Induction of RNA interference genes by double-stranded RNA; implications for susceptibility to RNA interference

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ABSTRACT

Gene silencing by RNA interference (RNAi) can be a useful reverse genetics tool in eukaryotes. However, some species appear refractory to RNAi. To study the role of the differential expression of RNAi proteins in RNAi, we isolated partial *dicer-2*, *argonaute-2 translin*, *vasa intronic gene* (*VIG*) and *tudor staphylococcus/micrococcal nuclease* (*TSN*) genes from the tobacco hornworm, *Manduca sexta*, a well-studied insect model which we have found to be variably sensitive to RNAi. We found that the RNAi gene, *translin*, was expressed at minimal levels in *M. sexta* tissue and that there is a specific, dose-dependent upregulation of *dicer-2* and *argonaute-2* expression in response to injection with dsRNA, but no upregulation of the other genes tested. Upregulation of gene expression was rapid and transient. In order to prolong the upregulation we introduced multiple doses of dsRNA, resulting in multiple peaks of *dicer-2* gene expression. Our results have implications for the design of RNAi experiments and may help to explain differences in the sensitivity of eukaryotic organisms to RNAi.

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1. Introduction

Gene silencing by RNA interference (RNAi) is a useful tool for the functional characterisation of genes. 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, however, not all species are equally susceptible to RNAi. Despite recognition in the research community that species (or higher taxa) 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 (Terenius et al., 2011; Bellés, 2010).

Insects are one taxon for which differences insensitivity to RNAi have been relatively well documented. Although RNAi experiments have successfully been conducted in a number of insect species including members of the Blattodea (Martín et al., 1995), Diptera (Misquitta and Paterson, 1999; Attardo et al., 2003), Coleoptera (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004), Hemiptera (Jaubert-Possamai et al., 2007), Hymenoptera (Lynch and Desplan, 2006; Amdam et al., 2003), Isoptera (Zhou et al., 2006), Lepidoptera (Rajagopal et al., 2002), Neuroptera (Konopova and Jindra, 2008) and Orthoptera (Miyawaki et al., 2004; Dong and Friedrich, 2005), it is considered that members of the Lepidoptera (butterflies and moths) are relatively insensitive to RNAi and Terenius et al. (2011) found that relatively large amounts of dsRNA (the RNAi trigger) are used in many RNAi experiments in lepidopteran insects and that lepidopteran species vary extensively in their sensitivity to RNAi.

Bellés (Bellés, 2010) has suggested some potential causes of RNAi insensitivity, including the possibility that there is a low response (upregulation) of core RNAi genes after dsRNA treatment. Core RNAi genes include *dicer-2*, which encodes an RNase III-like enzyme that recognises long dsRNAs and cleaves them into shorter dsRNA molecules termed short interfering RNAs (siRNAs; Elbashir et al., 2001a, 2001b; Zamore et al., 2000); *argonaute-2*, which codes for the endonuclease component of the RNA-induced silencing complex (RISC) that cleaves target RNAs (Elbashir et al., 2001b; Hammond et al., 2000; Nykänen et al., 2001) and a number of genes coding for RISC components or proteins with auxiliary functions in RISC loading and activation {for example *translin*, *vasa intronic gene* (*VIG*) and *tudor staphylococcus/micrococcal nuclease* (*TSN*) (Liu et al., 2009; Caudy et al., 2003; Shin et al., 2008)}.

In this study we commence an investigation into the role of RNAi gene responsiveness in insect RNAi by asking: are RNAi components regulated by dsRNA challenge in insects? We know that the RNAi machinery can be induced in some taxa. For instance, in the fish

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Gobiocypris rarus, *dicer* mRNA levels were found to be significantly elevated during infection with the Grass carp reovirus (Su et al., 2009) and in the marine invertebrate, *Litopenaeus vannamei dicer-2* was upregulated following challenge with viral particles or synthetic dsRNA analogues (Chen et al., 2011). However, there is, to our knowledge, no evidence for a similar response to dsRNA in insects.

In order to investigate the responsiveness of RNAi genes to dsRNA we analysed gene expression in the tobacco hornworm, *Manduca sexta*, a model lepidopteran insect in which RNAi has been previously achieved (for example Levin et al., 2005; Eleftherianos et al., 2009), but which we have found to be variably sensitive to RNA. There is evidence that other researchers have experienced difficulties achieving RNAi in *M. sexta*. Terenius et al. (2011), undertook a metastudy which integrated published and unpublished results from RNAi experiments, revealing that a high degree of silencing (a subjective measure of silencing as provided by the author) was observed in less than half of RNAi experiments conducted in *M. sexta* (42%; 14/33).

In this study we cloned five *M. sexta* RNAi genes and investigated their basal expression levels as well as their expression in response to dsRNA. These experiments have not only allowed us to investigate the degree to which the genes involved in RNAi are responsive to dsRNA, but also suggest some possible reasons as to why *M. sexta* and other lepidopteran insects are relatively insensitive to RNAi.

2. Materials and methods

2.1. Insects

Larvae of the tobacco hornworm, *M. sexta* (Lepidoptera: Sphingidae), were reared according to the instructions of Bell and Joachim (1976) and Reynolds et al. (1985). Caterpillars were kept at 25 °C, 50% humidity and a photoperiod of 17 h light: 7 h dark. Artificial diet was prepared according to the recipe of Yamamoto (1969) as modified by Bell and Joachim (1976).

