIU et al., 2012). However, the specific mechanisms involved in systemic RNAi and its persistence are still not well understood. In this study continuous feeding of neonate larvae with 10 µg of dsRNA was tested (data not shown) and did not resulted in an improved gene silencing. However, previous studies with *Ostrinia nubilalis* reported a reduction chitinase expression by of 60% with a continuous feeding (KHAJURIA et al., 2010).

In this study, larvae treated with cadherin dsRNA followed by Cry1F exposure did not exhibited decreased susceptibility to Cry1F compared to controls. However, other studies reported that cadherin dsRNA affected the susceptibility of *S. exigua* to Cry1Ca and Cry1Ac toxins (REN et al., 2013), and *Mythimna separata* to Cry1Ab (WANG et al., 2013). In a different study, a cadherin *S. frugiperda* fragment increased toxicity to Cry1F in *S. frugiperda* larvae in a synergism bioassay experiment indicating that cadherin might be a receptor (RAHMAN et al., 2012). However, under our experiment conditions and for this specific cadherin sequence, the dsRNA did not affect the susceptibility of *S. frugiperda* larvae to Cry1F. One of the possibilities in the differentiation of results might be a diferente part of the gene used to make the dsRNA or the Cry1F protein used.

The bioassays performed in this study demonstrated efficient delivery of dsRNA to *S. frugiperda* neonates with a decrease in cadherin expression after 24 hours of exposure. This type of bioassays could be used in future RNAi experiments with *S. frugiperda* to understand the resistance mechanism of Bt toxins or to determine the function of a candidate gene.

Competing interests

The authors declare that they have no competing interest.

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Table 1. General information of the primers for initial PCR analyzes

Gene name	Primer Name	Primer Sequences for qRT-PCR (5´-3´)	Product length (bp)	Tm (°C)
Cadherin	Cadh.2-F	(T_7) GCACACGACAGAGATGAACC	348	60
	Cadh.2-R	(T_7) CTATGGCACGCTCCCTAGAA		
GFP	GFP-F	(T_7) GGTGATGCTACATACGGAAAG	375	60
	GFP-R	(T_7) TTGTTTGTCTGCCGTGAT		

Table 2. General information of the primers for qRT-PCR analyzes

Gene name	Primer Name	Primer Sequences for qRT-PCR (5´-3´)	Product length (bp)	Tm (°C)	Eff (%)	R^2
Cadherin	Cadh.2-F	GCACACGACAGAGATGAACC	348	60	95.3	0.997
	Cadh.2-R	CTATGGCACGCTCCCTAGAA				
β-Actin*	Actin-F	CGGTATCGTGCTGGACTCCGGTG	150	60	93.0	0.999
	Actin-R	GAGTAACCCCTCTCGGTGAGGATC				

*Rodríguez-Cabrera et al (2010).
E: Amplification efficiency; R^2: Correlation Coefficients

Score	Expect	Identities	Gaps	Strand
411 bits(222)	4e-111	288/321(90%)	0/321(0%)	Plus/Minus

```
Query  1     GGTTGAGCCAGGAGTTGGGAACACAAACACCGGTGAATGGAAGTTGTATGGCCTGACGGT  60
             ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3510  GGTTGAGCCAGGCGTTGGGAACACAAACACCGGTGAATGGAAGTTGTATGGCCTGACGGT  3451

Query  61    TAGTTGATAGGTCTCGAATGAATCCAGCATCGGGACACCGTGGTCAAACGCACGTATCTC  120
             ||||| |||||||||||| ||||| |||   |||  ||||||||||||||||||||||| ||
Sbjct  3450  TAGTTCATAGGTCTCGATTGAATACAGTGGCGGCTCACCGTGGTCAAACGCACGTATTTC  3391

Query  121   GACATCATAGGTGCCCCAGTATCCTCTAAGGTCCATGGTAGTCTCCAACTCTCCAACGAA  180
             ||| ||||||||||||||||||||||||||||||||||||||| |||||||||| |||||
Sbjct  3390  GACTTCATAGGTGCCCCAGTATCCTCTAAGGTCCATGGTAGTCACCAACTCTCCTACGAA  3331

Query  181   TTTCCAGGTATCGAGATCATCAATCGTTATTATTTTGAATGGATCTTGAGGAAGCTCGAT  240
             | ||||||| |||| || |  |||||||  |||||||||||||||||||||||| || ||
Sbjct  3330  TCTCCAGGTTTCGAAATTACTAATCGTTTCTATTTTGAATGGATCTTGAGGAAGTTCAAT  3271

Query  241   GTCTCTATTGATCAATTTGATCGATCGAATTTCATATCCCACGCGAGAGTTGTCGTTGAA  300
              ||||||||||||||| |||  ||| |||||||||||| |||||||||||||    |||
Sbjct  3270  CTCTCTATTGATCAATGTGACTGATAGAATTTCATATCCGACGCGAGAGTTGTTTGAGAA  3211

Query  301   TGGTTCATCTCTGTCGTGTGC  321
             |||||||||||||||||||||
Sbjct  3210  TGGTTCATCTCTGTCGTGTGC  3190
```

