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Tyrosine hydroxylase, a potential target for the RNAi-mediated management of diamondback moth (Lepidoptera: Plutellidae)

R. Ellango^{1,*}, R. Asokan^{1,*}, G. Sharath Chandra¹, N. K. Krishna Kumar², Riaz Mahmood³, and V. V. Ramamurthy⁴

Abstract

Cruciferous vegetables are severely damaged by infestation of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). Successful utilization of RNA interference (RNAi) in insect pest management programs depends on various factors, such as target gene, mode of double-stranded RNA (dsRNA) delivery, frequency of application, and development stage of the target insect. Among these factors, selection of the target gene is crucial to the success of RNAi-based programs. In the present investigation, an attempt was made to assess the potential of tyrosine hydroxylase (*TH*) as a target gene, which is a key regulator in the biosynthesis of 3,4-dihydroxyphenylalanine (DOPA). DOPA is a precursor for agents that function in neurotransmission, melanization, sclerotization of the cuticle, and immune responses in insects. Here, we provided to diamondback moth larvae 3 concentrations (1.04, 2.08, and 3.12 µg/cm²) of cognate dsRNA coated on discs of cabbage leaf, the natural diet for the larvae. We recorded the influence of the dietary dsRNA on *TH* transcript levels, larval growth, and larval survival rate. The dietary dsRNA led to reduced target gene transcript level and larval feeding level, and caused larval mortality in a concentration-dependent manner. These results demonstrate that the *TH* gene has potential as a target gene for RNAi-mediated management of *P. xylostella*.

Key Words: RNA interference; tyrosine hydroxylase; double-stranded RNA; *Plutella xylostella*; pest management

Resumen

Las verduras crucíferas están dañadas gravemente por la infestación de la polilla de la col, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). La utilización exitosa de ARNi en los programas de manejo de plagas de insectos depende de varios factores, tales como el gen blanco, el modo de entrega de dsARN, la frecuencia de aplicación, y el estadio de desarrollo del insecto blanco. Entre estos factores, la selección del gen blanco es crucial para el éxito de los programas basados en ARNi. En la presente investigación, se hizo un intento de evaluar el potencial de la tirosina hidroxilasa (TH) como un gen blanco en la biosíntesis de 3,4-dihidroxifenilalanina (DOPA). DOPA es un precursor para los agentes que funcionan en la neurotransmisión, melanización, esclerotización de la cutícula, y la respuesta inmuna en los insectos. Aquí, provemos 3 concentraciones (1,04, 2,08 y 3,12 mg/cm²) de ARN de doble cadena (dsARN) cognado para larvas de la polilla de la col recubiertas sobre discos de hojas de col, la dieta natural de las larvas. Se registró la influencia de dsARN dietético sobre el nivel de transcripción de *TH*, el crecimiento de las larvas y la tasa de sobrevivencia. El ARN dietético resultó en un nivel reducido de transcripción del gen blanco y en el nivel de alimentación de las larvas y causó mortalidad en las larvas de una manera dependiente de la concentración. Estos resultados demuestran el gen *TH* tiene potencial como un gen blanco para el manejo de *P. xylostella* por medio de ARNi.

Palabras Clave: interferencia de ARN; tirosina hidroxilasa; ARN de doble cadena; *Plutella xylostella*; control de plagas

Plutella xylostella (L.) (Lepidoptera: Plutellidae) is a highly migratory cosmopolitan pest of cruciferous vegetables and several ornamentals. Management of *P. xylostella* is daunting due to its nature and development of resistance to insecticides and to transgenic crops expressing *Bacillus thuringiensis* toxins (Sayyed et al. 2005; Grzywacz et

al. 2010; Zalucki et al. 2012). There is a need to find effective, alternative approaches for managing this pest (Ellango et al. 2014b)

RNA interference (RNAi) is an endogenous natural defense mechanism against invading viruses and pathogens. RNAi is achieved by delivery of long double-stranded RNA (dsRNA) or small-interfering RNA

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(siRNA) that mediates sequence-specific degradation of target mRNA (Mao & Zeng 2012). This tool has shown potential to combat insect pests and to characterize newly identified genes (Baum et al. 2007; Mao et al. 2011; Zha et al. 2011; Firmino et al. 2013; Ramaseshadri et al. 2013; Xiong et al. 2013). However, the efficacy of RNAi varies among species and target genes (Terenius et al. 2011; Asokan et al. 2014). Therefore, identification of a potential target gene for RNAi is important in order to control *P. xylostella*.

