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Research paper

Phenotypic screen for RNAi effects in the codling moth *Cydia pomonella*

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ABSTRACT

RNAi-based technologies have the potential to augment, or replace existing pest management strategies. However, some insect taxa are less susceptible to the induction of the post-transcriptional gene silencing effect than others, such as the Lepidoptera. Here we describe experiments to investigate the induction of RNAi in the codling moth, *Cydia pomonella*, a major lepidopteran pest of apple, pear, and walnut. Prior to a knockdown screen, fluorescently labeled small interfering RNA (siRNA) and double-stranded RNA (dsRNA) derived from green fluorescent protein (GFP) coding sequence were delivered to the surface of artificial diet to which neonate larvae were introduced and subsequently examined for the distribution of fluorescence in their tissues. Fluorescence was highly concentrated in the midgut but its presence in other tissues was equivocal. Next, dsRNAs were made for *C. pomonella* genes orthologous to those that have well defined deleterious phenotypes in *Drosophila melanogaster*. A screen was conducted using dsRNAs encoding *cullin-1* (*Cpcul1*), *maleless* (*Cpmle*), *musashi* (*Cpmsi*), a homeobox gene (*CpHbx*), and *pumilio* (*Cppum*). The dsRNAs designed from these target genes were administered to neonate larvae by delivery to the surface of the growth medium. None of the dsRNA treatments affected larval viability, however *Cpcul1*-dsRNA had a significant effect on larval growth, with the average length of larvae about 3 mm, compared to about 4 mm in the control groups. Measurement of *Cpcul1* transcript levels by quantitative real-time PCR (qRT-PCR) revealed a dose-dependent RNAi effect in response to increasing amount of *Cpcul1*-dsRNA. Despite their reduced size, *Cpcul1*-dsRNA-treated larvae molted normally and matured to adulthood in a manner similar to controls. In an additional experiment, *Cpcul1*-siRNA was found to induce similar stunting effect as that induced by *Cpcul1*-dsRNA.

1. Introduction

The codling moth, *Cydia pomonella*, is a major pest of apple, pear, and nut tree crops throughout much of the world (Witzgall et al., 2008). It is consequently the target of repeated insecticide applications and the cause of much of pest management costs for these crops. Due to increasingly strict regulatory pressure restricting insecticide use and the evolution of genetically based resistance, new approaches to control this pest are both necessary and desirable.

The discovery of RNA interference (RNAi) has opened up new avenues to pursue forward genetic investigations in non-model organisms (Fire et al., 1998) and the potential of using it for practical purposes in novel crop protection strategies. The latter was initially suggested by a landmark study demonstrating effective gene silencing after feeding double stranded (dsRNA) in artificial diet to larvae of the light brown apple moth, *Epiphyas postvittana* (Turner et al., 2006). Subsequently, two independent investigations demonstrated the feasibility of conferring resistance to feeding damage by insect pests by transgenic expression of dsRNAs *in planta*, targeting the expression of genes of the western corn rootworm *Diabrotica virgifera* (Baum et al., 2007) and the cotton bollworm *Helicoverpa armigera* (Mao et al., 2007). Despite these successes suggesting the development of novel tools for pest management, the ability to experimentally induce RNAi effects varies greatly among different insect species. Although strong RNAi effects have been induced in some insects (Whyard et al., 2009; Gu and Knipple, 2013; Prentice et al., 2015), in others RNAi caused low rates of visible phenotypic changes, or none at all (Saleh et al., 2006; Iga and Smagghe, 2010; Marcus, 2005). A recent review of successful and unsuccessful attempts to affect RNAi-induced gene silencing in lepidopteran species discussed variable efficiency of RNAi related to species, tissue and gene function (Terenius et al., 2011). Furthermore, differences in the RNAi effect induced by dsRNA as opposed to small interfering RNA (siRNA) have also been shown among different insect species (Wang et al., 2013).

