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Research Article

On the Effectivity of Gene Silencing Induction in Fall Armyworm (*Spodoptera Frugiperda*) Using Specific dsRNA

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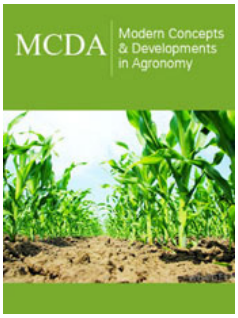

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Abstract

The fall armyworm, *Spodoptera frugiperda* (JE Smith) is a polyphagous pest of maize and horticultural crops in the Americas, but it recently was found in Africa as well. Main control strategies are based on the use of chemical insecticides or transgenic crops expressing *Bacillus thuringiensis* (*Bt*) toxins. However, in the last years, resistance of *S. frugiperda* field populations to *Bt* corn transgenic was detected in different Latin American countries. The available genomic information of *S. frugiperda* allows the development of new control technologies based on the use of RNA interference (RNAi). Three different *S. frugiperda* target genes were amplified and cloned to perform *in vivo* synthesis of specific dsRNA (ribosomal, trehalase and tubulin genes). Second to fourth larval instars were either fed, injected or soaked in dsRNA solutions and the effect on the target gene transcripts was evaluated by reverse transcriptase quantitative PCR (RT-qPCR). Only soaking second-instar larvae in dsRNA solution of ribosomal RNA gene sequence showed a statistically significant knockdown of target mRNA levels compared to control treatment. In contrast, no significant differences were registered on third and fourth larval stages. Therefore, the results showed that RNAi responses by soaking method depend of life stages, the method of inoculation and target gene sequence. Further research is needed in order to evaluate the use dsRNA as sprayable pesticide in fall armyworm strategies.

Keywords: RNA interference; Fall armyworm; Soaking method; Pest control; Maize

Introduction

The fall armyworm (FAW), *Spodoptera frugiperda* (*Lepidoptera: Noctuidae*), is a tropical insect and one of the most important pests in the Americas. Its distribution spans from Argentina to the United States [1-3]. Recently, FAW was reported in India [4] and the equatorial region of Africa [5,6]. *S frugiperda* larvae exhibit polyphagous behavior enabling them to attack many cultivated plant species [7,8]. Main plant hosts include maize, sorghum and important others crop such as cotton and soybean [9-11]. At present, FAW is mainly controlled by applying chemical pesticides and/or cultivating transgenic crops expressing *Bacillus thuringiensis* entomotoxins (*Bt*). In recent years, the emergence of genetic resistance of *S. frugiperda* populations to Bt transgenic corn was reported in Puerto Rico, Brazil and Argentina [12-14]. Therefore, the evaluation of new control strategies for FAW control has gained renewed interest. RNAi has emerged as a promising biotechnological tool for the control of pests [15,16]. RNAi is a post-transcriptional gene silencing mechanism that involves double-stranded RNA (dsRNA) directed against a target gene or its promoter region. It is a conserved cellular mechanism described in eukaryotic organisms [17].

Despite the successful examples of RNAi uses in different insects, some reports showed that dsRNA administration fails in producing knockdown of specific target genes [18]. Most of the coleopteran's insects showed an efficient RNAi response by dsRNA feeding or injection. In contrast, RNAi is less efficient in lepidopteran insects displaying varying effects in different species [19]. Therefore, a much larger amount of dsRNA is required to trigger RNAi response in lepidopteran insects compared to coleopteran species [18]. In previous studies, RNAi strategy was successfully used in *S. frugiperda* to evaluate gene functions by dsRNA microinjec-

tions [20] although more recent experiments demonstrated that accumulation of dsRNA in midgut cells endosomes contributes to inefficient RNAi effect in FAW [21]. In this study, the effect of dsRNA against FAW targeted genes of *S. frugiperda* was evaluated by feeding, microinjection and soaking dsRNA delivery methods. The present findings suggest that dsRNA soaking could be an effective method to produce gene suppression in FAW and represents a potential strategy for the control of this insect pest.

Material and Methods

Spodoptera frugiperda rearing and midgut RNA isolation

Table 1: Primer sequences for *S. frugiperda* gene fragments RT-PCR amplification and β-actin RT-qPCR.

