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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,

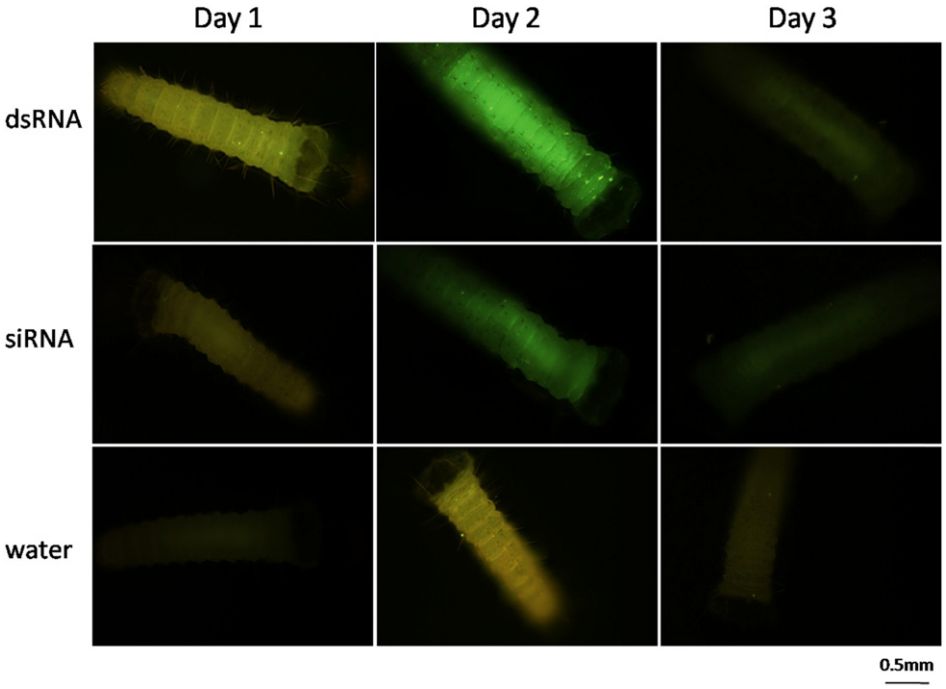

Fig. 1. Photographs of first instar larvae 2 h (Day 1), 24 h (Day 2) and 48 h (Day 3) after feeding on fluorescently labeled dsRNA- and siRNA-containing diet.

1994). Following a significant p value for the ANOVA, differences between means were separated by Tukey's test.

3. Results

3.1. dsRNA and siRNA incorporation and persistence in the codling moth larval body

To determine whether dsRNA and siRNA administered to neonate larvae can be transferred to larval tissues, we treated neonate larvae with fluorescently-labeled *GFP*-dsRNA or *GFP*-siRNA (Materials and Methods 2.4 and 2.5) and monitored fluorescence over three days (Fig. 1). One day after treatment, first instar larvae exhibited fluorescence over the entire outline of the body in both fluorescently-labeled treatment groups, but fluorescence was stronger and more uniform in the dsRNA treatment group; no fluorescence was observed in the control group. Two days after treatment, fluorescence was observed concentrated in the midgut in both fluorescently-labeled treatment groups, with clear differentiation (less or no fluorescence) of the foregut and the hindgut. In contrast to the Day 1 control group, autofluorescence over the entire outline of the body was observed in the Day 2 control group. Overall fluorescence diminished rapidly in both treatment groups after Day 2 but was still faintly present in the midgut on Day 3 (Fig. 1).