2.2. Molecular cloning of core RNAi machinery components

Several *M. sexta* expressed-sequence-tag (EST) and 454 sequencing libraries (found at insectacentral.org) were interrogated for *dicer-2*, *argonaute-2*, *translin*, *VIG* and *TSN* sequences using a tBLASTn search. Apart from *argonaute-2*, which was cloned by 3′ RACE-PCR using a SMARTer RACE cDNA Amplification Kit (ClonTech), partial sequences were obtained by nested reverse transcription PCR (RT-PCR) using primers based on the EST library data (for primer sequences see Supplementary Information: Table S1 in Appendix S1). Total RNA was extracted from larval fat body, haemocyte and midgut tissue by phenol–chloroform extraction with TRI reagent (Sigma). Extracted RNA was pooled, treated with RNase-free DNaseI (Ambion) and reverse transcribed using MMLV reverse transcriptase (Promega) with random hexadeoxynucleotide primers. PCR reactions were performed using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) under standard conditions. Amplified PCR products were cloned into the pCR®II-TOPO® vector (Invitrogen) and sequenced using ABI 3730xl technologies.

2.3. Sequence analysis

The ExPASy translate tool (found at http://expasy.org/tools/dna.html) was used to translate nucleotide sequences. Sequence alignments were performed using the CLUSTALW program (Thompson et al., 1994). Phylogenetic analysis was performed using the neighbour-joining method (Saitou and Nei, 1987) and phylogram trees were rooted using NJplot (Perrière and Gouy, 1996). Conserved domains were identified using the ExPASy ScanProsite conserved domain search (http://expasy.org/tools/scanprosite/).

2.4. dsRNA synthesis

dsRNA for eGFP was synthesised by PCR and *in vitro* transcription as described in Clemens et al. (2000). PCR using primers with terminal 5′ T7 promoter sites (eGFP_T7_F: **5′-TAA TAC GAC TCA CTA TAG GGA GA** CCT GAA GTT CAT CTG CAC CA-3′ and eGFP_T7_R: **5′-TAA TAC GAC TCA CTA TAG GGA GA** GAA CTC CAG CAG GAC CAT GT-3′) generated a product used as a template for *in vitro* transcription using the T7 "Megascript" kit (Ambion). Transcription was performed as per the kit instructions except that the reaction was allowed to proceed overnight. dsRNA was treated with DNase, precipitated with LiCl and resuspended in DEPC-treated water.

2.5. Injection experiments

Newly emerged fifth instar *M. sexta* larvae were used for all injection experiments. Insects were anaesthetised and immobilised by placing them on, and covering them in, ice for 10–15 min. They were surface sterilised with 70% ethanol and injected with 50 μl of 1 μg dsRNA for eGFP suspended in 50 μl DEPC-treated water or with 50 μl DEPC-treated water using a disposable 1 ml polycarbonate 30-gauge hyperdermic needle. In the dose–response experiment five doses of dsRNA were injected (4 μg, 400 ng, 40 ng, 4 ng and 0.4 ng). Injected larvae were incubated in the insectarium (at 25 °C, 50% humidity) until the time of their dissection. Dissections were carried out using standard techniques. To separate haemocytes from hemolymph plasma larval haemolymph was centrifuged at 1000 × g for 8 min at 4 °C.

2.6. q-RT-PCR

Total RNA (obtained as in section 2.2) was quantified with a Qubit® 2.0 Fluorometer (Invitrogen) and reverse transcription was conducted as described in section 2.2. Real-time PCR was carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) and iTaq SYBR Green Supermix (Bio-Rad) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye. PCR reactions were carried out in duplicate using 7.5 pmol specific primers and approximately 5 ng cDNA (equivalent of 5 ng RNA in RT reaction) in a total volume of 15 μl. Ribosomal protein S3 (rpS-3; GI: 527679) was used as the internal control. Primer pairs (presented in the Supplementary Information: Table S2 in Appendix S1) were validated by standard curve analysis. Full details of qPCR methods are included in the Supplementary Information (Appendix S1) in full compliance with MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines.

2.7. Bacterial injections

Escherichia coli strain DH5α was prepared for injection by inoculating 5 ml LB liquid media with 5 μl of a 20% glycerol stock culture of *E. coli* and incubating for 16 h in a shaking incubator at 37 °C and ~220 rpm. The density of the bacterial culture was determined using spectrophotometry at 600 nm, the bacterial cells were washed with sterile phosphate buffered saline (PBS) and diluted to a concentration of 2000 colony forming units (CFU)/ml. For bacterial challenge experiments, insects were injected with 50 μl of this dilution and each insect therefore received a dose of approximately 1×10^5 CFU. The number of injected bacterial cells was confirmed by making serial dilutions of the injection solution and plating the dilutions onto 1.5% agar plates, followed by incubation at 37 °C overnight.

3. Results

3.1. Isolation of RNAi gene cDNA

Sequences for *dicer-2*, *argonaute-2*, *translin*, *VIG* (*vasa intronic gene*) and *TSN* (*tudor staphylococcus/micrococcal nuclease*) were identified in a *M. sexta* EST database and partial cDNA clones obtained using reverse transcription-PCR (RT-PCR) and RACE-PCR.

