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Source: Journal of Insect Science, 4(15) : 1-9

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.004.1501

Fabrick JA, Kanost MR, Baker JE. 2004. RNAi-induced silencing of embryonic tryptophan oxygenase in the Pyralid moth, *Plodia interpunctella*. 9pp. *Journal of Insect Science*, 4:15, Available online: insectscience.org/4.15

Journal of Insect Science

insectscience.org

RNAi-induced silencing of embryonic tryptophan oxygenase in the Pyralid moth, *Plodia interpunctella*

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Received 27 October 2003, Accepted 16 February 2004, Published 17 May 2005

Abstract

Gene silencing through the introduction of double-stranded RNA (RNA interference, RNAi) provides a powerful tool for the elucidation of gene function in many systems, including those where genomics and proteomics are incomplete. The use of RNAi technology for gene silencing in Lepidoptera has lacked significant attention compared to other systems. To demonstrate that RNAi can be utilized in the lepidopteran, *Plodia interpunctella*, we cloned a cDNA for tryptophan oxygenase, and showed that silencing of tryptophan oxygenase through RNAi during embryonic development resulted in loss of eye-color pigmentation. The complete amino acid sequence of *Plodia* tryptophan oxygenase can be accessed through NCBI Protein Database under NCBI Accession # AY427951.

Keywords: Insect; Lepidoptera, double-stranded RNA; eye pigmentation; ommochrome; Indianmeal moth

Abbreviation:

RNAi	RNA interference
PCR	polymerase chain reaction
RT-PCR	reverse transcription-PCR

Introduction

Eye-color mutations in insects provide convenient and reliable genetic markers, and the genes responsible for eye pigmentation are frequently utilized as transgenic markers. Mutations that effect insect eye-color involve either the biosynthesis or transport of ommochrome (brown) or pteridine (red) screening pigments. Dark eye-color in insects has been attributed to the ommochrome pigment pathway, in which the initial step involves the conversion of tryptophan to N-formylkynurenine by tryptophan oxygenase (EC 1.13.1.11). The *vermilion* gene encodes tryptophan oxygenase in *Drosophila sp.* (Walker et al., 1986; Searles and Voelker, 1986; Searles et al., 1990; Begun and Aquadro, 1995; Stephan et al., 1998; Begun and Whitley, 2000) and in *Tribolium castaneum* (Lorenzen et al., 2002). Tryptophan oxygenases have also been cloned from mosquitoes, including *Anopheles gambiae* (Mukabayire et al., 1996) and *Aedes aegypti* (Fang and Li, 2001).

Silencing of genes by RNA interference (RNAi) through the delivery of a specific double-stranded RNA is a powerful method for the functional analysis of specific gene products in an impressive array of organisms, including several orders of insects. However to date, RNAi has only been successfully demonstrated in a small number of Lepidoptera, including *Spodoptera litura* (Rajagopal et al., 2002), *Hyalophora cecropia* (Bettencourt et al., 2002), *Bombyx mori* (Quan et al., 2002), and in cultured *Manduca sexta* neuronal cells (Vermehren et al., 2001). In this work, we obtained a cDNA encoding the first tryptophan oxygenase from a lepidopteran insect, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), and demonstrated that a phenotype lacking larval eyespots can be obtained upon delivery of tryptophan oxygenase double-stranded RNA into preblastoderm embryos.

Materials and Methods

Insects

A strain of *P. interpunctella* was collected from shelled corn in El Paso, Illinois, in November of 1998 and has been reared in the laboratory on a diet of cracked wheat, wheat shorts, wheat germ, Brewer's yeast, honey, glycerin, and water. Cultures are maintained at 27° C and 50-55% relative humidity in a 12 h photophase. Mated *Plodia* adults were aspirated into a pint jar containing a piece of corrugated filter paper and eggs were collected after 30 minutes.

Preparation of P. interpunctella Genomic DNA

Genomic DNA was obtained from single 5th instar

wandering stage *P. interpunctella* larvae by homogenization in 0.6 ml of Nuclei Lysis Solution (Promega, www.promega.com) with a Kontes homogenization pestle and tube. After the homogenate was incubated at 65° C for 30 minutes, 12 µg of RNase A was added, mixed, and incubated at 37° C for 30 minutes. Lysates were cooled to room temperature and 0.2 ml of Protein Precipitation Solution (Promega) was added to each tube. Samples were chilled on ice for 5 minutes and centrifuged at 13,000 x g for 1 minute. Supernatants were placed into sterile microtubes and the DNA was precipitated with isopropanol and washed with ethanol. DNA pellets were allowed to briefly air dry and were resuspended in 100 µl of sterile water. Genomic DNA samples were quantified from A_{260} values.

Partial Cloning of Tryptophan Oxygenase by PCR

Fully-nested PCR was used to obtain a partial clone encoding the *vermilion* gene (tryptophan oxygenase) from *P. interpunctella*. First-round PCR was conducted from ~100 ng of genomic DNA template with the degenerate PCR primers VerF (5' TAYGARYTNTGGTTYAARCA 3') and VerR4 (5' CATTKCKTTGCACCATSAWMACRTGATTRT 3') in a Biometra (www.biometra.de)T Gradient thermocycler. Touchdown PCR was performed by using TaKaRa ExTaq DNA polymerase with cycles of denaturation (30 seconds at 94° C), followed by the first of fifteen cycles beginning 15° C above the Tm of the primer set and dropping 1° C for each annealing step, and extension (1 minute at 72° C). Following the first fifteen cycles, 25 cycles of denaturation (30 seconds at 94° C), annealing (30 seconds at primer set Tm), and extension (1 minute at 72° C) completed the first round of PCR. A second round of PCR was conducted on 1 µl of template from the first round of PCR by using fully-nested PCR primers, VerF3 (5' TGCAGTTCCGKYTSHTSGAGAACA 3') and VerR (5' CCNGGNGTNCKYTCNARCC 3') and a total of 45 cycles. Control PCR reactions containing sterile water in place of template were conducted for both rounds of PCR. PCR products were separated on 1% agarose gels in 1 x TBE containing 0.5 µg/ml ethidium bromide and analyzed over UV light. PCR-generated bands were gel-purified by using the Quantum Prep Freeze-N-Squeeze DNA Gel Extraction kit (BioRad, www.bio-rad.com) and cloned into the pCR4-TOPO vector (Invitrogen) following the manufacturers instructions. TOP10 OneShot *E. coli* (Invitrogen, www.invitrogen.com) were transformed and plasmid DNA was recovered by using a QIAprep Spin Mini Prep kit (Qiagen, www.qiagen.com). An aliquot of plasmid DNA was digested with EcoRI (Stratagene, www.stratagene.com) to confirm size of insertion and then plasmid DNA was sequenced at the Kansas State University DNA Sequencing and Genotyping Facility by using a T7 vector primer.