The biochemical 3,4-dihydroxyphenylalanine (DOPA) acts in several aspects of insect biology including circadian rhythms, neurotransmission, tanning of cuticle, immunity, sclerotization, melanization, and wound repair in insects (Birman et al. 1994; Gorman et al. 2007). DOPA biosynthesis is regulated by a key rate-limiting enzyme, tyrosine hydroxylase (*TH*). Therefore, we envisioned that silencing the gene encoding *TH* might impact individual *P. xylostella* larvae and result in reduced populations.

Achievement of RNAi through diet-mediated delivery (Turner et al. 2006) of dsRNA is promising, compared with delivery of dsRNA through a spray or transgenic plants. Here, we provided to *P. xylostella* larvae dsRNA coated on cabbage leaf discs as the larval natural diet.

Materials and Methods

INSECT CULTURE

Larvae of *P. xylostella* were collected from cabbage (*Brassica oleracea* L.; Brassicaceae) grown at Indian Institute of Horticulture Research, Bangalore, India. They were maintained on cabbage leaves at $28 \pm 1^\circ\text{C}$ and a 14:10 h L:D photoperiod in the laboratory. Eclosed moths were transferred to rearing cages (40 × 30 × 40 cm), where adults were provided 5% honey in water on cotton wads.

DOUBLE-STRANDED RNA (DsRNA) SYNTHESIS

The previously cloned *TH* gene of *P. xylostella* (Ellango et al. 2014a) was used for dsRNA synthesis. Off-target minimized region for the synthesis of dsRNA was selected using the online software “dsCheck” (Naito et al. 2005). The selected dsRNA region was analyzed for short perfect nucleotide matches with non-target organisms like pollinators, predators, and humans with NCBI-BLAST. The T7 promoter sequence was added at the 5' end of specific primers and used for synthesis of dsRNA template (Table 1). For non-target control, dsRNA derived from approximately 500 bp of dehydration responsive element binding protein 1A (*DREB1A*) of *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) was used. *DREB1A*-derived dsRNA had been used as a non-target control for RNAi experiments in the cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Asokan et al. 2013).

The template for dsRNA synthesis was obtained by polymerase chain reaction (PCR) performed in a reaction volume of 50 μL comprising 36.5 μL PCR-grade water, 5 μL 10X *Taq* buffer, 1.0 μL (10 mM) dNTP mix, 1.0 μL (10 mM) each of forward and reverse primers (Table 1), 2.5 μL 1:50 diluted plasmid clone as template, and 1.0 μL *Taq* polymerase (3 U/ μL) (Fermentas Life Sciences, USA). Amplification was performed with the following parameters: 94 $^\circ\text{C}$ for 3 min followed by 35 cycles of 94 $^\circ\text{C}$ for 30 s, 64 $^\circ\text{C}$ for 30 s, and 72 $^\circ\text{C}$ for 45 s and final extension at 72 $^\circ\text{C}$ for 10 min. PCR product was resolved in ethidium bromide pre-stained 1.2% agarose gel, and the desired band was excised and eluted with Nucleospin Extract II kit (Macherey-Nagel, Germany). We synthesized dsRNA by in vitro transcription of 2 μg of eluted PCR product with MEGAscript® kit (Ambion Life Technologies, USA), according to the manufacturer's instructions. Concentration and integrity of the dsRNA were assessed by NanoDrop™ 2000 (Thermo Scientific, USA) and agarose gel electrophoresis, respectively.

INSECT BIOASSAY WITH DsRNA

Fresh cabbage leaf discs (diameter 19.16 cm^2) were placed in a Petri dish containing 1% agar. Stock dsRNA was diluted with 0.1% diethylpyrocarbonate-treated water to yield concentrations of 20, 40, and 60 $\mu\text{g}/40 \mu\text{L}$. The final concentration of dsRNA on each leaf disc was approximately 1.04, 2.08, and 3.12 $\mu\text{g}/\text{cm}^2$ in 20, 40, and 60 μg treatments, respectively. These 3 dilutions were applied individually on the surface of the leaf disc with a clean soft paint brush and allowed to dry. We used the highest concentration, 3.12 $\mu\text{g}/\text{cm}^2$ (60 μg), of *DREB1A* dsRNA for non-target control. Five-day-old *P. xylostella* larvae were transferred to each leaf disc and maintained until the end of the experiment. There were 4 replicates, and each replicate contained 5 larvae (20 total) for each concentration of cognate dsRNA treatment and for non-target and negative (water) controls. Observations on the larval mortality rates were recorded on subsequent days up to 5 d (larval mortality was confirmed by prodding). Leaf area eaten by larvae was calculated with LI-3100C Area Meter (LI-COR Biosciences, USA) by following the manufacturer's protocol.