RT-PCR isolated 2085 bps of *dicer-2* nucleotide sequence, which contained an open reading frame (ORF) encoding 695 amino acid residues. The ExPASy ScanProsite conserved domain search predicted the putative *M. sexta* Dicer protein to encode two helicase domains and a dicer double-stranded RNA-binding fold domain, making it similar to other insect Dicer-2 proteins (but not Dicer-1 proteins) in its domain architecture (Fig. S1a in Supplementary Information: Appendix S2). Furthermore, the *M. sexta* Dicer protein sequence shows high amino acid identity with conserved regions of Dicer-2 proteins from other insect species (Fig. S1b), has amino acid identity of 65% with the recently identified *Bombyx mori* Dicer-2 protein and falls within the Dicer-2 clade in a neighbour-joining tree of insect Dicer-1 and Dicer-2 proteins (S2, Fig. S1c).

Using a 3′ rapid amplification of cDNA ends PCR (RACE-PCR) strategy a 546 bp clone of *argonaute-2* cDNA was obtained. The sequence contained a 384 bp ORF encoding 128 aas, as well as 162 bp of 3′ untranslated region. The deduced amino acid sequence was predicted by the Expasy ScanProsite search to encode a P-element-induced wimpy testis (PIWI) domain (Supplementary Information, Appendix S2, Fig. S2a), a domain characteristic of Argonaute proteins. Comparison with Argonaute 1, Argonaute 2 and Argonaute 3 proteins from other insect species confirmed the identity of the partially cloned *M. sexta* protein as Argonaute-2; the protein has 83% amino acid identity with the *B. mori* Argonaute-2 protein (Fig. S2b) and also falls within the Argonaute-2 clade in a neighbour-joining tree of insect Argonaute proteins (Fig. S2c).

Details of the bioinformatic analysis of the *translin*, *VIG* and *TSN* cDNAs are given in the Supplementary Information (Appendix S2, Figs. S3–S5 and Appendix S3).

3.2. Resting expression levels of RNAi genes

We analysed the relative expression of the newly identified *M. sexta dicer-2*, *argonaute-2*, *translin*, *VIG* and *TSN* genes in larval tissue using q-RT-PCR. Transcript levels were calculated relative to *M. sexta* ribosomal protein S3 (rpS-3; GI: 527679) in all experiments. We found that mRNAs from all five genes are present in fat body, haemocytes and midgut (Fig. 1). There are clear differences in the resting expression levels of these genes, with *TSN* transcripts being comparatively abundant at levels comparable to those of the reference standard, rpS-3. mRNA encoding *argonaute-2* was only slightly less abundant. *VIG* transcript levels varied according to tissue, being comparatively abundant in fat body, but less so in haemocytes and gut tissue. Transcripts of *dicer-2* were less abundant, being present at levels only about 1% of *TSN* and 10% of *argonaute-2*. By contrast, although it was clearly detectable, *translin* is expressed at only very low levels, approximately 0.1% of that of *TSN* (Fig. 1; note the log scale).

3.3. Expression of RNAi genes in response to dsRNA

In order to investigate the transcriptional regulation of core RNAi genes during an RNAi experiment, the expression of the five newly identified RNAi pathway genes following dsRNA injection was determined. The amount of dsRNA injected was 1 μg, which is within the range of doses of dsRNA that have been used in RNAi experiments (Terenius et al., 2011) and the sequence used was
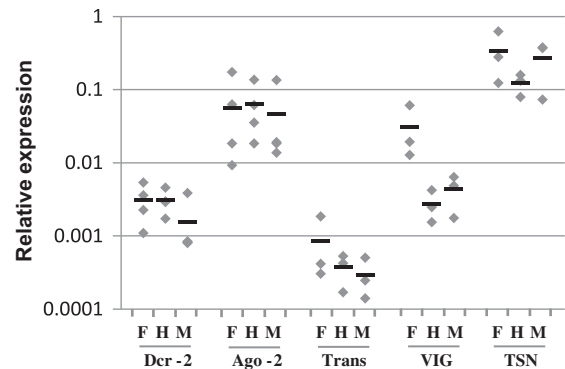
Fig. 1. Relative expression of RNAi genes. Resting expression levels of *M. sexta Dicer-2* (Dcr-2), *Argonaute-2* (Ago-2), *Translin*, *VIG* (*vasa intronic gene*) and *TSN* (*tudor staphylococcus/micrococcal nuclease*) genes in fat body (F), haemocytes (H) and midgut (M). Newly ecdysed 5th instar larvae were injected with water 6 h before fat body, haemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels relative to the internal control, rpS-3. The values for the three replicates (four for Dcr-2 and Ago-2) are shown as grey diamonds and the mean is depicted with a black dash.

a 541 bp section of the enhanced green fluorescent protein (eGFP) gene (not present in the *M. sexta* genome). Responses were measured at 6 and 18 h after injection with dsRNA.

dicer-2 mRNA levels were significantly increased 6 h after injection (relative to the control) in all three tissues tested (Fig. 2a, *t*-tests on inverse transformed data: fat body $t = 2.777$, $p = 0.0321$; haemocytes $t = 4.867$, $p = 0.00280$; midgut $t = 4.062$, $p = 0.00664$). Although mRNA levels in dsRNA-injected insects were still higher than controls at 18 h after dsRNA injection, the difference was no longer statistically significant (Fig. 2a, *t*-tests on inverse transformed data: fat body $t = 2.068$, $p = 0.0841$; haemocytes $t = 2.390$, $p = 0.054$; midgut $t = 2.363$, $p = 0.0561$). Further experiments revealed that the upregulation of *dicer-2* was rapid and transient (Fig. 2b) and that *dicer-2* responded to dsRNA in a dose-dependent manner (Fig. 2c). The *dicer-2* mRNA values vary relative to those of the reference gene in the experiments shown in Fig. 2a–c. We have noticed this discrepancy and suggest that this variation is likely attributable to a rather variable basal level of *dicer-2* expression, since expression levels do not greatly vary when they are compared to the H_2O controls, being 362, 100 and 170 respectively in Fig. 2a–c.