Cloning of Full-length Plodia tryptophan oxygenase by RACE

The missing 5'- and 3' ends of *P. interpunctella* tryptophan oxygenase were obtained by using the GeneRacer™ kit (Invitrogen). Total RNA from ten larvae was obtained as described previously (Fabrick et al., 2003). RNA was ligated to the GeneRacer™ RNA oligo as described by the manufacturer and reverse transcription was performed by using Avian Myeloblastosis Virus Reverse Transcriptase with the GeneRacer™ oligo-dT primer. The 5' end of *P. interpunctella* tryptophan oxygenase was obtained through two-rounds of PCR using semi-nested primers with the first round conducted using the GeneRacer™ 5' Primer and VerR5 (5' GCGCAACGCCTGTTTTAGCCCCAA 3'). The first round of PCR consisted of a total of 40 cycles and the annealing temperature was 5° C below the Tm of the primer pair. The second round of PCR was conducted over 40 cycles by using the template from first round PCR with the 5' GeneRacer™ Nested primer and VerR5. The 3' end of *P. interpunctella* tryptophan oxygenase was obtained from two rounds of fully-nested PCR with the first round of PCR conducted using VerF7 (5' GCAGCTATGGACGCCTTGCACAAATCA 3') and the GeneRacer™ 3' Primer over a total of 40 cycles. The first 15 cycles were performed with decreasing annealing temperatures (as outlined above for touchdown PCR). The second round of PCR was conducted using VerF6 (5' GGCGCTGATCGAGCGGTGGCTAGAACGAA 3') and the GeneRacer™ 3' Nested Primer over a total of 25 cycles. Both first and second rounds of PCR were conducted under gradient temperature conditions to empirically determine optimum annealing temperatures. PCR products from 5'- and 3'-RACE were inserted into cloning vectors as outlined previously and both strands of DNA was sequenced using T3 and T7 vector primers.

A full-length cDNA encoding the open reading frame (ORF) of *Plodia* tryptophan oxygenase was obtained by PCR from 1st strand cDNA template by using the PCR primers VerF8 (5' ATGGCCTGTCCTATGAGGTCAATG 3') and VerR6 (5' AGCCAACTCCAATTTCAACATGCAGTT 3'). The 1.2-kb PCR product was gel-purified and cloned as described above. Purified plasmid DNA was digested with EcoRI to confirm insert size and plasmid DNA was sequenced as above.

Computer Analysis of Sequence Data

Comparison of the *P. interpunctella* tryptophan oxygenase cDNA sequence against the non-redundant public sequence database was made using BLASTX (Altschul et al., 1990). Sequence analysis tools of the ExPASy (www.expasy.ch) Molecular Biology Server of Swiss Institute of Bioinformatics, including Translate, Compute pI/MW and SignalP were used to analyze the deduced tryptophan oxygenase protein sequence. Similar protein sequences were aligned using PRETTY with the BLOSUM90 scoring matrix and sequence identities and similarities were calculated using BESTFIT from SeqWeb (Version 2) (Accelrys, www.accelrys.com). A phylogenetic tree was generated from TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) (version 1.6.6) with SeqWeb's GrowTree data (Accelrys) by using Kimura distance correction method, Neighbor joining tree construction, and the BLOSUM90 scoring matrix.

Synthesis of double-stranded RNA

The MEGAscript™ RNAi kit (Ambion, www.ambion.com) was used to generate double-stranded RNA corresponding to nucleotides 680 to 1,336 of the *Plodia* tryptophan oxygenase. T7 promoter sites were added to the PCR primers VerF5 (5' CTGGCGCTGATCGAGCGGTGG 3') and VerR6 to generate the primers T7VerF5 (5' TAATACGACTCACTATAGGGACTGGCGCTGATCGAGCGGTGG 3') and T7VerR6 (5' ATTATGCTGAGTGATATCCCTAGCCAACTCCAATTTCAACATGCA

3'). Two separate PCR reactions were conducted to generate complementary templates with a single T7 promoter site (T7VerF5+VerR6 and VerF5+T7VerR6). T7 RNA polymerase was used to transcribe single-stranded RNA from each DNA template over 4 h at 37° C. double-stranded RNA was produced by mixing solutions containing equivalent amounts of complementary ssRNA, incubating at 75° C for 5 minutes, and allowing the solution to cool to room temperature. DNA and single-stranded RNA were removed from the solution by digestion with DNase I and RNase at 37° C for 1 h. The double-stranded RNA was purified using provided purification cartridges as per the manufacturer's instructions. Double-stranded RNA was eluted with two successive 50 µl washes of 95° C pre-heated 10 mM Tris-HCl pH 7 containing 1 mM EDTA (ethylenediamine tetraacetate). Total double-stranded RNA was quantified from A_{260}.

RNA Interference

P. interpunctella eggs were collected into a 100 µm nylon cell strainer (Falcon 2360) and washed for 10 seconds in 2.5% bleach solution followed by distilled water for 1 minute. A fine brush dipped in water was used to position eggs (~100 per slide) onto double-sided tape attached to a glass microscope cover slip. Controls included observations of late stage embryos and newly hatched larvae from eggs that were not injected, and presence or absence of eyespots in eggs injected with water or buffer. *Plodia* tryptophan oxygenase double-stranded RNA (~1 mg ml^{-1}) was injected into 1- to 3-h old embryos (injection volume ~100 pl/ embryo) using a pulled glass needle beveled at a 45° angle. Embryos were covered with Halocarbon oil (series 700) and incubated at 30° C in a humidified, oxygenated chamber (Billups-Rothenberg, www.brincubator.com). Late-stage embryos or newly hatched 1st-instar larvae were checked daily for development and eyespot pigmentation.

RT-PCR

Relative tryptophan oxygenase RNA transcript levels were measured following RT-PCR from single newly hatched *P. interpuntella* larvae using Ambion's Cells-to-cDNA II kit. Individual larvae were collected into sterile, nuclease-free Kontes homogenization tubes and washed with 100 µl ice-cold phosphate buffered saline. The buffer was replaced with 100 µl Lysis Buffer (Ambion) and each larva was briefly homogenized on ice with Kontes homogenization pestles. Following heating at 75° C for 10 minutes, 2 µl of DNase I was added to homogenates and allowed to incubate at 37° C for 15 minutes. DNase I was inactivated by heat-treatment at 75° C for 5 minutes. Reverse transcription of RNA was carried out on 10 µl of each homogenate using random decamer primers (provided in kit from Ambion) and M-MLV reverse transcriptase at 42° C for 50 minutes. Semi-nested PCR was conducted on cDNA templates by using the primer pairs VerF8+VerR6 and VerF7+VerR6 in first and second rounds of PCR, respectively. First-round PCR consisted of an initial 2 minutes at 94° C followed by 35 cycles of 30 seconds at 94° C, 30 seconds at 55° C, and 30 seconds at 72° C, with a final extension of 5 minutes at 72° C. Semi-nested PCR was conducted on first-round templates over 15, 23, or 40 cycles and consisted of an initial 2 minutes at 94° C, followed by cycles of 30 seconds at 94° C, 30 seconds at 57° C, and 60 seconds at 72° C, and a final 10 minute extension at 72° C. Controls included a negative reverse transcription reaction that lacked reverse transcriptase and PCR without template. RT-PCR control reactions for ribosomal protein S7 (RPS7) (Fabrick et al. 2003) were performed on the *P. interpunctella* RNA extracts with 35 cycles as outlined above.