ASSESSMENT OF GENE SILENCING

Total RNA was extracted from single larvae of *P. xylostella* on the 3rd day of dsRNA treatment by using the Isolate II RNA mini kit (Bio-line Reagents Ltd., United Kingdom), according to the manufacturer's instructions. RNA integrity was analyzed by agarose gel (1.2%) electrophoresis, and concentration was determined with NanoDrop™ 2000. Complementary DNA (cDNA) was synthesized by reverse transcribing 2 μg total RNA in the Tetro cDNA Synthesis kit (Bio-line Reagents Ltd., United Kingdom) by following the manufacturer's protocol.

Table 1. List of primers used in this study.

Serial No.	Primer ID	GenBank accession no.	Primer sequence 5' to 3'	Tm (°C)	Amplicon length (bp)	Purpose
1	Px-dsRNA- <i>TH</i> For	JN410829	taatacactcactatagggCAGACCAAGCAAAGCGTT	72.3	500	dsRNA synthesis
2	Px-dsRNA- <i>TH</i> Rev		taatacactcactatagggCGGAGAGATCGGATGAGTTG	71.8		
3	<i>DREB1A</i> -ds For	DQ018385	taatacactcactatagggTTTCGTGAGACTCGTCACCCA	72.7	423	dsRNA synthesis
4	<i>DREB1A</i> -ds Rev		taatacactcactatagggAGCCAACAAACTCGGCATCTC	72.4		
5	Px-qPCR- <i>TH</i> For	JN410829	TGTTCCGCATCAAGAAGTC	59.2	110	Real-time PCR
6	Px-qPCR- <i>TH</i> Rev		TGCTTGGTCTGCTTGACTAC	61.3		
7	Px-qPCR-Actin For	JN410820	GACCGTGACAACCTCCATCA	60.7	124	
8	Px-qPCR-Actin Rev		GATCTCCTTCTGCATCTGTGTC	61.1		

Note: Lower case letters are the sequence of the T7 promoter.

The extent of gene silencing was assessed by quantitative PCR (qPCR) (Light Cycler 480II-Roche Diagnostics Pvt. Ltd. Switzerland). The qPCR assays were designed according to the Minimum Information for Publication of Quantitative PCR Experiments (MIQE) guidelines (Bustin et al. 2009). In the present study, β -actin was used as a reference gene for normalization of qPCR data (Teng et al. 2012; Mao & Zeng 2012). Initially, we assessed the expression stability of 5 reference genes under dsRNA-treated conditions (unpublished data) and results were similar to those of Teng et al. (2012), who found that β -actin was a suitable reference gene for *P. xylostella* gene expression studies. PCR amplification efficiency of reference and target genes were assessed in 4 dilutions, approximately 25, 2.5, 0.25, and 0.025 ng cDNA of control and of 20 and 60 μg *TH* dsRNA-treated samples. The qPCR mix comprised 10 μL SYBR® Green JumpStart™ Taq Ready-Mix™ (Sigma-Aldrich, USA), 0.5 μL each of forward and reverse primers (Table 1), and 3 μL (1:5) diluted cDNA template, and the final volume was brought to 20 μL with molecular biology-grade water. All qPCR assays were performed in triplicates for each sample. Amplification was performed by using the following parameters: 94 °C for 3 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Amplification specificity was analyzed by generating melting curves. The relative expression of *TH* was calculated with the $2^{-\Delta\Delta\text{CT}}$ method (Livak & Schmittgen 2001).

STATISTICAL ANALYSES

The extent of target gene silencing was statistically analyzed by *t*-tests with *C_q* (quantification cycle) values of 3 independent biological replicates. Larval mortality data were analyzed by ANOVA at $P = 0.05$. Leaf area eaten was statistically analyzed by paired *t*-tests. Pearson's correlation analysis was performed to evaluate the relationship between the concentration of dsRNA and percentage of silencing. Analyses were performed with GraphPadPrism v.5 (GraphPad Software, Inc., USA).