Figure 1. Cadherin sequence from *S. frugiperda* was indentity in 90% with *S. litura* Sequence ID: gb|JN687590.1| on Blast.

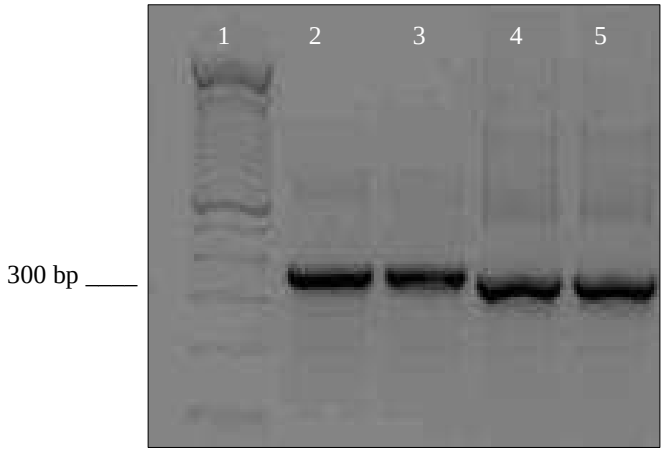

Figure 2. dsRNA quality in 1% agarose gel. Molecular maker of 1Kb from Invitrogen (lanes 1); dsRNA of purified GFP (lanes 2 and 3); dsRNA of purified Cadherin (lanes 4 and 5).

A B C

Figure 3. Cadherin dsRNA (2µg/µL) droplet feeding. A: Petri dish with paper filter, droplets of dsRNA; B: Droplet with blue food dye; C: *S. frugiperda* larvae with blue gut after drinking dsRNA solution with blue dye.

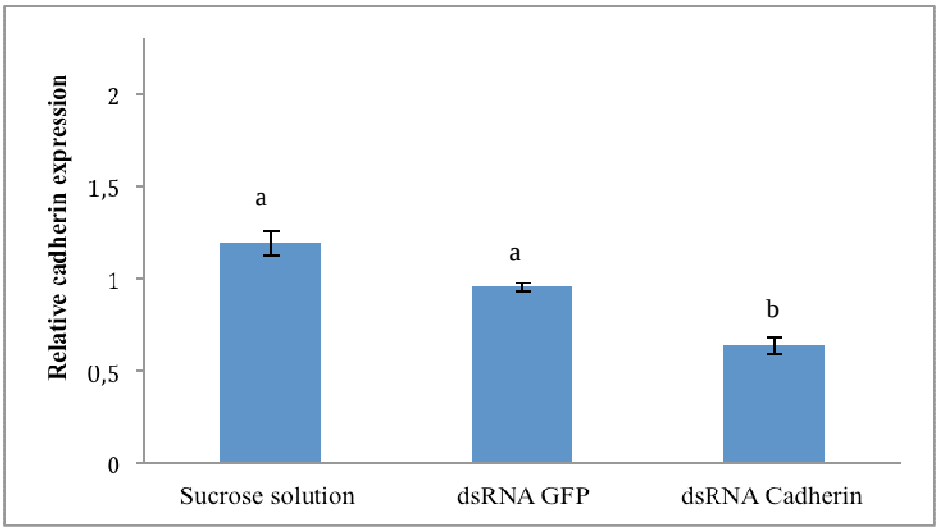

Figure 4. Relative *Cadherin* expression after 24 hours of dsRNA exposure. *β-actin was used as housekeeping gene. Values represent the means and the error bars indicate the standard deviation of four replicates of samples containing 10 neonates. Different letters indicate significant differences in the expression level (ANOVA, Tukey Test, $p<0.050$).