Results

Plodia tryptophan oxygenase cDNA

Members of the tryptophan oxygenase family are highly conserved, which allowed for the design of degenerate primers successfully used in PCR. A partial genomic clone of 179 bp encoding a tryptophan oxygenase from *P. interpunctella* was obtained from fully-nested PCR by using two sets of degenerate primers. By using RACE, we determined the 5'- and 3'-sequences and a full-length cDNA consisting of 1,474 bp was obtained by RT-PCR. The 179 bp genomic clone did not span any intron sequence, as determined by comparison with the full-length cDNA (and which was predicted by comparison with genomic sequences from *Drosophila melanogaster, A. gambiae,* and *T. castaneum*). The 1,474 bp cDNA contains an open reading frame of 1,212 bp that encodes 404 amino acid residues and contains a 124 nucleotide 5' double-stranded RNA and a 138 nucleotide 3' double-stranded RNA (Fig. 1).

BLASTX, multiple sequence alignment, and phylogenetic tree analysis of the cDNA indicate that the deduced protein is closely related to other tryptophan oxygenases (Fig. 2A, B). *P. interpunctella* tryptophan oxygenase shares high sequence identity (>72%) and similarity (>76%) with all other insect tryptophan oxygenases currently available in the public database. The *P. interpunctella* tryptophan oxygenase is also very similar to tryptophan oxygenases from *Caenorhabditis elegans* (56.6% identical, 60.4% similarity) and mammals (>55% identity, >61% similarity). The predicted molecular weight and pI of *P. interpunctella* tryptophan oxygenase are 46,587 Da and 6.0, respectively. *P. interpunctella* tryptophan oxygenase was not predicted to be a secreted protein as determined by SignalP.

RNAi

Wild-type *P. interpunctella* larvae normally possess six dark brown eyespots (stemmata) on each side of the head. Often the individual eyespots are so darkly pigmented that it is difficult to visualize the individual stemmata and they therefore appear as a smeared concave crescent at the lower proximal portion of the head capsule (Fig. 3A). An eyespot in the *P. interpunctella* embryo is typically the only dark coloration visible inside the egg (other than the head capsule itself) and is normally visible through the chorion approximately one day prior to hatching.

Both newly hatched larvae and fully-developed embryos were examined from the uninjected controls for eyespots. Of 2,270 1st instar larvae examined from batches of eggs laid by 8 different cohorts of *P. interpunctella*, all had the wildtype phenotype. However, when we checked egg hatch of the El Paso strain used in this study, only 38% or 415/1083 embryos developed when no injection was performed. This hatch rate is compared with a hatch rate of 98% for a laboratory strain of *P. interpunctella* (McGaughey

```
1   GGACACTGACATGGACTGAAGGAGTAGAAAGTATCGAGTGACCACGCGCCCGGCAAGTGCGCGCGCGTCCTAATTCGAAA    80
    TTCAAACTTTATTTATTTTTTAGTGTTTAACTTAGAACTACAAAATGGCCTGTCCTATGAGGTCAATGAACGACTCGTCG   160
                                          1  M  A  C  P  M  R  S  M  N  D  S  S        12

    CAGGACGGCAGCTGCCTGGGCAACGAAGCCGGCATGCTGTACGGGGAGTACCTGATGCTGGACAAGCTCCTCGCAGCACA   240
    Q  D  G  S  C  L  G  N  E  A  G  M  L  Y  G  E  Y  L  M  L  D  K  L  L  A  A  Q    39

    GCGGATGCTCAGCGCCGAGTCCTCGCGGCCGGCGCACGACGAGCATCTGTTCATTGTCACCCATCAAGCGTACGAGTTAT   320
     R  M  L  S  A  E  S  S  R  P  A  H  D  E  H  L  F  I  V  T  H  Q  A  Y  E  L  W    66

    GGTTCAAGCAGATAATATATGAAGTGGACTCTGTCAGGTCGTTGCTGGACGTTGAAGGTCTGGACGAGAGCCACACCATG   400
      F  K  Q  I  I  Y  E  V  D  S  V  R  S  L  L  D  V  E  G  L  D  E  S  H  T  M     92

    GAGATACTGAAGCGGCTAAACAGGATTGTGTTAATTCTTAAGCTTCTTGTGGACCAAGTCATGATCTTAGAGACCATGAC   480
    E  I  L  K  R  L  N  R  I  V  L  I  L  K  L  L  V  D  Q  V  M  I  L  E  T  M  T   119

    CCCTCTGGACTTCATGGACTTCCGGAACTACCTGAGGCCAGCTTCCGGCTTCCAGAGCCTTCAGTTCCGGCTATTGGAAA   560
     P  L  D  F  M  D  F  R  N  Y  L  R  P  A  S  G  F  Q  S  L  Q  F  R  L  L  E  N   146

    ATAAGTTGGGGCTAAAACAGGCGTTGCGCGTGAAGTACAATCAGAATTACCAGACAGTGTTTGGGGATGACCCTGCAGCT   640
      K  L  G  G  L  K  Q  A  L  R  V  K  Y  N  Q  N  Y  Q  T  V  F  G  D  D  P  A  A   172

    ATGGACGCCTTGCACAAATCAGAAGAAGAACCAGCGCTCCTGGCGCTGATCGAGCGGTGGCTAGAACGAACCCCAGGGCT   720
    M  D  A  L  H  K  S  E  E  E  P  A  L  L  A  L  I  E  R  W  L  E  R  T  P  G  L   199

    GCAAACGCACGGCTTCAACTTCTGGGGCAAGTTCCAGGCCGCCGTCAACAAAACTATTACAGACGACATTGAGGCCGCTA   800
     Q  T  H  G  F  N  F  W  G  K  F  Q  A  A  V  N  K  T  I  T  D  D  I  E  A  A  M   226

    TGACGGAACCCAACGAAACAGTCCGTCGTCACAGGCTGCAGGACGCGGAAAACCGACGCGAGATCTACCGGTCTATCTTC   880
      T  E  P  N  E  T  V  R  R  H  R  L  Q  D  A  E  N  R  R  E  I  Y  R  S  I  F     252

    GATCCAGCCGTGCACGATGCCCTCAGATCCAGGGGGGAAAGGAGACTGTCGCACCGCGCGCTGCAAGGCGCCATCATGAT   960
    D  P  A  V  H  D  A  L  R  S  R  G  E  R  R  L  S  H  R  A  L  Q  G  A  I  M  I   279

    CACGATCTACCGCGACGAGCCGCGTTTCTCGCAGCCGCACCAGCTGCTCACGCTGCTCATGGACATCGACAGCCTCATCA  1040
     T  I  Y  R  D  E  P  R  F  S  Q  P  H  Q  L  L  T  L  L  M  D  I  D  S  L  I  T   306

    CCAAGTGGCGCTATAACCACGTGATCATGGTGCAGCGTATGATTGGCTCGCAGCAACTCGGCACGGGCGGCTCCTCAGGG  1120
      K  W  R  Y  N  H  V  I  M  V  Q  R  M  I  G  S  Q  Q  L  G  T  G  G  S  S  G    332

    TACCAGTACCTTCGCTCCACTCTCAGTGACCGTTACAAGGTATTCTTGGATCTATTTAACCTCTCAACCTTCCTCCTCCC  1200
    Y  Q  Y  L  R  S  T  L  S  D  R  Y  K  V  F  L  D  L  F  N  L  S  T  F  L  L  P   359

    CCGCTCCCTCATCCCGCCCCTCGACGACGGCATGAAGAAGAGTCTCAACCTCACCTGGGGCGACAACGCCAGGGAGAATG  1280
     R  S  L  I  P  P  L  D  D  G  M  K  K  S  L  N  L  T  W  G  D  N  A  R  E  N  G   386

    GGAAAGAAACACACGACGGAGAGCTTAATAACTGCATGTTGAAATTGGAGTTGGCTTGATCTGATTATGTCATGTACTGT  1360
      K  E  T  H  D  G  E  L  N  N  C  M  L  K  L  E  L  A  *                         404

    ATATAATACTGTAAGAGAGGTAATCTGGGGGCTACCATGAAAAATAAAACACGTAGTGCGTCATTACGTCTAAAAAAAAA  1440
    AAAAAAAAAACACTGTCATGCCGCTTACGTAGCG                                                 1474
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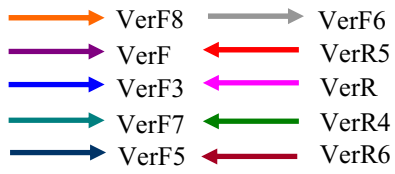

Figure 1. Nucleotide and deduced amino acid sequence of *Plodia interpunctella* tryptophane oxygenase.
The cDNA nucleotide sequence (1-1474) is shown above the deduced amino acid sequence (1-404). A putative polyadenylation sequence, AATAAA, is double underlined, and the termination codon TGA is marked with an asterisk. PCR primer positions are indicated with colored arrows and direction of arrows indicates sense/antisense orientation. The complete amino acid sequence of *P. interpunctella* tryptophane oxygenase can be accessed through NCBI Protein Database under NCBI Accession # AY427951.