None of the other RNAi pathway genes tested showed such markedly elevated expression levels following dsRNA injection. *Argonaute-2* mRNA levels were somewhat elevated in response to injection with dsRNA, with the mean expression 6 h post dsRNA injection being 8, 22 and 27 times the mean control level in the fat body, haemocytes and midgut respectively (Fig. 3a). These changes in gene expression were non-significant (*t*-tests on inverse transformed data, fat body: $t = -1.365$, $p = 0.2211$; haemocytes: $t = -1.530$, $p = 0.1768$ and midgut: $t = -1.354$, $p = 0.2244$), but further time course and dose–response experiments confirmed that *argonaute-2* expression is significantly, although moderately, induced following dsRNA injection (Fig. 3b and c).

Unlike *dicer-2* and *argonaute-2*, the levels of *translin*, *VIG* and *TSN* mRNAs did not change in response to injected dsRNA (Fig. 4).

3.4. Excluding the possibility of bacterial contamination

It was important to rule out the possibility that the observed increases in core RNAi gene expression were caused by contamination of the injected dsRNA. Since the reagents used to synthesise
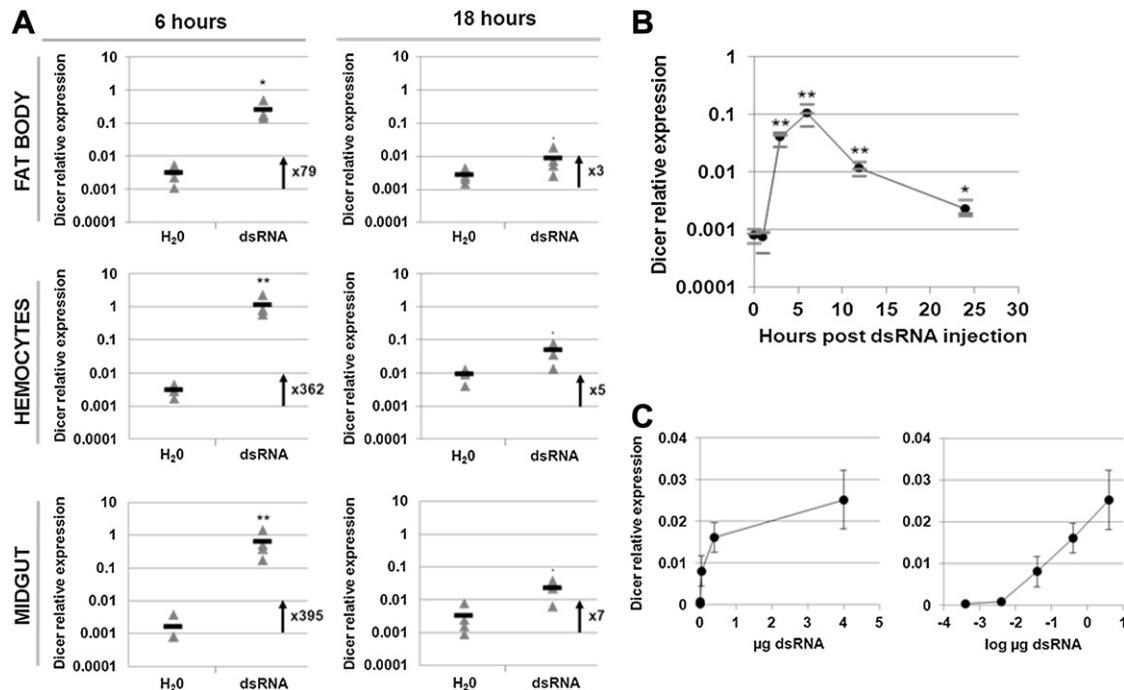
Fig. 2. Relative expression of *dicer-2* mRNA in response to dsRNA. (a) Expression of *M. sexta dicer-2* mRNA in control water-injected (H$_2$0) and eGFP dsRNA-injected (dsRNA) insects. Newly ecdysed 5th instar larvae were injected with water or 1 μg dsRNA ($n = 4$). Six hours or 18 h post injection fat body, haemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels relative to the internal control, rpS-3. Plotted values are the four replicates (grey triangles) and the mean (black dash). The fold difference in the means is written next to a black arrow. Significant differences in transcript levels between the two treatments (water-injected and dsRNA-injected) are denoted using the following terminology for the *p*-values obtained from *t*-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**) and 0.001 (***). (b) Time course of *M. sexta dicer-2* mRNA expression following injection with dsRNA. Newly ecdysed 5th instar larvae were injected with 1 μg eGFP dsRNA and dissected for haemocytes a number of hours later ($n = 3$). Relative mRNA levels were quantified in haemocytes using q-RT-PCR. Plotted values are the three replicates (grey dashes) and the mean (black circle). Significant differences in transcript levels from time zero are denoted using the following terminology for the *p*-values obtained from *t*-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**) and 0.001 (***). (c) Response of *M. sexta dicer-2* mRNA to different doses of dsRNA. Newly ecdysed 5th instar larvae were injected with a range of doses of eGFP dsRNA ($n = 4$). Six hours post injection haemocytes were dissected and subjected to q-RT-PCR to quantify mRNA levels. The data are plotted for clarity on both linear (left) and semi-logarithmic (right) scales.