Primers RT-qPCR	Sequence	Amplicon Size (bp)	GenBank Accession Number
qtubulin fw qtubulin rv	CCCTACAACTCCATCCTCAC GATGAGACGGTTCAGGTTG	144	HQ008728.1
qRibosomal protein fw qRibosomal protein rv	TCAAGAGTACGTGAAACCGTTCAGG TGAAACGACCGAAGGCATCG	117	HQ178613.1
qTrehalas fw qTrehalase rev	ACTTCATGGACATCGTCGAGAGG GGGGAGGCTGTGATCTCATAGC	81	EU872435.1
qActin rv qActin fw	CCATCACCGGAGTCCAAGACG CCTCAACCCTAAGGCCAACAGG	147	[26]
Primers Target Genes	**Sequence**	**Amplicon Size (bp)**	**GenBank Accession Number**
Tubulin Sf fw Tubulin Sf rv	GTGAGTGCATCTCAGTACACGTTGG TTGGCCGCATCTTCCTTACC	359	HQ008728.1
Trehalase Sf fw Trehalase 5 Sf rv	GTGTGTTTTTGTTGCGATACTGG TTCCTGAATATCCGCTTTGG	308	EU872435.1
Ribosomal protein Sf fw Ribosomal protein Sf rv	ACCAGGATTTCCTTAGTAGCGGC TTGTTCGCTATCGGTCTCGC	359	HQ178613.1

S. frugiperda larvae were reared at the Institute of Microbiology and Agricultural Zoology (IMYZA), INTA bioterium. Larvae were raised in artificial diet without formalin [22]. RNA extractions were performed on sets of 5 midguts from 3rd instar *S. frugiperda* larvae. Each midgut section was individually dissected and gently rinsed to eliminate the midgut diet residues. Total RNA was isolated using TRIZOL reagent kit (Invitrogen, USA) according to the manufacturer's instructions. Extracted RNA was stored in 20µl double distilled water and its concentration was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA) (Table 1).

Reverse transcription PCR and gene fragments cloning

Total RNA extracted from midguts were treated with RQ1 RNase-free DNase (Promega, UK) in order to eliminate any residual DNA. Reverse transcription (RT) reactions were performed with Random primers (Invitrogen, USA) and 2µg RNA were used for complementary DNA (cDNA) synthesis with M-MLV reverse transcriptase enzyme (Promega, UK) according to the manufacturer's guidelines. *S. frugiperda* genes fragments (ribosomal protein, trehalase and tubulin genes) were amplified using specific primers and PCR products ranging between 300 and 360 base pairs (bp) (Supplementary material). PCRs were performed under the following reaction conditions: 92 °C for 5 minutes (min), 35 cycles of 92 °C for 1min, 55 °C annealing temperature for 40 seconds (sec), 72 °C for 1min and 72 °C for 5min. The obtained PCR fragments were purified using Inbio Highway Kit® (Argentina) and individually cloned in pGemT vector (Promega, UK), according to the manufacturer's guidelines. The cloned fragments were sequenced using T7 and Sp6 promoter primers to verify correct amplification. The fragments cloned in pGemT vector were released with restriction enzymes and cloned in dsRNA synthesis plasmid (pL4440) according methodology described in Gamboa et al. [23]. A plasmid pL4440-GFP with green fluorescent protein (GFP) gene fragment was used as control [23].

Bioassays

Bacterially expressed dsRNA

Colonies of *E. coli* HT115 bacteria containing pL4440 recombinant plasmids were individually grown and IPTG-induced under conditions described by Tian et al. [24]. The dsRNA expression in bacteria was verified by analyzing 1 milliliter (ml) of each culture. Each 1ml aliquot was centrifuged and resuspended in 200µl of TRIZOL reagent (Invitrogen™) to obtain total RNA, according to the manufacturer's instructions. The extracted RNA was dissolved in 30µl of distillated water and dsRNA expression product was confirmed by agarose gel electrophoresis (1%). After dsRNA expression verification, induced bacteria cells were collected and diluted with different volumes of ddH2O. The cell concentrations

expression dsRNA of each target gene (ribosomal protein, trehalase and tubulin) were designated as 250x, 50x and 10x based on the dilution factors.

Feeding assays

In order to evaluate the effect of dsRNA bioassays were carried out by feeding of 10x, 50x, and 250x to newly molted 2nd, 3rd and 4th instars *S. frugiperda* larvae, after 12h starvation. Larvae were fed using the droplet-feeding method described by Hughes and Wood [25]. After feeding confirmation, larvae were placed in individual containers with artificial diet during 8 days.

