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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.

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 (Clon-Tech), 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 *p*mol 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.