```
                  1                                                                                                           120
M. musculus 1     MSGCPFAGNS VGYTLKNLSM EDNEEDRAQT GVNRASKG.G LIYGNYLQLE KILNAQELQS EVKGNKIHDE HLFIITHQAY ELWFKQILWE LDSVREIFQN GHVRDERNML KVIARMHRVV
M. musculus 2     MSGCPFAGNS VGYTLKNVSM EDNEEDRAQT GVNRASKG.G LIYGNYLQLE KILNAQELQS EVKGNKIHDE HLFIITHQAY ELWFKQILWE LDSVREIFQN GHVRDERNML KVIARMHRVV
R. norvegicus     MSGCPFSGNS VGYTLKNLSM EDNEEDGAQT GVNRASKG.G LIYGDYLQLE KILNAQELQS EIKGNKIHDE HLFIITHQAY ELWFKQILWE LDSVREIFQN GHVRDERNML KVMTRMHRVV
H. sapiens        MSGCPFLGNN FGYTFKKLPV EGSEEDKSQT GVNRASKG.G LIYGNYLHLE KVLNAQELQS ETKGNKIHDE HLFIITHQAY ELWFKQILWE LDSVREIFQN GHVRDERNML KVVSRMHRVS
D. ananassae      MS.CPYAG.S .G.N...... .....DHDDA AVPLSTEV.G KIYGEYLMLD KLLDAQCMLS KEDKRPVHDE HLFIITHQAY ELWFKQIIFE FDSIRDML.D AEVIDETKTL EIVKRLNRVV
D. pallidosa      MS.CPYAG.S .G.N...... .....DHDDA AVPLSTEV.G KFYGEYLMLD KLLDAQCMLS KEDKRPVHDE HLFIITHQAY ELWFKQIIFE FDSIRDML.D AEVIDETKTL EIVKRLNRVV
D. melanogaster   MS.CPYAG.N .G.N...... .....DHDDS AVPLTTEV.G KIYGEYLMLD KLLDAQCMLS EEDKRPVHDE HLFIITHQAY ELWFKQIIFE FDSIRDML.D AEVIDETKTL EIVKRLNRVV
D. simulans       MS.CPYAG.N .G.N...... .....DHDDS AVPLTTEV.G KIYGEYLMLD KLLDAQCMLS EEDKRPVHDE HLFIITHQAY ELWFKQIIFE FDSIRDML.D AEVIDETKTL EIVKRLNRVV
D. yakuba         MS.CPYAG.N .G.N...... .....EHDDS AVPLTTEV.G KIYGEYLMLD KLLDAQCMLS EEDKRPVHDE HLFIITHQAY ELWFKQIIFE FDSIREML.D AEVIDETKTL EIVKRLHRVV
A. aegypti        MS.CPVGNHN .G........ .....DP.QG GQRLGSEA.G MLYGEYLMLD KVLSAQRMLS VESNKPVHDE HLFIVTHQAY ELWFKQIIFE LDSIRSLF.S TEHMEESRTL EILKRLNRIV
A. gambiae        MS.CPM..RS .GFV...... .....DSVQG GHHLGSEA.G MLYGEYLMLD KVLSAQRMLS VEGKKPVHDE HLFIVTHQAY ELWFKQIIFE LDSIRDLF.S TEHIEESRTL EILKRLNRIV
P. interpunctella MA.CPM...R .SMN...... .....DSSQD GSCLGNEA.G MLYGEYLMLD KLLAAQRMLS AESSRPAHDE HLFIVTHQAY ELWFKQIIYE VDSVRSLL.D VEGLDESHTM EILKRLNRIV
T. castaneum      MS.CPLR.PS .EAQ...... .....EGDQ. ...LSEEC.G MLYGEYLMLD KILEAQRLLS EQNNQPVHDE HLFIVTHQAY ELWFKQIIYE LDSIRNIF.. SDVLEESQTL EILKRLNRVV
C. elegans        MA.CPYLG.S GELTHRVTFM EGGEE..CQQ GVNKVEMGFG QTYSEYLQLD KILTAQRLKS EADGQRVDDE HLFIVIHQAH ELWFKQIIFD LDNVRKLLNN T.IVDETKTL KIVSGLDRMT
Consensus         M--CP----- ---------- ---------- ---------G --Y--YL-L- K-L-AQ---S --------DE HLFI--HQA- ELWFKQI--- -D--R----- -----E---- -------R--
                  121                                                                                                         240
M. musculus 1     VIFKLLVQQF SVLETMTALD FNDFREYLSP ASGFQSLQFR LLENKIGVLQ SLRVPYNRKH YRDNFGGDYN EL..LLKSEQ EQTLLQLVEA WLERTPGL.. ..EPNGFNFW GKFEKKILKG
M. musculus 2     VIFKLLVQQF SVLETMTALD FNDFREYLSP ASGFQSLQFR LLENKIGVLQ SLRVPYNRKH YRDNFGGDYN EL..LLKSEQ EQTLLQLVEA WLERTPGL.. ..EPNGFNFW GKFEKNILKG
R. norvegicus     VIFKLLVQQF SVLETMTALD FNDFREYLSP ASGFQSLQFR LLENKIGVLQ SLRVPYNRKH YRDNFEGDYN EL..LLKSEQ EQTLLQLVEA WLERTPGL.. ..EPHGFNFW GKFEKNILKG
H. sapiens        VILKLLVQQF SILETMTALD FNDFREYLSP ASGFQSLQFR LLENKIGVLQ NMRVPYNRRH YRDNFKGEEN EL..LLKSEQ EKTLLELVEA WLERTPGL.. ..EPHGFNFW GKLEKNITRG
D. ananassae      LILKLLVDQV PILETMTPLD FMDFRKYLAP ASGFQSLQFR LIENKLGVLT EQRVRYNQK. YSDVFGGDED ALTAIRSSEQ EPSLLELVQR WLERTPGL.. ..EESGFNFW EKFQQSV...
D. pallidosa      LILKLLVDQV PILETMTPLD FMDFRKYLAP ASGFQSLQFR LIENKLGVLT EQRVRYNQK. YSDVFGGDED ALTAIRSSEQ EPSLLELVQR WLERTPGL.. ..EESGFNFW EKFQQSV...
D. melanogaster   LILKLLVDQV PILETMTPLD FMDFRKYLAP ASGFQSLQFR LIENKLGVLT EQRVRYNQK. YSDVF.SDEE ARNSIRNSEK DPSLLELVQR WLERTPGL.. ..EESGFNFW AKFQESV...
D. simulans       LILKLLVDQV PILETMTPLD FMDFRKYLAP ASGFQSLQFR LIENKLGVLT EQRVRYNQK. YSDVF.SDEE ARNSIRNSEK DPSLLELVQR WLERTPGL.. ..EETGFNFW AKFQESV...