3.2. RNAi screen for visible or lethal phenotypes in C. pomonella

Five codling moth genes orthologous to those that have well defined deleterious phenotypes in *D. melanogaster* were chosen for use in RNAi experiments. These genes with known molecular and/or biological functions included *cullin-1*, *maleless*, *musashi*, a *homeobox* gene, and *pumilio* (Table 2, Supplemental Fig. 1). Coding sequences of these five genes were used to generate dsRNAs, which were layered onto the diet of first instar larvae (Materials and Methods 2.5). One of the five dsRNAs, *Cpcul1*-dsRNA, induced a visible phenotypic change evident after three days, a significant stunting of larval growth (Figs. 2, 3), which is discussed more fully below. However, *Cpcul1*-dsRNA had no effect on viability after continuous exposure in the diet for eight days (Table 3), nor did any of the other four dsRNA treatments (data not shown).

3.3. Treatment with Cpcul1-dsRNA stunts larval growth, but has no effect on molting or viability

To further characterize the effects of *Cpcul1*-dsRNA, we measured the length of the larvae after exposure to dsRNA in the diet for 3 days and found a significant stunting effect relative to controls. The average length of the larvae treated with dsRNA was about 3 mm compared to the control groups that were about 4 mm ($p = 0.016$, Fig. 3). The stunting effect persisted through Day 8 ($p = 0.027$, Fig. 3), with treated larvae having an average length of 6.0 mm compared to control larvae having an average length of 7.8 mm. Treatment with *Cpcul1*-dsRNA did not cause mortality, even at delivered concentrations as high as 250 ng/μl (Table 3, one way ANOVA, $p = 0.687$). Although it was difficult to establish the exact time of molting, most of the larvae in the *Cpcul1*-dsRNA treatment group molted after 3 days (data not shown).

Table 2
C. pomonella genes used in this study.

Gene name	GenBank accession number	Biological/molecular process	cDNA fragment size (bp)
cullin-1	KP236737	Proteolysis, component of 28S proteasome	2385
homeobox	KP236738	Transcription factor, segment identity	792
maleless	KP236739	RNA binding, sex determination	3231
musashi	KP236740	Cell fate determination, nervous system	1062
pumilio	KP236741	RNA binding, pole cell migration	3723

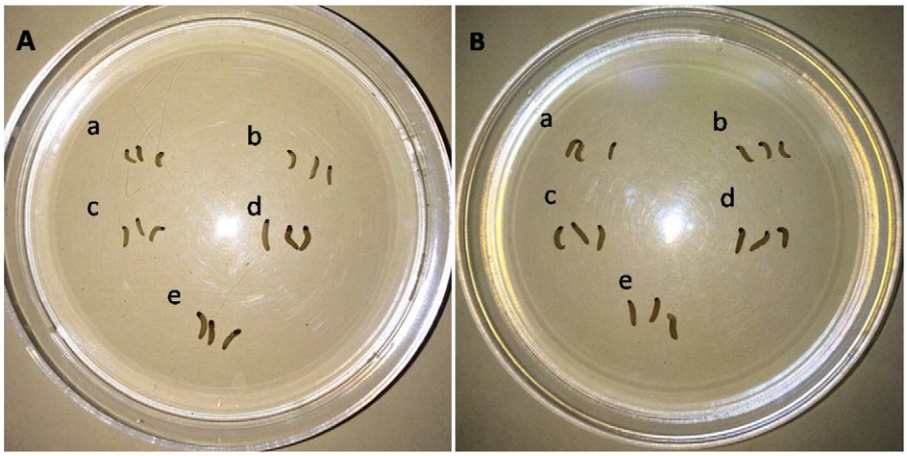

Fig. 2. Photographs of larvae showing stunting phenotype induced following three days of treatment with *Cpcul1*-dsRNA and *Cpcul1*-siRNA: (A) comparison of larval phenotypes induced by *Cpcul1*-dsRNA *vs.* controls, a–e representing the larvae treated with (a) 250 ng/μl, (b) 100 ng/μl, (c) 50 ng/μl *Cpcul1*-dsRNA, (d) *GFP*-dsRNA and (e) water; and, (B) comparison of larval phenotypes induced by *Cpcul1*-dsRNA *vs. Cpcul1*-siRNA, a–e representing larvae treated with (a) *Cpcul1*-siRNA (b) *Cpcul1*-dsRNA, (c) *GFP*-siRNA, or (d) *GFP*-dsRNA, and (e) water.