Soaking assays

Newly molted 2nd, 3rd and 4th instars *S. frugiperda* larvae were individually soak in dsRNA solution during 10 seconds three times a week. After soaking, each larva was placed in individual container with artificial diet during 8 days. A 40ng/ul dsRNA solution of each target gene was used in all experiments. DsRNA GFP solution was used as control.

Microinjection assays

The direct injection of dsRNA solution into hemolymph using an insulin syringe was evaluated on newly molted 2nd, 3rd and 4th instars *S. frugiperda* larvae. After injection, each larva was placed in individual container with artificial diet during 8 days. A 40ng/µl dsRNA solution of each target gene was used in all experiments. The dsRNA GFP solution was used as control.

Quantitative real time-PCR

The effect of specific dsRNA administration on the steady state transcriptional levels of target genes was evaluated by three delivery methods in 2nd, 3rd and 4th instars *S. frugiperda* larvae during 8 days. On the 8th day, total RNA was extracted from pools of 5 dissected midguts using Trizol reagent (Invitrogen, USA). cDNA was synthesized using MMLV reverse transcriptase (Invitrogen, USA) and random primers (Invitrogen, USA) according to the manufacturer's instructions. The cDNAs were diluted 10 folds and used as template in qRT-PCR using the TransStart Green qPCR Super Mix (Transgen Biotech, China) and the following primers: qRib-fw 5´TCAAGAGTACGTGAAACCGTTCAGG 3´, qRib-rv 5´TGAAACGACCGAAGGCATCG3´; qTreh-fw 5´CACAGCCTCCCCTCCTAATTCC3´; qTreh-rv 5´ATCCCGACGTCTGCCAGATTCC3´; qTub-fw 5´CCCTACAACTCCATCCTCAC3´, qTub-rv 5´GATGAGACGGTTCAGGTTG3´ and actin as housekeeping: qActin-fw 5´CCTCAACCCTAAGGCCAACAGG´, qActin-rv 5´CCATCACCGGAGTCCAAGACG3´.

The PCR program used for all samples was as follows: 95 °C for 30sec, 40 cycles of 95 °C for 20sec, 55 °C for 20sec, 60 °C for 30sec followed by melting curve steps 95 °C for 15sec, 60 °C for 15sec and a final step at 95 °C for 15s. All qRT-PCR experiments included three biological replicates and three technical repetitions. The qPCRs were performed in Eppendorf Realplex2 equipment and the threshold cycle (CT) values were calculated using Eppendorf software. The CT values of ß-actin were used as reference [26].

Results and Discussion

In order to evaluate the effect of dsRNA on *S. frugiperda* target genes, we first amplified and cloned three DNA fragments into the plasmid pL4440 in order to synthesize them in the bacterial strain HT115 (DE3). The selected target fragments belong to genes that are involved in digestion processes (trehalase) and structural components (tubulin and ribosomal protein). Each HT115 bacterium containing the specific recombinant plasmid apparently synthesized the corresponding dsRNA in agreement with the expected molecular lengths ranging between 500 and 600bp as evidenced by agarose gel electrophoresis analysis (Figure 1). The obtained dsRNA, whose concentrations ranged between 100 and 300ng/µl, were used in feeding, soaking and microinjection bioassays. In feeding bioassays, no significant reduction in relative expression of target genes mRNA level was detected in 2nd, 3rd and 4th instars *S. frugiperda* larvae (data not shown).

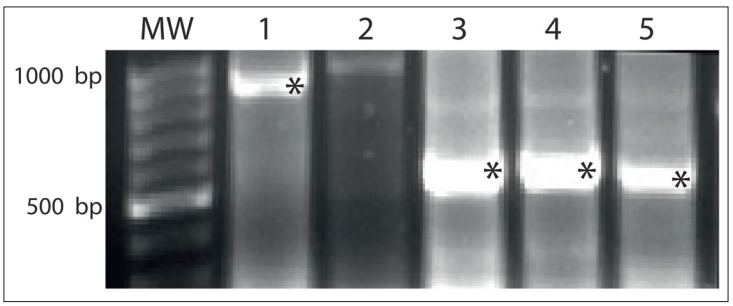

Figure 1: Bacterially expressed dsRNA. Total RNA was extracted from IPTG induced bacteria HT115(DE3) containing the recombinant plasmids. MW: Molecular weight 1000 base pairs (bp).
1. dsRNA GFP (control).
2. pL4440 plasmid empty (control).
3. Ribosomal dsRNA.
4. Tubulin dsRNA.
5. Trehalase dsRNA.
6. Asterisks indicate the dsRNA bands.