D. yakuba         LILKLLVDQV PILETMTPLD FMDFRKYLAP ASGFQSLQFR LIENKLGVLT EQRVRYNQK. YSDVF.SDEE ARNSIRNSEK DPSLLELVQR WLERTPGL.. ..EESGFNFW AKFQESV...
A. aegypti        MILKLLVDQV PILETMTPLD FMDFRDFLSP ASGFQSLQFR LLENKLGVKT EHRVKYNQK. YSEVFASDPC AIERLSITES EPSLADLVQK WLERTPGL.. ..ETNGFNFW GKFEESV...
A. gambiae        MILKLLVDQV PILETMTPLD FMDFRDYLSP ASGFQSLQFR LLENKLGVKS EHRVKYNQK. YTEVFASDPG AIERIGTTET EPSLADLVQK WLERTPGL.. ..EQDGFNFW GKFQESV...
P. interpunctella LILKLLVDQV MILETMTPLD FMDFRNYLRP ASGFQSLQFR LLENKLGLKQ ALRVKYNQN. YQTVFGDDPA AMDALHKSEE EPALLALIER WLERTPGL.. ..QTHGFNFW GKFQAAV...
T. castaneum      LILKVLVDQV MILETMTPLD FMDFRCYLRP ASGFQSLQFR LLENKLGVRQ ENRVKYNQN. YSKVFGNDEK ALEQIAKSEK EPSLTDLVQR WLERTPGL.. ..ELEGFNFW GKYQKAV...
C. elegans        KILSLLTEQI TLLDTMSPLD FVDFRKYLTP ASGFQSLQFR VLENKLGVRQ ERRIKYNAQH YKNVF.NDTD .LKTLNVTEE EKSLLTLIES WLERTPGLKS TSEDEG..FW IKYEKSVNKY
Consensus         -I---L--Q- --L-TM--LD F-DFR--L-P ASGFQSLQFR --ENK-G--- --R--YN--- Y---F----- --------E- ---L--L--- WLERTPGL-- -----G--FW -K--------
                  241                                                                                                         360
M. musculus 1     LEEEFLKIQA K..KDSEEKE EQM.AEFR.. ...KQKEVLL CLFDEKRHDY LLSKGERRLS YRALQGALMI YFYREEPRFQ VPFQLLTSLM DIDTLMTKWR YNHVCMVHRM LGTKA.GTGG
M. musculus 2     LEEEFLRIQA K..TDSEEKE EQM.AEFR.. ...KQKEVLL CLFDEKRHDY LLSKGERRLS YRALQGALMI YFYREEPRFQ VPFQLLTSLM DIDTLMTKWR YNHVCMVHRM LGTKA.GTGG
R. norvegicus     LEEEFLKIQA K..KDSEEKE EQM.AEFR.. ...KQKEVLL CLFDEKRHDY LLSKGERRLS YRALQGALMI YFYREEPRFQ VPFQLLTSLM DIDTLMTKWR YNHVCMVHRM LGSKA.
H. sapiens        LEEEFIRIQA K..EESEEKE EQV.AEFQ.. ...KQKEVLL SLFDEKRHEH LLSKGERRLS YRALQGALMI YFYREEPRFQ VPFQLLTSLM DIDSLMTKWR YNHVCMVHRM LGSKA.GTGG
D. ananassae      ..DKFLEAQV H..SAMEEPV ERA.KNYRLM DIEKRREVYR SIFDPAVHDA LVRRGDRRFS HRALQGAIMI TFYRDEPRFS QPHQLLTLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
D. pallidosa      ..DKFLEAQV H..SAMEEPV ERA.KNYRLM DIEKRREVYR SIFDPAVHDA LVRRGDRRFS HRALQGAIMI TFYRDEPRFS QPHQLLTLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
D. melanogaster   ..DRFLEAQV Q..SAMEEPV EKA.KNYRLM DIEKRREVYR SIFDPAVHDA LVRRGDRRFS HRALQGAIMI TFYRDEPRFS QPHQLLTLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
D. simulans       ..DRFLEAQV Q..SAMEEPV EKA.KNYRLM DIEKRREVYR SIFDPAVHDA LVRRGDRRFS HRALQGAIMI TFYRDEPRFS QPHQLLTLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
D. yakuba         ..DRFLAAQV Q..SAMEEPV EKA.KNYRLM DIEKRRRVYR SIFDPAVHDA LVRRGDRRFS HRALQRAIMI TFYRDEPRFS QPHQLLTLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
A. aegypti        ..EQLLADQE A..SAMEEEH ENV.KNYRLM DIEKRREVYK SIFDASVHDA LVARGDRRFT HRALQGAIMI TFYRDEPRFS QPHRLLMLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
A. gambiae        ..EQLLAEQE A..SAMSEEH ENV.REYRLM DIDKRREVYK SIFDAQVHDA LVARGERRFT HKALQGAIMI TFYRDEPRFS QPHQLLMLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
P. interpunctella ..NKTITDDI E..AAMTEPN ETV.RRHRLQ DAENRREIYR SIFDPAVHDA LRSRGERRLS HRALQGAIMI TIYRDEPRFS QPHQLLTLLM DIDSLITKWR YNHVIMVQRM IGSQQLGTGG
T. castaneum      ..EKLLTEQK E..LAEKEEA ETL.KRYKLN DLEKRREVYE SIFKVEVHEA LMSRGERRFS HKALQGAIMI TFYRDEPRFS QPHQILTLLM DIDSLITKWR YNHVLMVQRM IGSSQLGTGG
C. elegans        LAD..LAKQA ADPSNTEEIA KQLTAEYH.. ...KTADAFQ SILDPRQHEQ HIRNGNRLLS HDATKGAMMI YFYRDMPRFS QPYQILTFLM DIDSLFTKWR YNHVLLVQRM LGAKQ.GTGG
Consensus         ---------- -------E-- ---------- ---------- -------H-- ----G-R--- --A---A-MI --YR--PRF- -P---L--LM DID-L-TKWR YNHV--V-RM -G----GTGG
                  361                                                            436   % Ident.  % Similar.