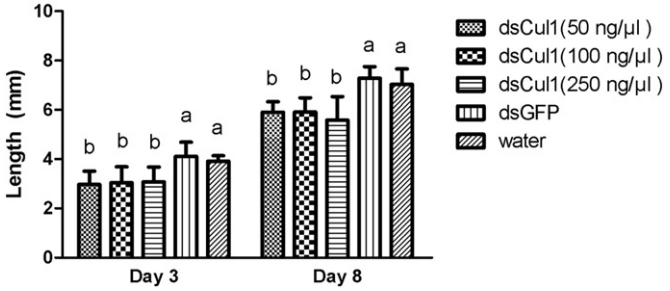

Fig. 3. Lengths of *C. pomonella* larvae following treatment with various concentrations of *Cpcul1*-dsRNA (dsCul1) compared to control larvae treated with *GFP*-dsRNA (dsGFP) and water. Three replications were tested, and each replication contained 10 larvae. The error bars represent SD of means and different letters indicate a significant difference at the 0.95 probability level.

To determine if *Cpcul1*-dsRNA directly affected *Cpcul1* RNA levels through an RNAi effect, we measured the steady state levels of *Cpcul1* RNA by qRT-PCR on Day 8 of treatment. An RNAi effect was in fact confirmed, with *Cpcul1* RNA levels decreasing with increasing *Cpcul1*-dsRNA dosage ($p = 0.001$, Fig. 4). At the maximum dose (delivered concentration of 250 ng/μl) *Cpcul1* RNA levels were reduced by two-fold relative to the control groups. In contrast, the control group treated with *GFP*-dsRNA showed no significant difference compared to the control group treated with water.

3.4. Treatment with Cpcul1-siRNA produces similar phenotypic effects as Cpcul1-dsRNA

Because there was only a two-fold transcript reduction using *Cpcul1*-dsRNA, we wanted to determine if using siRNA would have a greater effect. *Cpcul1*-siRNA was generated by digesting *Cpcul1*-dsRNA with *dicer* and administered to larvae by adding it to the diet surface. As with the *Cpcul1*-dsRNA treatment, *Cpcul1*-siRNA did not induce lethality (Table 3, one way ANOVA, $p = 0.875$). However, treatment with *Cpcul1*-siRNA produced a stunting effect similar to that induced by *Cpcul1*-dsRNA (Figs. 2B, 5, $p < 0.001$). Furthermore, treatment with *Cpcul1*-siRNA produced the same suppression effect of *Cpcul1*-dsRNA levels relative to controls as treatment with *Cpcul1*-dsRNA (Fig. 6, $p = 0.03$).

3.5. Cullin1 proteins are conserved among insects

A partial cDNA sequence of *Cpcul1* was obtained by PCR using degenerate primers. The cDNA was 1234 bp in length and encoded 412 amino acids. We subsequently identified a longer 2385 nt sequence encoding 795 amino acids by using BLAST analysis of RNASeq data (data not shown), which contained a perfect match to the 1234 bp sequence obtained from the cloned *Cpcul-1* PCR product. We used the longer amino acid sequence to query the databases of other insects and found Cpcul1 to have high sequence identity and similarity to other insect cullin1 proteins (Fig. 7A). The phylogenetic tree obtained using the Maximum Likelihood method shows that Cpcul1 is in the same branch as cullin1 proteins from other lepidopteran species (Fig. 7B).

4. Discussion

The application of RNAi holds promise as a means to develop novel tools for insect pest management, but this phenomenon has been investigated in only a small number of insect pest species and is not well characterized. Here we used five dsRNAs to target the expression of specific genes in the non-model lepidopteran species, *C. pomonella*, screening for visible phenotypic effects and effects on viability. We showed that treatment with *Cpcul1*-dsRNA induced a significant stunting of larval growth, but had no effect on viability, while none of the other four dsRNAs had any apparent effect in our assay.