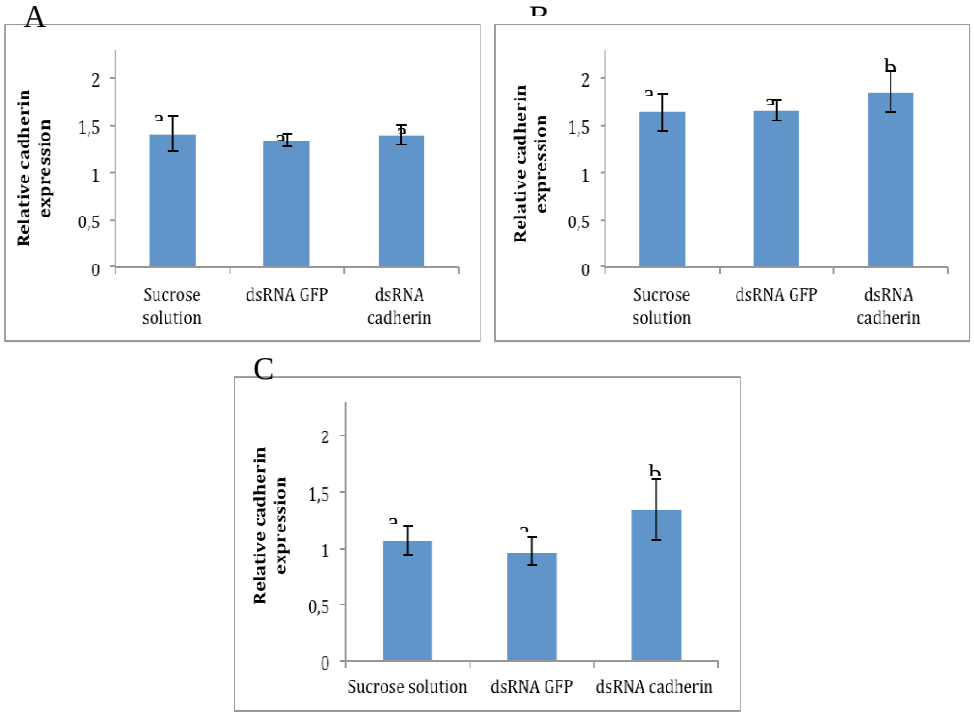

Figure 5. Relative *Cadherin* expression after 16 (A), 36 (B) and 48 hours (C) of dsRNA exposure. *β-actin was used as housekeeping gene. Values represent the means and the error bars indicate the standard deviation of four replicates of samples containing 10 neonates. Different letters indicate significant differences in the expression level (ANOVA, Tukey Test, $p<0.050$).