M. musculus 1     SSGYHYLRST VSDRYKVFVD LFNLSTYLVP RHWVPKMNPI IHKFLYTAEY SDSSYFSSDE SD~~~~~~~~ ~~~~~~      55.5       62.0
M. musculus 2     SSGYHYLRST VSDRYKVFVD LFNLSTYLVP RHWVPKMNPI IHKFLYTAEY SDSSYFSSDE SD~~~~~~~~ ~~~~~~      55.5       62.0
R. norvegicus     SSGYYYLRST VSDRYKVFVD LFNLSSYLVP RHWIPKMNPI IHKFLYTAEY SDSSYFSSDE SD~~~~~~~~ ~~~~~~      55.8       62.3
H. sapiens        SSGYHYLRST VSDRYKVFVD LFNLSTYLIP RHWIPKMNPT IHKFLYTAEY CDSSYFSSDE SD~~~~~~~~ ~~~~~~      55.1       61.4
D. ananassae      SSGYQYLRST LSDRYKVFLD LFNLSTFLIP REAIPPLDES IRKKLINKNV ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~      73.0       77.3
D. pallidosa      SSGYQYLRST LSDRYKVFLD LFNLSTFLIP REAIPPLDES IRKKLINKSV ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~      73.0       77.3
D. melanogaster   SSGYQYLRST LSDRYKVFLD LFNLSTFLIP REAIPPLDET IRKKLINKSV ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~      72.6       77.0
D. simulans       SSGYQYLRST LSDRYKVFLD LFNLSTFLIP REAIPPLDET IRKKLINKSV ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~      72.6       77.0
D. yakuba         SSGYQYLRST LSDRYKVFLD LFNLSTFLIP REAIPALDET IRKKLINKSV ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~      72.8       77.3
A. aegypti        SSGYQYLRST LSDRYKVFID LFNLSTFLIP RGSIPPLTCE MQKAL.NLAW GSPVHQAKQI NYAAK~~~~~ ~~~~~~      72.8       76.8
A. gambiae        SSGYQYLRST LSDRYKVFLD LFNLSTFLIP RQSIPPLTNE MQKAL.NLAW GSPAHFARNG SLH~~~~~~~ ~~~~~~      73.3       78.2
P. interpunctella SSGYQYLRST LSDRYKVFLD LFNLSTFLLP RSLIPPLDDG MKKSL.NLTW GDNARENGKE THDGELNNCM LKLELA      100        100
T. castaneum      SSGYQYLRST LSDRYKVFVD LFNLSTFLIP RSYIPPLSTS MRSHLCNWGS ANSTNIVSNG NN~~~~~~~~ ~~~~~~      73.0       77.6
C. elegans        SSGYMYL.ST VSDRYKVFLD LFNLSTWLIP REYIPMLSPR MVKTL..SEH SNLSHSQSSE SD~~~~~~~~ ~~~~~~      56.6       60.4
Consensus         SSGY-YL-ST -SDRYKVF-D LFNLS--L-P R---P----- ----L----- ---------- -~~~~~~~~~ ~~~~~~       -          -
```

Figure 2a. Multiple sequence alignment and phylogenetic tree of *Plodia interpunctella* tryptophan oxygenase with other family members.
The deduced *P. interpunctella* tryptophan oxygenase protein sequence was aligned with tryptophan oxygenases from *Mus musculus* (1, P48776 and 2, NP_064295), *Rattus norvegicus* (P21643), *Homo sapiens* (P48775), *Drosophila ananassae* (AAC24239), *Drosophila pallidosa* (AAC24240), *Drosophila melanogaster* (A34780), *Drosophila simulans* (AAA81532), *Drosphila yakuba* (AAF68620), *Aedes aegypti* (AAL37360), *Anopheles gambiae* (AAC27659), *Tribolium castaneum* (AAL15464), and *Caenorhabditis elegans* (AAA6299). A consensus sequence generated for residues conserved in all 14 proteins is shown below the alignment. Percent identities and similarities are indicated following alignment.

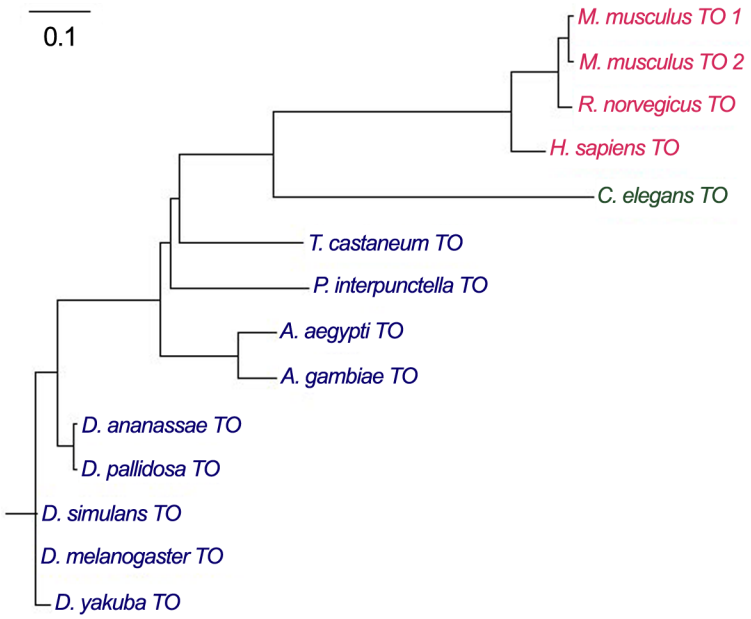

Figure 2b. Multiple sequence alignment and phylogenetic tree of *Plodia interpunctella* tryptophan oxygenase with other family members.
Phylogenetic distances were obtained from GrowTree data for the aligned sequences and a rooted tree was constructed using TreeView.

1978). Reasons for the poor hatch rate in the El Paso strain are unknown but it undoubtedly compounded the mortality produced by the invasive injection procedure. Despite the low hatch rate, all fully-developed embryos in the uninjected controls that did hatch had the wild-type phenotype.