dsRNA include recombinant proteins expressed in microbial cells, one possible type of contamination would be microbial pattern molecules, which are well known to elicit expression of immune-related genes in *M. sexta*, as in other insects (e.g. Eleftherianos et al., 2006). We therefore quantified *dicer-2* and *argonaute-2* transcript levels in *M. sexta* larvae in response to injection with bacterial cells (*E. coli*, DH5-α). Injection with bacteria (5 × 10^5 *E. coli* DH5α cells in 50 μl PBS) did not result in elevated expression levels of *dicer-2* (relative to a control injection) in fat body at 6, 12 and 24 h post injection (Supplementary Information, Fig. S6 in Appendix S4; 6 h $t = -1.905$, $p = 0.0859$; 12 h $t = -0.978$, $p = 0.3535$; 24 h $t = -0.213$, $p = 0.83556$) or in haemocytes at 6 h post injection (*t*-test: $t = -0.607$, $p = 0.5606$). There was also no significant increase in expression levels (relative to the control) of *argonaute-2* in bacteria-injected insects (Appendix S4, Fig. S6; fat body: 6 h $t = 1.403$, $p = 0.191$; 12 h $t = -0.141$, $p = 0.891$; 24 h $t = 1.563$, $p = 0.149$ and haemocytes: $t = -0.813$, $p = 0.4396$). Furthermore, injection with 50 μl of a 50 μg/ml solution of *Micrococcus luteus* peptidoglycan (Sigma), a bacterial pattern molecule, did not elicit a significant change in expression levels of *dicer-2* and *argonaute-2* at 6 h post injection (Supplementary Information, Fig. S7 in Appendix S4; *dicer-2*: $t = -0.132$, $p = 0.8976$, *argonaute-2*: $t = 1.625$, $p = 0.135$).

We were also keen to demonstrate that the observed upregulation of *dicer-2* and *argonaute-2* in response to dsRNA challenge was specific in nature and not a general response to nucleic acids. Our experiments revealed that an equivalent quantity of DNA bearing the eGFP sequence did not elicit an upregulation in gene expression. Although single-stranded RNA (ssRNA) did stimulate

significantly increased expression of *dicer-2* (but not *argonaute-2*), the increase was to a lesser degree than that elicited by dsRNA (Supplementary material, Fig. S8 in Appendix S4). These results indicate *dicer-2* and *argonaute-2* are specifically upregulated in response to dsRNA. Even though there was some upregulation in *dicer-2* expression by ssRNA, this may have been caused by aberrant dsRNA molecules in the ssRNA preparation, as the RNA polymerases used for preparation of RNAs are known to produce some molecules with a double-stranded structure (Fire et al., 1998).