Our results are consistent with previous reports where dsRNA fed to lepidopteran larvae escapes dsRNAse digestion, enters midgut cells but it is not processed into small interference RNA [27]. Thus, gene silencing failed to occur in midgut cells. More recent evidence reveals that accumulation of dsRNA in midgut endosomes contributes to inefficient RNA interference in FAW [21]. Furthermore, similar results were obtained by using a microinjection method (data not shown). There was no significant RNAi effect in any of the instars of *S. frugiperda* larvae when compared to control treatments. Thus, our results do not support previous research reporting that microinjection was an effective method to produce a knock down target genes in FAW [20] although the target genes used in this study and those used in the present report are different. In contrast with Rodriguez et al. [26], recent reports showed that microinjection of dsRNA in fat bodies induce an ineffective RNAi response in FAW [21]. Therefore, further studies need to be carried out in order to clarify if the effect of dsRNA microinjection in *S. frugiperda* larvae is dependent of specific conditions like target gene sequence, its relative accumulation of the mRNA and other specific or general factors. Second to fourth larval instars of FAW were soaked in dsRNA solutions and the effect on the target gene transcripts was evaluated by reverse transcriptase quantitative PCR (RT-qPCR).

Only second-instar larvae soaked in dsRNA solution of the ribosomal RNA gene sequence showed a statistically significant knockdown of target mRNA levels when compared to the control treatment (Figure 2A). In contrast, no significant differences were registered for third and fourth larval stages (Figure 2B & 2C). Therefore, the assay results showed that RNAi responses by soaking depend on specific life stages and target gene sequence. Our experiments thus demonstrate a distinct RNA effect in FAW by dsRNA in contact with larval tegument. This result is in agreement with recent studies reporting that sprayable dsRNAs have demonstrated the feasibility and efficacy of RNAi-based gene silencing through non-transformative delivery strategies in different systems [28].

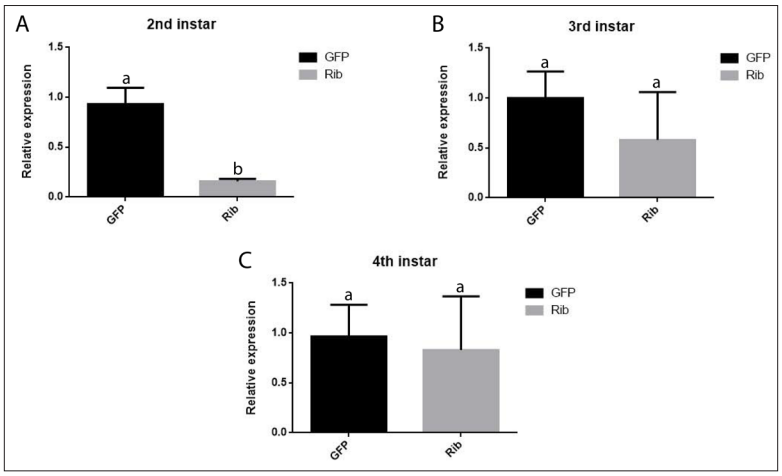

Figure 2: Relative target gene expression level in 2nd, 3rd and 4th instars *S. frugiperda* larvae soaked either in ribosomal (Rib) or GFP (control) dsRNAs. Bars (±SE) followed by the same letter are not statistically different at $p<0.05$.

Conclusion

This study demonstrated the effect of dsRNA targeted against expressed genes in *S. frugiperda* larvae. Here, different dsRNA delivery methods were used and RNAi effect on target genes was evaluated. In accordance with previous studies, oral and injection dsRNA administration failed to produce gene silencing on FAW. Our results indicate that a significant target gene knockdown could be achieved just only in second larval instars using a soaking method. This is the first report of such effect of gene silencing by soaking *S. frugiperda* larvae in dsRNA solutions. This study provides the basis for developing new FAW management methods based on RNAi. Further research is needed in order to evaluate the use dsRNA as sprayable pesticide in *S. frugiperda* control strategies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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