Table 3
The mortality of larvae among different treatments (% ± SD).

Treatment**	Day 1	Day 2	Day 4*	Day 6*	Day 8*
CpCul1-siRNA	9.13 ± 0.85	3.70 ± 6.41	0.00 ± 0.00	7.87 ± 6.85	8.47 ± 7.50
CpCul1-dsRNA	9.10 ± 12.87	0.00 ± 0.00	5.55 ± 7.85	0.00 ± 0.00	0.00 ± 0.00
GFP-siRNA	16.55 ± 9.26	5.00 ± 7.07	0.00 ± 0.00	5.55 ± 7.85	3.85 ± 0.74
GFP-dsRNA	12.87 ± 14.51	6.67 ± 11.55	3.33 ± 5.77	0.00 ± 0.00	3.70 ± 6.41
Water	9.10 ± 12.87	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

* The mortality of Day 4, Day 6, and Day 8 included that occurring during the prior day.
** All RNA concentrations were 250 ng/μl.

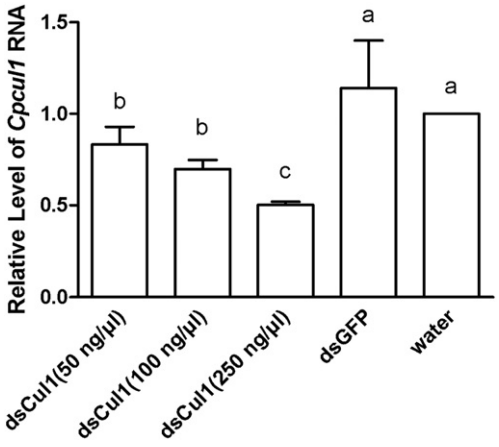

Fig. 4. *Cpcul1*RNA levels in response to treatment with various doses of *Cpcul1*-dsRNA (dsCul1) compared to *CpCul1*RNA levels in response to control treatments with *GFP*-dsRNA (dsGFP) and water. The error bars represent SD of means and different letters indicate a significant difference at the 0.95 probability level.

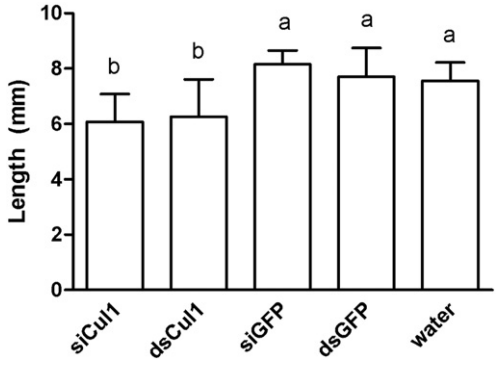

Fig. 5. Lengths of larvae treated with *Cpcul1*-siRNA (siCul1) and *Cpcul1*-dsRNA (dsCul1) compared to those treated with *GFP*-siRNA (siGFP), *GFP*-dsRNA (dsGFP) and water on Day 8 of treatment. Three replications were tested, and each replication contained 10 larvae. The error bars represent SD of means and different letters indicate a significant difference at the 0.95 probability level.