Results

EFFECT OF DsRNA ON TARGET GENE TRANSCRIPT LEVELS

The PCR amplification efficiency ranged from 95 to 97% for β -actin and *TH* indicating that the amplification efficiencies for the reference and target genes were similar (Table 2). We quantified the relative expression of *TH* on the 3rd day of dsRNA treatment. The highest silencing (83%) was observed in the 3.12 $\mu\text{g}/\text{cm}^2$ treatment followed by 55 and 13% in the 2.08 and 1.04 $\mu\text{g}/\text{cm}^2$ treatments, respectively (Fig. 1). A strong linear relationship ($R^2 = 0.9921$) between the dsRNA concentration and silencing was observed. Application of the non-target *DREB1A* dsRNA did not alter the expression of *TH* compared with the water control (Fig. 1).

EFFECT OF DsRNA ON LARVAL SURVIVAL RATE

We observed larval mortality onset on the 3rd day after treatment. The observed mortality was significantly higher compared with con-

Table 2. PCR amplification efficiency of reference (β -actin) and target genes in various treatments.

Gene	Treatment	Y-intercept	Slope	PCR efficiency
β -actin	Control	21.60	-3.509	96.40%
	20 μg dsRNA	21.51	-3.516	96.25%
	60 μg dsRNA	24.08	-3.44	97.65%
<i>TH</i>	Control	19.95	-3.567	95.35%
	20 μg dsRNA	21.51	-3.516	96.25%
	60 μg dsRNA	22.89	-3.529	96.00%

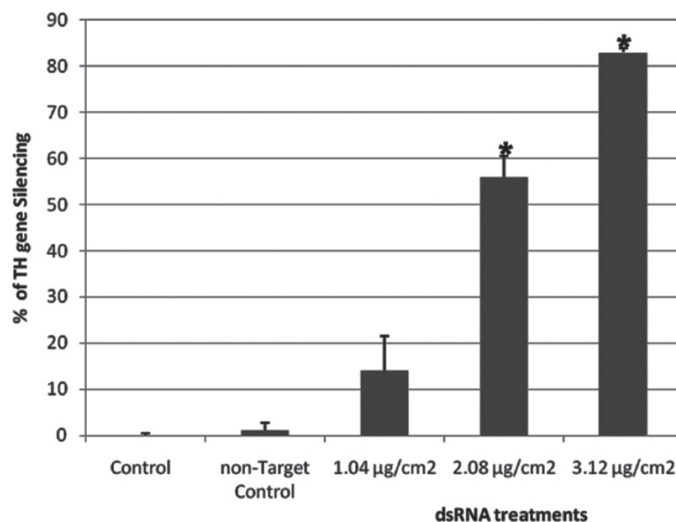


Fig. 1. Extent of *TH* silencing in the larvae treated with various concentrations of dsRNA. The expression levels of *TH* were analyzed on the 3rd day of feeding on various concentrations of cognate *TH* dsRNA and 3.12 $\mu\text{g}/\text{cm}^2$ non-target *DREB1A* (control) dsRNA. Error bars indicate the standard error of the mean of 3 replicates.

trols (water control and non-target *DREB1A* dsRNA). Larval mortality rates increased with increasing concentration of dsRNA. The highest mortality (90%) was recorded in the 3.12 $\mu\text{g}/\text{cm}^2$ treatment whereas mortality was 85 and 55% in the 2.08 and 1.04 $\mu\text{g}/\text{cm}^2$ treatments, respectively (Table 3). There was no significant difference between the non-target dsRNA treatment and the water control.

EFFECT OF DsRNA ON LARVAL FEEDING

Larvae in control (water control and *DREB1A*) treatments consumed a significantly greater (56 and 57%) leaf area compared with larvae in the *TH* dsRNA treatment (Fig. 2). The mean area of leaf eaten by larvae was inversely proportional to the concentrations of the target dsRNA.

Discussion

We report here that feeding dsRNA led to silencing of *TH* whereas non-target *DREB1A* dsRNA did not influence gene expression. Silencing of target gene expression via the feeding route appears to be a more attractive and simpler approach than other methods such as microinjection, droplet feeding, and soaking. Our results of the feeding approach suggest the possibility of developing dsRNA-expressing transgenic plants or products that can be applied in a manner similar to insecticides for field-level pest control (Bettencourt et al. 2002; Huvenne & Smaghe 2010; Mao et al. 2011; Terenius et al. 2011; Hunter et al.

Table 3. Percentage of mortality (\pm SE) of *Plutella xylostella* ($n = 20$) in treatments with various concentrations of dsRNA.