Here we describe investigations to evaluate a screen based on incorporation of dsRNA and siRNA in the insect diet to produce RNAi-induced

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; qRT-PCR, quantitative real-time PCR; cDNA, complementary DNA; ANOVA, analysis of variance; RNAi, RNA interference; dsRNA, double strand RNA; SD, standard deviation.

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phenotypic effects in *C. pomonella* and to identify genes that could be potentially useful for RNAi-based pest management.

2. Materials and methods

2.1. Insects

A codling moth colony was established with insects purchased from Benzon Research (Carlisle, PA) and maintained on an artificial diet (Toba and Howell, 1991) at 27 °C, 16:8 L:D, and 70% RH.

2.2. Molecular cloning, sequence analysis and phylogeny

Degenerate oligonucleotide primers were designed to amplify *cullin-1*, *maleless*, *musashi*, a *homeobox* gene, and *pumilio* and cDNA made from total RNA was isolated from embryos using the Qiagen RNA Extraction kit (Qiagen, Valencia, CA). RNA was converted to cDNA using the cDNA synthesis kit (QuantiTect® Reverse Transcription Kit, Qiagen, CA). PCR reactions using degenerate primers and embryonic cDNA as template were done using the following conditions: initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s and amplification at 72 °C for 1 min. A final extension step was done at 72 °C for 10 min. Following PCR, products were separated using agarose gel electrophoresis and bands of predicted sizes were isolated from agarose gels. Excised PCR products (along with a 591 bp fragment of green fluorescent protein (*GFP*) coding sequence as control) were purified with Amicon® Ultra-0.5 Centrifugal Filter Devices (Millipore, MA) and then individually cloned into pEASY-T3 plasmid vector. Cloned PCR products were verified by DNA sequencing, and the sequences were analyzed using BLASTX to establish orthology.

To obtain longer transcript sequence information, the cDNA sequences obtained above were used to mine RNAseq transcriptome data generated from codling moth whole body (unpublished) using MegaBlast. The resultant transcriptome sequences were aligned to those from seven representative insect species (*Bombyx mori*, *Papilio polytes*, *Acyrthosiphon pisum*, *Tribolium castaneum*, *Apis mellifera*, *Culex quinquefasciatus* and *Drosophila melanogaster*) using ClustalW2.1 (Sievers et al., 2011) and phylogenetic trees were constructed by MEGA 6 using the Maximum Likelihood method with the bootstrap value set as 1000. The transcriptome sequences used in this study are deposited in GenBank (accession numbers were listed in Table 2).

2.3. Preparation of dsRNA and siRNA

The plasmids containing the cloned cDNAs were used as templates for amplification by PCR using specific primers to which the T7 RNA polymerase promoter (5′-taatacgactcactataggg-3′, Table 1) was attached to the 5′ end. The PCR products were purified with Amicon® Ultra-0.5 Centrifugal Filter Devices and used as templates for dsRNA synthesis with the *Silencer*® siRNA Cocktail Kit (Ambion, TX). The synthesized dsRNAs were precipitated with isopropanol, resuspended in nuclease-free water, and quantified with a spectrophotometer (NanoDrop™ 1000) at 260 nm. The purity and integrity of dsRNAs were determined by agarose gel electrophoresis. To produce siRNA, the dsRNA was digested by the RNase III family member dicer *in vitro* with the kit described above according to the manufacturer's instructions.

2.4. Evaluation of dsRNA transmission to larval tissues

The Silencer® siRNA Labeling Kit with Cy®3 dye (Ambion, TX) was used to fluorescently label the control *GFP*-dsRNA and *GFP*-siRNA synthesized as above. 10 μl of 100 ng/μl fluorescently labeled *GFP*-dsRNA or *GFP*-siRNA was pipetted onto the surface of the artificial diet as described above. The newly hatched larvae were put on the diet and observed by fluorescence microscopy (Olympus SZX16) once each day for three days.

Table 1
Oligonucleotide primers used in this investigation.