We also performed experiments in an effort to assess the ability of dsRNAs and siRNAs to be incorporated into tissues beyond the midgut, which we consider to be the primary route of delivery in this assay design (Fig. 1). We nevertheless cannot preclude the possibility that

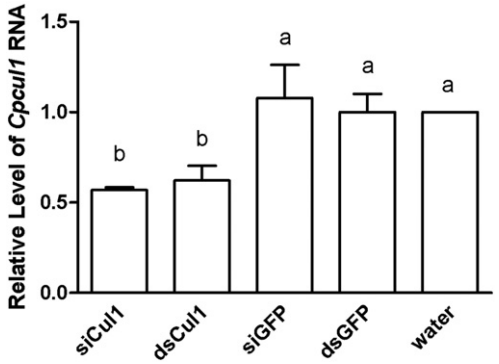

Fig. 6. *Cpcul1* RNA levels in response to treatment with *Cpcul1*-siRNA (siCul1) and *Cpcul1*-dsRNA (dsCul1) relative to *Cpcul1* RNA levels in response to control treatments with *GFP*-siRNA (siGFP), *GFP*-dsRNA (dsGFP), and water on Day 8 of treatment. The error bars represent the SD of means and different letters indicate a significant difference at the 0.95 probability level.

dsRNAs and siRNAs could also enter into larval tissues *via* other routes such as across the unsclerotized and untanned cuticle shortly after hatching from the egg or by infiltration of the tracheal system. Consistent with this idea, an earlier study showed that dsRNA can induce an RNAi effect when sprayed onto larvae of another lepidopteran species, the Asian corn borer *Ostrinia furnacalis* (Guenée) (Wang et al., 2011).

Unfortunately, the results of our experiments using fluorescently-labeled *GFP*-dsRNA and *GFP*-siRNA were inconclusive in addressing the question of whether these RNAs are incorporated into larval tissues beyond the midgut or not. Although uniform fluorescence was observed over the entire body of both fluorescently-labeled treatment groups on Day 1, this observation could be due to adhesion of the fluorescently-labeled RNAs to the immature cuticle, since the larvae were fully immersed in the treatment solutions immediately after hatching. There is also the issue of autofluorescence (Fig. 1, evident in Day 2 control and probably also in Day 1 dsRNA treatment), which we suggest is associated with maturation of the larval cuticle. Despite the limitations of interpretation of these results, it is unambiguous that fluorescence is concentrated in the midgut in Day 2 larvae of both fluorescently-labeled treatment groups. This fluorescence diminished significantly by Day 3, which suggests degradation of the RNAs.

Rapid degradation of dsRNA and siRNA in the midgut and the absence of unequivocal evidence for incorporation of dsRNA and siRNA in larval tissues beyond the midgut are consistent with the failure of the *Cpmle*-dsRNA, *Cpmsi*-dsRNA, *CpHbx*-dsRNA, and *Cppum*-dsRNA to produce RNAi-induced phenotypes. This could also account for the somewhat puzzling finding that *CpCul1*-dsRNA impaired larval growth but had no effect on larval viability, if this effect is attributable to suppression of *Cpcul1* gene expression in cells of the midgut where exposure to the dsRNA would be high initially, but diminished with time.

Another explanation for the findings that treatment with *CpCul1*-dsRNA had no effect on larval viability despite its dose-dependent effect on *Cpcul1* gene expression and its effect on larval growth even at the lowest dose of *CpCul1*-dsRNA (Figs. 2A, 3) could be related to the fact that most insects have five *Cullin* genes (Marín, 2009). The Cullin1 protein is evolutionarily conserved among insects, as illustrated by our comparative sequence analysis (Fig. 4). It functions as a component of the 28S proteasome, which plays a major role in protein degradation and cell cycle regulation (Kipreos et al., 1996; Petroski and Deshaies, 2005), influencing a range of biological processes, including cell growth, development, signal transduction, transcriptional control, genomic integrity and tumor suppression (Merlet et al., 2009). We suggest that the observed effect of treatment with *CpCul1*-dsRNA on growth but not on larval viability could be attributed to partial complementation of *CpCul1* function by one or more other members of the *Cullin* gene family.