Less than 5% (103/2488) of embryos injected with double-stranded RNA developed such that a knockdown or knockout phenotype could be determined. Of these 103 embryos, 3 displayed knockdown phenotypes and 2 displayed the knockout phenotype of complete loss of eye color pigmentation (Fig. 3B-3D). The remainder were wildtype. Figure 3D, which is an enlarged image of that found in Fig. 3C, illustrates an example of the knockout phenotype in which the individual stemmata appear as white or clear spots. The complete knockout phenotypes are compared to the dark stemmata in the wild-type phenotype (Fig. 3F). Interestingly, the 2 knockout embryos died shortly after hatching. Embryos injected with water alone also suffered high mortality, with only 7% hatching (44/600), again illustrating the difficulty of successfully injecting the small eggs. It should be noted, that in no case, during these and other studies, were any fully-developed embryos or newly hatched larvae observed that did not have the dark eyespots. As a result, we are confident that even though only 2 knockout phenotypes were observed among those treated, they were a direct result of the double-stranded RNA they received upon injection.

RT-PCR

Ambion's Cells-to-cDNA kit was used to extract RNA and generate semi-nested RT-PCR products with one of the embryos with the knockout phenotype. The tryptophan oxygenase transcript was not present in this larva (Fig. 4) indicating gene silencing, whereas RT-PCR products were clearly visible in three wild-type individuals. RT-RCR did not detect reduced levels of tryptophan oxygenase RNA from knockdown individuals. Although reduced levels of the tryptophan oxygenase transcript might have been present in these larvae, the corresponding PCR products were most likely saturated during the two rounds of semi-nested PCR. Control RPS7 RT-PCR products were detected in all larvae tested, indicating that the RNA was intact.

Discussion

Screening pigments in the insect eye function to prevent stray light from penetrating light-sensitive regions and thus preventing unwanted activation of photoreceptors (Linzen, 1974). The primary screening pigments in insects are the ommochromes and pteridines. They are responsible for the dark or red eye coloration, respectively. Tryptophan oxygenases have been identified and cloned from a number of non-lepidopteran insects and have long been known to play a key role in the ommochrome eye pigmentation (Summers et al., 1982). In addition to the synthesis of pigment, the primary function of tryptophan oxygenase may actually be to degrade excess tryptophan that occurs during times of high protein turnover (Sullivan and Kitos, 1976). The importance of tryptophan oxygenase for survival in *P. interpunctella*, as with many other insects (including those with known *vermilion* mutations), is unknown.

In this study, a cDNA encoding the first tryptophan oxygenase from a lepidopteran insect was obtained from the Indianmeal moth, *P. interpunctella*. *P. interpunctella* tryptophan oxygenase is an ortholog to the eye-color gene *vermilion* identified and characterized from *Drosophila*, *Tribolium*, and *Anopheles*. Pairwise sequence comparisons of the *P. interpunctella* tryptophan oxygenase with other sequences revealed very high conservation with >55% identity and >61% similarity with a nematode tryptophan oxygenase and mammalian tryptophan oxygenases.