Primer name	5′–3′ sequence	PCR product (bp)
dicer-F	CAYGAYAAYTGGYTNCCNCC	828 and 870*
dicer-R	ARNGCYTTNGGNACYTCNAC	
maleless-F	ACNGGNTGYGGNAARACNAC	809
maleless-R	ARNGCRAADATNARRTTCCANCC	
musashi-F	MGNGGNTTYGGNTTYATHAC	384
musashi-R	CNGCYTCYTTNGGYTGNG	
pumilio-F	GACNGAYGTNTTYGGNAAYTAYG	702
pumilio-R	GCRWAYTGRTCYTTCATCATNAC	
cullin1-dsRNA-F	T7 + TAACAGCAACGCCGTGA	1117
cullin1-dsRNA-R	T7 + CCGCCTGGATAAGCATC	
homeobox-dsRNA-F	T7 + AGCCATGACGACAACCA	625
homeobox-dsRNA-R	T7 + CATCATTCGGAGTCAACC	
maleless-dsRNA-F	T7 + CATCATTCGGAGTCAACC	707
maleless-dsRNA-R	T7 + TGTTCAGGGAGCCGATGTAC	
musashi-dsRNA-F	T7 + CGCAAGGAACACACGAAT	239
musashi-dsRNA-R	T7 + TGAAACGTGACGAAACCG	
pumilio-dsRNA-F	T7 + CAGAAGGCACTCGAATC	436
pumilio-dsRNA-R	T7 + CTCGACTACGTTGGAGGC	
GFP-dsRNA-F	T7 + GACCAAGGAGATGACCATGAA	591
GFP-dsRNA-R	T7 + GTCAGCTTGTGCTGGATGAA	
cullin1-qPCR-F	ATCTATCTCGTGCGATGAA	124
cullin1-qPCR-R	TGACTGTATCTGTTATGTCCTAT	
actin-qPCR-F	TCGGTGAGTGCTGTTGTCTC	148
actin-qPCR-R	TGAGAAACGTGCCATGTTGC	

T7 sequence: TAATACGACTCACTATAGGG.
Degenerate nucleotide symbols: N (G + C + A + T), Y (C + T), R (A + G), M (A + C), W (A + T), D (A + G + T), and H (A + C + T).
* The 828 and 870 bp amplification products encoded amino acid sequences with identity/similarity >80% to regions of *D. melanogaster* proteins cul-1 and proteins containing Antennapedia/fushi tarazu homeobox domains.

2.5. Insect bioassay

Individual neonate larvae were placed on the surface of fresh artificial diet contained in 1.5 ml centrifuge tubes to which 50 μl dsRNA at various concentrations from 50 ng/μl to 250 ng/μl was added to the surface immediately after the diet had cooled and solidified. After two days, an additional 20 μl dsRNA was pipetted on the top of the diet. To determine effects on viability, 20–30 larvae were analyzed for each treatment group and counted daily. The lengths of ten larvae selected at random from each treatment group were measured at Day 3 and Day 8 with three replications performed. The data were analyzed using one-way ANOVA to test the effects of experimental treatments compared to treatments with *GFP*-dsRNA and milliQ water controls.

2.6. Quantitative real-time PCR (qRT-PCR) analyses of target-gene expression

Total RNA was extracted from pools of five treated larvae using Qiagen RNA Extraction kit (Qiagen, CA) and 1 μg total RNA was used to synthesize cDNA by using the QuantiTect® Reverse Transcription Kit (Qiagen, CA) with DNAse treatment. The primers used in this experiment are listed in Table 1. The qRT-PCR was conducted using SsoAdvanced™ universal SYBR® Green supermix (BioRad, CA) on the BioRad CFX96 qPCR System. Expression of the β-actin gene was used as a reference. In summary, cDNA template equivalent to 1.0 ng of input total RNA was used, in addition, two controls included addition of no template (to ensure no primer products were being produced) and a no reverse transcriptase RNA template (to ensure RNA was free of genomic DNA). After PCR reactions were complete, melting curve analysis was performed to ensure a single amplified product was produced. Final data were analyzed using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001). Statistical analyses were performed using SPSS 18.0 (Norušis,