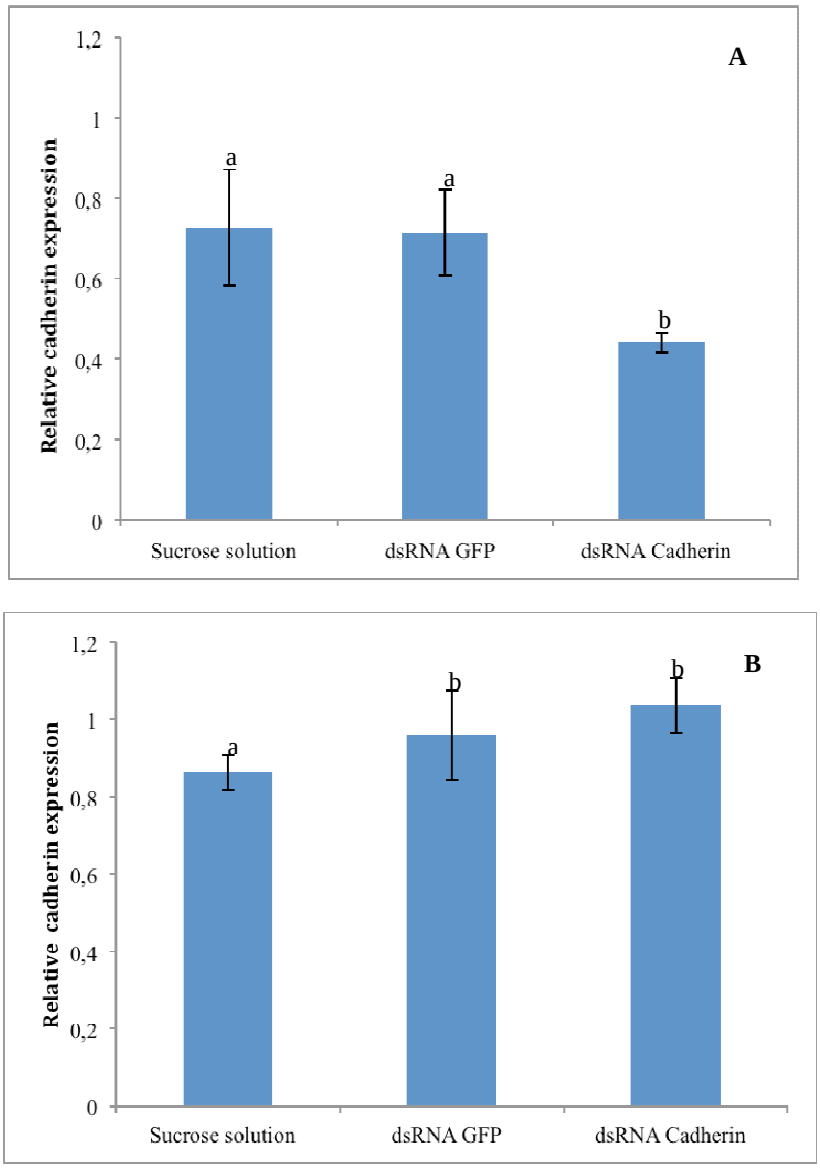

Figure 6. After 24 hours of exposure with dsRNA, larvae with a blue integument were individually placed to wells with artificial diet without treatment and collected after 24 hours (**A**) and 48 hours (**B**) for qRT-PCR analysis expression**.** Values represent the means and error bars indicate the standard deviation of four replicates of samples containing 10 neonate larvae. Different indicate significant differences in expression levels (ANOVA of t-test, $p<0.050$).

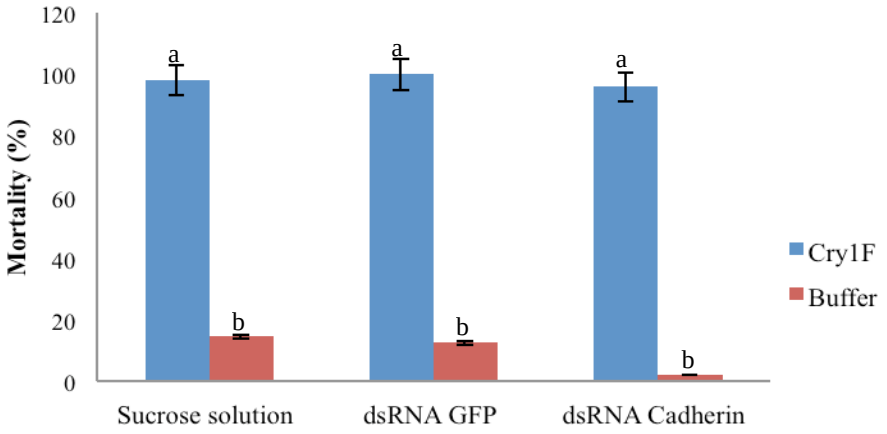

Figure 7. Cry1F mortality after exposure to *Cadherin* dsRNA. Mortality was recorded 24 hours after exposure to Cry1F. Values represent the means and error bars indicate the standard deviation of three replicates of samples containing 16 larvae. Different indicate significant differences in expression levels (ANOVA of t-test, $p<0.050$).