### 3.5. Expression of dicer-2 in response to multiple dsRNA injections

With the aim of finding a way to prolong the observed transient upregulation of *M. sexta dicer-2* mRNA (Fig. 2b) we were interested to determine whether multiple injections of dsRNA could induce a long-lasting increase in *dicer-2* expression. An experiment was designed to compare *dicer-2* mRNA expression in haemocytes from insects injected with either i) two doses of water at $t = 0$ and $t = 24$ h, ii) a dose of dsRNA at $t = 0$ h and a dose of water at $t = 24$ h, iii) a dose of water at $t = 0$ h and a dose of dsRNA at $t = 24$ h and iv) two doses of dsRNA at $t = 0$ and $t = 24$ h (the experimental design is outlined in Fig. 5a). Transcript levels in haemocytes were analysed at $t = 0$, $t = 6$, $t = 24$, $t = 30$ and $t = 48$ h. Insects from the control treatment (two water injections) did not display an upregulation of *dicer-2* mRNA at any time following injection (Fig. 5b), whilst insects from the single dsRNA injection treatments had elevated *dicer-2* mRNA levels 6 h after dsRNA injection (injection at $t = 0$, Fig. 5c, $t = 4.834$, $p = 0.002900$ and injection at $t = 24$, Fig. 5d, $t = 2.231$, $p = 0.0671$). In each case, the response had largely
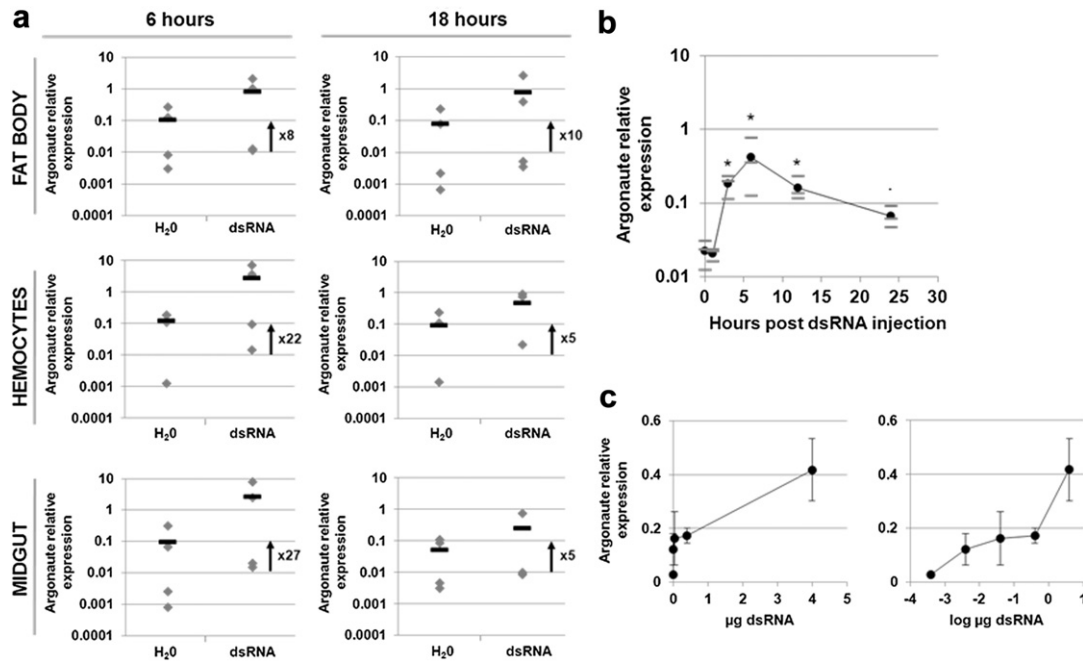
Fig. 3. Relative expression of *argonaute-2* mRNA in response to dsRNA. (a) Expression of *M. sexta argonaute-2* mRNA in control water-injected (H_2O) and eGFP dsRNA-injected (dsRNA) insects. Newly ecdysed 5th instar larvae were injected with water or 1 μg dsRNA ($n = 4$). Six hours or 18 h post injection fat body, haemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels relative to the internal control, rpS-3. Plotted values are the four replicates (grey triangles) and the mean (black dash). The fold difference in the means is written next to a black arrow. (b) Time course of *M. sexta argonaute-2* mRNA expression following injection with dsRNA. Newly ecdysed 5th instar larvae were injected with 1 μg eGFP dsRNA and dissected for haemocytes a number of hours later ($n = 3$). Relative mRNA levels were quantified in haemocytes using q-RT-PCR. Plotted values are the three replicates (grey dashes) and the mean (black circle). Significant differences in transcript levels from time zero are denoted using the following terminology for the *p*-values obtained from *t*-tests