To document gene silencing, we injected double-stranded RNA corresponding to tryptophan oxygenase into *P. interpunctella* embryos and screened embryos/larvae for eye pigmentation, an easily discernible loss-of-function phenotype. We observed several double-stranded RNA-injected individuals that lacked obvious eye coloration or had significantly reduced eye coloration. RT-PCR confirmed that tryptophan oxygenase RNA had in fact been completely silenced in an individual displaying a knockout phenotype. Thus, despite the low hatch rate in the El Paso strain of *P. interpunctella* and the invasive microinjection procedure, we demonstrated that RNAi can be utilized to decipher gene function in this moth species. Nevertheless, even though RNAi itself is not completely efficient, it is clear that the methodology for application of RNAi for this species needs to be optimized, including injection volumes, concentration of double-stranded RNA injected, age of embryo when injected, and in particular, selection of the moth strain used. Optimization of techniques will be especially required for functional analysis of genes not involved in synthesis of visible markers.

The complete knockout of eye pigmentation from our results suggests that synthesis of ommochromes via tryptophan oxygenase

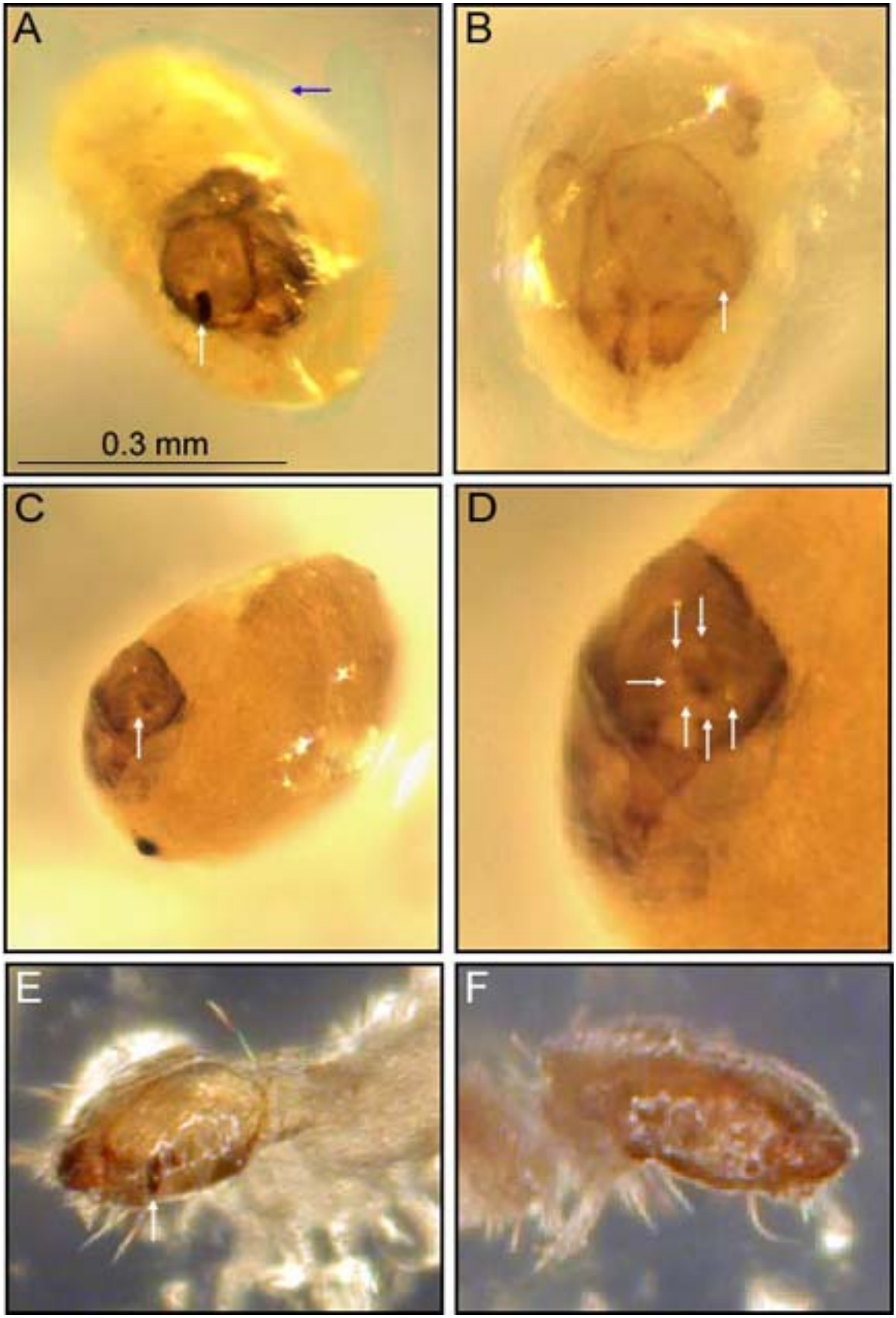

Figure 3. Effect of RNA interference on eyespot pigmentation in *Plodia interpunctella*.
(A) Dark, pigmented stemmata are visible (white arrow) through the egg chorion on the head capsule of a late-stage wild-type embryo. The blue arrow indicates the outer edge of the oval-shaped egg. The pigmented eyespots are reduced or are absent in the tryptophan oxygenase double-stranded RNA-injected embryos (B-D). An embryo with reduced eye pigmentation (knockdown phenotype) is shown in (B), whereas the embryo in (C) is completely lacking eyespot pigmentation (knockout). An enlarged image of the head capsule for the knockout embryo from (C) is shown (D), clearly illustrating the six white, unpigmented stemmata (as marked by white arrows). (E) and (F) illustrate newly hatched first-instar larvae with wild-type and knockout phenotypes, respectively. The blue asterisk shown in (C) denotes the double-stranded RNA injection site, and most likely represents a melanization wounding response.

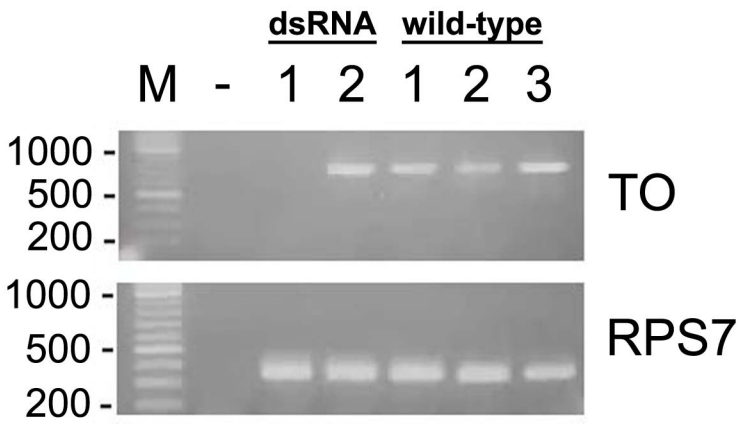

Figure 4. RT-PCR of double-stranded RNA-injected *Plodia interpunctella* Relative transcript levels of tryptophan oxygenase (top gel) and RPS7 (bottom gel) were determined through semi-nested RT-PCR. RT-PCR products from single *P. interpunctella* larva extracts that exhibited knockout or knockdown phenotypes following double-stranded RNA injection are shown in double-stranded RNA lanes 1 and 2, respectively. RT-PCR products obtained from three wild-type individuals (without injection) are shown in wild-type lanes 1-3. A PCR negative control is shown as "-". DNA marker is shown in Lane M with indicated sizes (in bp).

is the primary eye-color pigment pathway in *P. interpunctella*. Similarly, Beard et al. (1995) and Lorenzen et al. (2002) demonstrated that only the ommochromes contribute to eye coloration in mosquitoes and beetles, respectively.

In summary, we demonstrated that *P. interpunctella*, an economically important insect pest, utilizes tryptophan oxygenase to produce eye pigmentation in developing larvae. With both somatic and germline transformation in *P. interpunctella* currently being explored for the purpose of controlled production of double-stranded RNAs (P. Shirk, personal communication), the use of tryptophan oxygenase from this study and other eye-color genetic markers may provide an effective and convenient means to monitor the efficacy of the system.

Acknowledgements

We thank Marcé Lorenzen for thoughtful advice, technical assistance with RNAi, and critical review of this work. We also thank Maureen Gorman for critical reading of a previous version of this manuscript. This is contribution (04-125-J) from the Kansas Agricultural Experimental Station and a cooperative investigation between USDA-ARS and KSU.

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