Treatment	Mortality (%)
1.04 $\mu\text{g}/\text{cm}^2$ of <i>TH</i> dsRNA	55 \pm 4.78b
2.08 $\mu\text{g}/\text{cm}^2$ of <i>TH</i> dsRNA	85 \pm 5.45c
3.12 $\mu\text{g}/\text{cm}^2$ of <i>TH</i> dsRNA	90 \pm 2.90c
Control (water)	15 \pm 4.79a
3.12 $\mu\text{g}/\text{cm}^2$ of <i>DREB1A</i> dsRNA	10 \pm 2.92a

Note: Same lower case letters indicate no significant differences in mortality caused by various concentrations of dsRNA analyzed using 1-way ANOVA at $P = 0.05$.

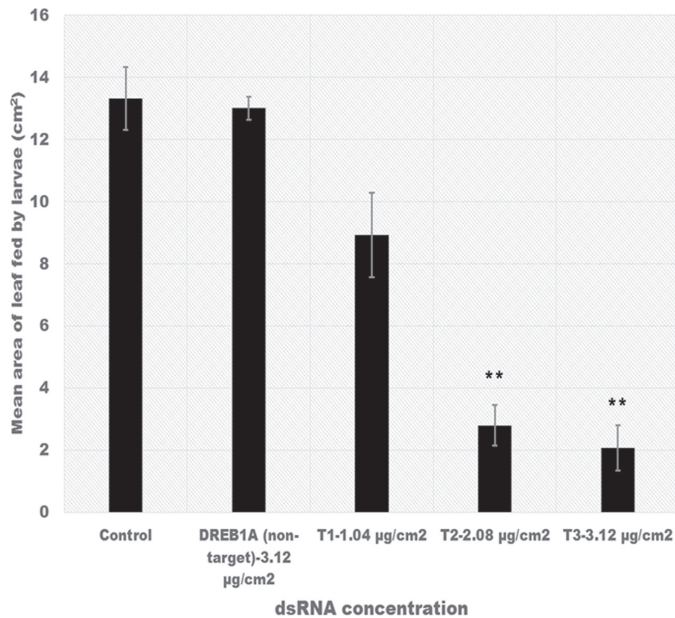


Fig. 2. Larval feeding rate in treatments with various concentrations of dsRNA. Here, T1, T2, and T3 are 3 concentrations of *TH* dsRNA. The initial leaf disc area was 19.16 cm².

2012). Leaf-coated dsRNA reduced *TH* transcript levels and eventually led to larval mortality. Diet-delivered dsRNA caused systemic silencing of non-midgut genes in *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) (Turner et al. 2006; Tomoyasu et al. 2008), and our results show that the non-midgut gene *TH* was silenced with diet-delivered dsRNA. Together, these results demonstrate that diet-delivered dsRNA can be used to silence non-midgut genes that are suitable targets for RNAi-mediated pest control.

In our experiment, the target gene silencing was strongly correlated with dsRNA concentrations. Similarly, a strong positive correlation was observed between the concentration of dsRNA and silencing of a target gene encoding a digestive protease, chymotrypsin (Asokan et al. 2013). On the other hand, the same study reported negative correlation between dsRNA and expression of a gene encoding juvenile hormone acid methyl transferase (*jhamt*) in *H. armigera* (Asokan et al. 2013). These findings indicate that the target gene response to dsRNA is highly variable among genes and species. Our results show that higher concentrations of dsRNA were required to achieve optimal silencing. Higher dsRNA concentrations required to silence any given gene may impact the success of pest management programs at the field level, depending on the ability of transgenic crops to produce the silencing constructs.

In this study, larval feeding was reduced by *TH* dsRNA treatments compared with controls. In this regard, the sensory organs such as dorsal and ventral organs, terminal organs, and 3 pharyngeal organs are connected by dopaminergic neurons that are involved in triggering aversive (initial recognition and distinguishing of food) and appetitive memory. In *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), the blocking of dopaminergic neuron output resulted in the loss of appetitive memory that consequently led to reduced feeding (Selcho et al. 2009). Reduced *TH* levels eventually reduced the DOPA levels, from which we infer a strong correlation between the levels of *TH* and DOPA (Jaber et al. 1999). Thus, silencing of *TH* might have reduced the DOPA levels and consequently impaired the aversive and appetitive memory that resulted in lesser feeding by the larvae.

We observed larval mortality, which may be directly due to reduced *TH* transcript levels. Given the wide range of DOPA-mediated physiological functions in insects, we can only speculate to which of these many functions the proximal causes of the mortality were related. The *TH* ultimately influences many areas of insect physiology; it has potential for RNAi-mediated management of *P. xylostella*.

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