In conclusion, previous work has demonstrated that the ability to experimentally induce RNAi effects in lepidopteran species is highly variable (Terenius et al., 2011). The results reported here provide another example of the difficulties of inducing RNAi effects in lepidopteran species. At present, the reasons for this are still not clear. A possible explanation is that lepidopteran species appear to lack clear functional homologs of the two types of genes required for systemic RNAi in *Caenorhabditis elegans*, *i.e.*, the canonical RdRP and the RNA transporter sid-1 (Tomoyasu et al., 2008). Another possibility is that some lepidopteran species possess a mechanism to rapidly degrade dsRNAs and siRNAs before they can induce the RNAi pathway, as suggested by the present investigation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.07.006.

Conflict of interest

None.

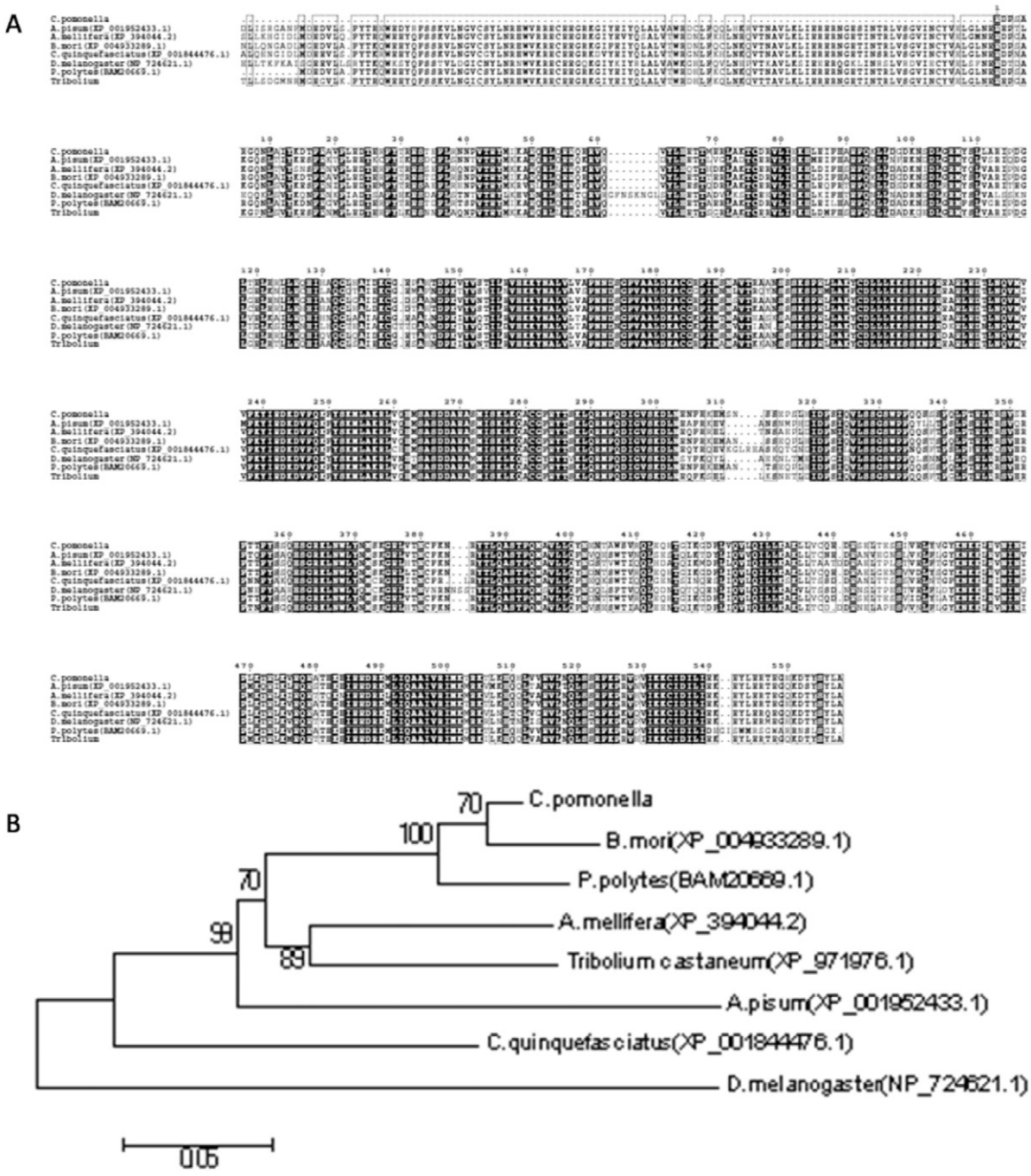

Fig. 7. Multiple sequence alignment and phylogenetic relationship analysis of predicted amino acids for *Cpcul1*: (A) amino acid sequence alignment of Cullin1 proteins from eight insect species, the black areas representing the regions of amino acid sequence identity; and, (B) phylogram showing the relationship between *C. pomonella* Cullin1 and Cullin1 proteins from the other selected insect species.

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References

Baum, J.A., et al., 2007. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. 25, 1322–1326.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Gu, L., Knipple, D.C., 2013. Recent advances in RNA interference research in insects: implications for future insect pest management strategies. Crop. Prot. 45, 36–40.

Iga, M., Smagghe, G., 2010. Identification and expression profile of Halloween genes involved in ecdysteroid biosynthesis in *Spodoptera littoralis*. Peptides 31, 456–467.

Kipreos, E.T., Lander, L.E., Wing, J.P., He, W.W., Hedgecock, E.M., 1996. *cul-1* is required for cell cycle exit in *C. elegans* and identifies a novel gene family. Cell 85, 829–839.

Mao, Y.B., et al., 2007. Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat. Biotechnol. 25, 1307–1313.