performed on the transformed data: 0.1 (.), 0.05 (*), 0.01 (**) and 0.001 (***). (c) Response of *M. sexta argonaute-2* mRNA to different doses of dsRNA. Newly ecdysed 5th instar larvae were injected with a range of doses of eGFP dsRNA ($n = 4$). Six hours post injection haemocytes were dissected and subjected to q-RT-PCR to quantify mRNA levels. The data are plotted for clarity on both linear (left) and semi-logarithmic (right) scales.
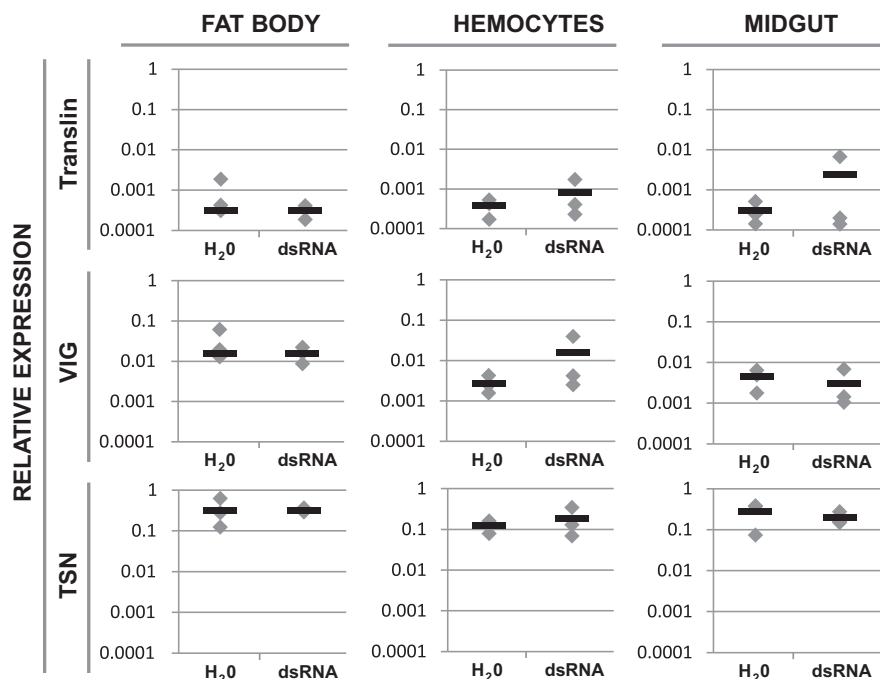
Fig. 4. Relative expression of *translin*, *Vig* and *TSN* in response to dsRNA. Expression of *M. sexta translin*, *VIG* (*vasa intronic gene*) and *TSN* (*tudor staphylococcus/micrococcal nuclease*) mRNA in control water-injected (H_2O) and eGFP dsRNA-injected (dsRNA) insects. Newly ecdysed 5th instar larvae were injected with water or 1 μg dsRNA ($n = 4$). Six hours post injection fat body, haemocytes and midgut were dissected and subjected to q-RT-PCR to quantify mRNA levels relative to the internal control, rpS-3. Plotted values are the three replicates (grey diamonds) and the mean (black dash). There were no significant differences in transcript levels between the two treatments.
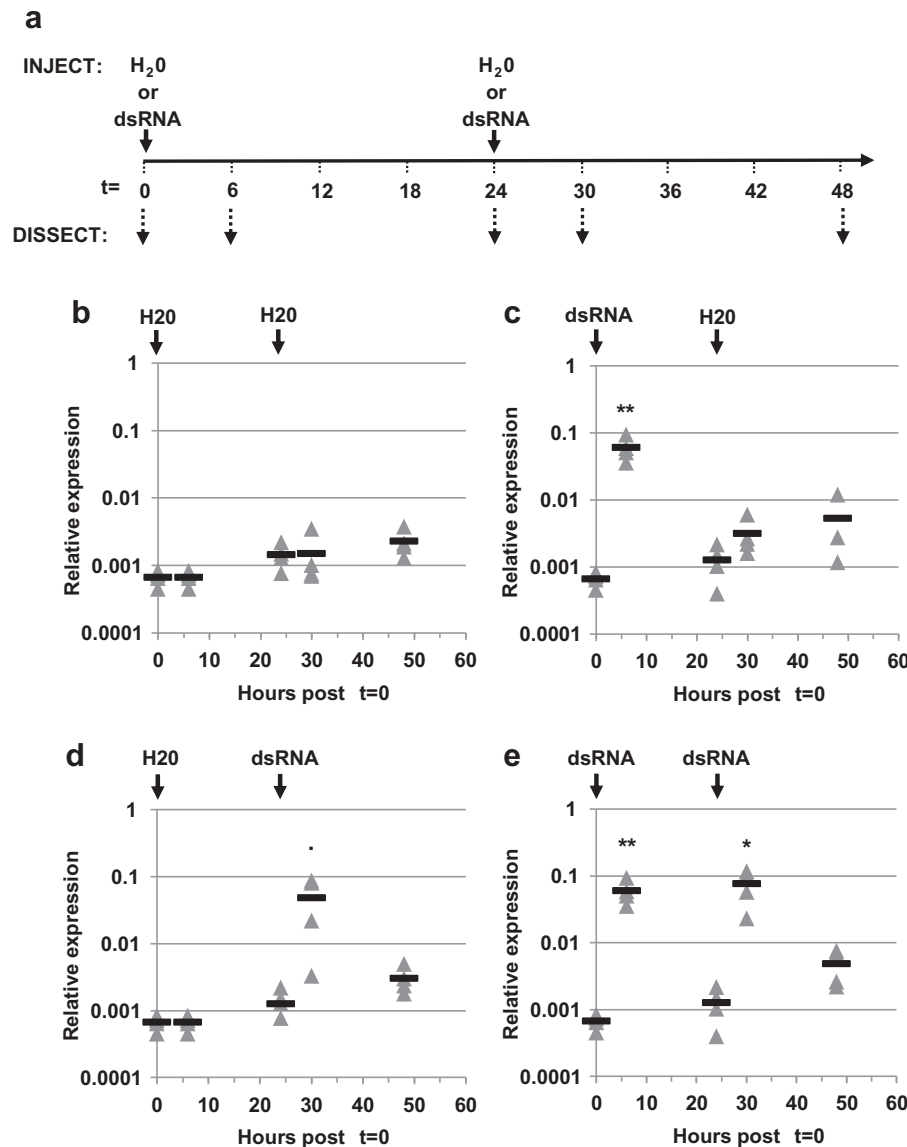
Fig. 5. *Dicer-2* mRNA levels respond to multiple dsRNA injections. Newly ecdysed 5th instar larvae were injected with two doses of either DEPC-treated water or 1 µg dsRNA for eGFP. Animals were dissected for hemolymph at a number of time points throughout the experiment and haemocytes were recovered and subjected to q-RT-PCR to quantify mRNA levels relative to the internal control, rpS-3. (a) Design of the experiment showing times at which insects were injected with dsRNA or water and the times when insects were dissected for haemocytes. Expression of *dicer-2* mRNA in insects (b) injected with two doses of water, (c) injected with a dose of dsRNA and then a subsequent injection with water, (d) injected with water and then with dsRNA and (e) injected with two doses of dsRNA. For parts (b) to (e) the values for the four replicates are shown as grey triangles and the mean is depicted with a black dash. Significantly increased transcript levels compared to levels in control insects (twice injected with H_2O) are denoted using the following terminology for the *p*-values obtained from *t*-tests performed: 0.1 (.), 0.05 (*), 0.01 (**) and 0.001 (***).