Marcus, J.M., 2005. Jumping genes and AFLP maps: transforming lepidopteran color pattern genetics. Evol. Dev. 7, 108–114.

Marín, I., 2009. Diversification of the cullin family. BMC Evol. Biol. 9, 267.

Merlet, J., Burger, J., Gomes, J.E., Pintard, L., 2009. Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization. Cell. Mol. Life Sci. 66, 1924–1938.

Norušis, M.J., 1994. SPSS advanced statistics 6.1. SPSS Advanced Statistics 6.1. SPSS Inc.

Petroski, M.D., Deshaies, R.J., 2005. Function and regulation of cullin-RING ubiquitin ligases. Nat. Rev. Mol. Cell Biol. 6, 9–20.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Prentice, K., et al., 2015. Transcriptome analysis and systemic RNAi response in the African sweetpotato weevil (*Cylas puncticollis*, Coleoptera, Brentidae). PLoS One 10 (1).
Saleh, M.C., et al., 2006. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat. Cell Biol. 8, 793–802.
Sievers, F., et al., 2011. Fast, scalable generation of high quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.
Terenius, O., et al., 2011. RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. J. Insect Physiol. 57, 231–245.
Toba, H., Howell, J., 1991. An improved system for mass-rearing codling moths. J. Entomol. Soc. B. C. 88, 22–27.
Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D., Bucher, G., 2008. Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome Biol. 9, R10.
Turner, C., Davy, M., MacDiarmid, R., Plummer, K., Birch, N., Newcomb, R., 2006. RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Mol. Biol. 15, 383–391.
Wang, Y., Zhang, H., Li, H., Miao, X., 2011. Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PLoS One 6, e18644.
Wang, J.D., Wu, M., Wang, B.J., Han, Z.J., 2013. Comparison of the RNA interference effects triggered by dsRNA and siRNA in *Tribolium castaneum*. Pest Manag. Sci. 69, 781–786.
Whyard, S., Singh, A.D., Wong, S., 2009. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem. Mol. Biol. 39, 824–832.
Witzgall, P., Stelinski, L., Gut, L., Thomson, D., 2008. Codling moth management and chemical ecology. Annu. Rev. Entomol. 53, 503–522.