decayed by 24 h after the dsRNA injection. When insects were injected twice with dsRNA, the second injection resulted in a significant elevation of *dicer-2* expression 6 h later when compared to mRNA levels at that time point in the control treatment (Fig. 5e, $t = 3.393$, $p = 0.0146$). The second response was similar in magnitude to the first.

4. Discussion

In this study we were interested to investigate whether RNAi machinery components are upregulated in response to dsRNA in insects. Our interest in this question stemmed from the suggestion that deficiency in intracellular dsRNA processing might be responsible for the apparent inefficacy of RNAi in some insects and the variable efficiency of RNAi experiments (Terenius et al., 2011).

We hypothesised that the expression of core RNAi genes might vary, being modulated in response to the presence of dsRNA. Several key components of the cellular RNAi pathway, *dicer-2*, *argonaute-2*, *translin*, *VIG* and *TSN*, were therefore identified in the *M. sexta* transcriptome and their basal expression and expression in response to dsRNA characterised using q-RT-PCR.

Our experiments revealed that all five genes were expressed in larval *M. sexta* tissue, with the level of expression of the different genes varying considerably (Fig. 1). Our results are consistent with the study of Swevers et al. (2011), which also found that the silkmoth-derived Bm5 cell line was deficient in expression of *translin*.

Our dsRNA-injection experiments revealed that *dicer-2* mRNA levels and, to a lesser extent, *argonaute-2* mRNA levels were elevated following injection with dsRNA in a specific and dose-

dependent manner (Figs. 2 and 3). Interestingly, the expression of the other RNAi genes tested, *translin*, *VIG* and *TSN*, was not responsive to exogenous dsRNA (Fig. 4). This is the first study of which we are aware to report the enhanced expression of core RNAi genes in response to dsRNA in insects.

Our results are consistent with studies of gene expression in other taxa (for instance in shrimp; Chen et al., 2011 and fish Su et al., 2009), which reported elevated levels of *dicer-2* mRNA in response to challenge with dsRNA or viral particles. However, these studies lacked data regarding RNAi machinery components other than *dicer-2*, so we are unable to make any comparisons regarding the expression of *argonaute-2*, *translin*, *VIG* and *TSN*. Furthermore, the degree of upregulation observed in these studies was much less than that observed in *M. sexta*: in our study *dicer-2* transcript levels increased 79, 362 and 395 fold in *M. sexta* fat body, haemocytes and midgut respectively, whereas the authors in Chen et al. (2011) reported a maximum of 7 fold and 12 fold increases in mRNA levels following challenge with the synthetic dsRNA analogue Poly(C-G) and White Spot Syndrome Virus respectively in the shrimp *Litopenaeus vannamei*.

Our experiments were prompted by the desire to understand the well-known interspecific and intraspecific variability of responses in RNAi experiments. We have ourselves experienced such variability in RNAi success in *M. sexta* (unpublished). Our data suggest that this insect should be responsive to RNAi, since it upregulates key pathway components after the dsRNA trigger. Deficiency in RNAi in this insect may be caused by lower resting expression levels of one or more core RNAi genes (possibly *translin*, or another RNAi factor that has not been tested), or alternatively, the restricted duration of elevated expression following exposure to dsRNA (i.e. the machinery may be under tighter control). Of course, a comparison of the expression profile in *M. sexta* with that from an insect highly susceptible to RNAi would be greatly advantageous and it remains possible that other factors (such as the efficient degradation of dsRNA in the haemolymph or the deficient amplification and spreading of the RNA signal (Bellés, 2010)) could be responsible for the variable response to RNAi observed in *M. sexta*.

These experiments also contribute to our knowledge regarding RNAi as an antiviral immune response. Whilst the role of RNAi in defence against viral pathogens is becoming clearer (see Ding, 2010 for a review), the transcriptional response to viral molecular patterns, such as dsRNA, has not (to our knowledge) been comprehensively studied in insects. Our experiments show that *dicer-2* and *argonaute-2* are upregulated in response to the presence of dsRNA in *M. sexta*, whilst *translin*, *VIG* and *TSN* were not responsive to dsRNA. The explanation for these results may be that Dicer-2 and Argonaute-2 have roles in addition to their function in processing intracellular dsRNAs, for example as immune pattern recognition receptors (PRRs). Evidence for a role of Dicer-2 as a viral PRR was provided by Deddouche et al. (2008), who found that the induction of the *Drosophila melanogaster* gene *vago*, the product of which participates in the control of viral load, was dependent on Dicer-2. An alternative explanation is that levels of Dicer-2 and Argonaute-2 are limiting during the processing of dsRNA. There is some evidence that Argonaute may be limiting during RNAi experiments: Diederichs et al. (2008) found that ectopic expression of Argonaute-2 enhanced silencing by short hairpin RNA constructs (shRNAs) in human lung cancer cells.

One way to potentially prolong the elevated expression of *dicer-2* and *argonaute-2* could be to inject multiple doses of dsRNA. Indeed, many successful RNAi experiments in insects have been conducted using multiple introductions of dsRNA (e.g. Araujo et al., 2006; Zhou et al., 2008; Maestro et al., 2009). Here, we investigated whether multiple injections of dsRNA result in multiple peaks of

dicer-2 expression and found that injecting a second dose of dsRNA does indeed cause a second peak of *dicer-2* expression (Fig. 5). This may explain why using multiple dsRNA injections is effective in achieving an RNAi knockdown of a target gene. It may be useful to try multiple dsRNA treatments in cases where trial RNAi experiments have proved to be unsuccessful.

5. Conclusion

In summary, we have identified *M. sexta dicer-2*, *argonaute-2*, *translin*, *VIG* and *TSN* genes and analysed the resting expression levels of these core RNAi genes as well as their transcriptional response to the presence of exogenous dsRNA. It is evident that in this insect the presence of dsRNA induces expression of some RNAi machinery components, indicating that the antiviral function of RNAi is not constitutive in this insect. Differences in the extent of basal and induced expression of core RNAi genes may therefore explain the observed differences between eukaryotic species in their susceptibility to viral infection, as well as species differences in the success of experimental RNAi procedures. Further, because prolonging the duration of contact with exogenous dsRNA has been shown to effectively prolong the period during which core RNAi gene transcripts are present at elevated levels, multiple dsRNA injections may prove to be useful in RNAi protocols.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2012.05.001.

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