A transferrin gene associated with development and 2-tridecanone tolerance in Helicoverpa armigera

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Abstract

The full-length cDNA (2320 bp) encoding a putative iron-binding transferrin protein from Helicoverpa armigera was cloned and named HaTrf. The putative HaTrf sequence included 670 amino acids with a molecular mass of approximately 76 kDa. Quantitative PCR results demonstrated that the transcriptional level of HaTrf was significantly higher in the sixth instar and pupa stages as compared with other developmental stages. HaTrf transcripts were more abundant in fat bodies and in the epidermis than in malpighian tubules. Compared with the control, the expression of HaTrf increased dramatically 24 h after treatment with 2-tridecanone. Apparent growth inhibition with a dramatic body weight decrease was observed in larvae fed with HaTrf double-stranded RNA (dsRNA), as compared with those fed with green fluorescent protein dsRNA. RNA interference of HaTrf also significantly increased the susceptibility of larvae to 2-tridecanone. These results indicate the possible involvement of HaTrf in tolerance to plant secondary chemicals.

Keywords: transferrin, Helicoverpa armigera, RNA interference, 2-tridecanone.

Introduction

Insects employ various strategies to increase their performance and fitness on host plants. Plants have also developed efficient strategies to defend against specific insects (Schuler, 2011). Herbivorous insects obtain nutrition from their host plants, and plants have evolved to reduce insect damage (Howe & Jander, 2008). One way that plants reduce the damage from herbivorous insects is through natural defence compounds (Howe & Jander, 2008).

Transferrins are multifunctional proteins with a prominent role in iron transport in serum. Insect transferrin was initially isolated from Manduca sexta (Bartfeld & Law, 1990). Transferrins are known to be regulated by juvenile hormone in insects (Ampasala et al., 2004; Nascimento et al., 2004). Insect transferrins have been proposed to play important roles in iron transport and to function as antibiotic agents, antioxidants and vitellogenic proteins (Geiser & Winzerling, 2012). However, the potential roles of transferrins in tolerance mechanisms to plant secondary chemicals remain unknown in insects.

Iron is essential for the survival of almost all organisms. It is critical for respiration, energy generation, oxygen transport, gene regulation, DNA biosynthesis and immunity (Dunkov & Georgieva, 2006; Ong et al., 2006; Munoz et al., 2009). Additionally, iron is involved in cuticle formation, tanning, melanization and wound healing in insects (Locke & Nichol, 1992). Iron is an important prosthetic biocatalyst or electron carrier, as it can readily change oxidation and coordination states. Despite these critical functional roles, excessive or misallocated iron is highly destructive as it can generate oxygen radicals (Hower et al., 2009; Kell, 2009).

Transferrins can also contribute to adaptation of insects to various stresses (Kim et al., 2008a; Kim et al., 2008b; Lee et al., 2006; Geiser & Winzerling, 2012). For example, under normal physiological conditions, H2O2 is commonly produced in mitochondria and peroxisomes as a by-product of oxidase-catalysed reactions. Transferrin may play a role in the defence against the formation of these hydroxyl radicals by sequestering iron, and thereby reducing damage to DNA, membrane lipids and proteins (Kim et al., 2008a). Reduction of transferrin by RNA interference (RNAi) caused an increase in iron and H2O2 levels.
in haemolymph and resulted in the induction of apoptotic cell death in fat bodies in Protaetia brevitarsis (Kim et al., 2008b). Under heat stress (37 °C), Apriona germari transferrin can inhibit apoptotic cell death in the fat bodies of larvae that typically live in more temperate climates (25 °C; Lee et al., 2006). In Apr. germari, transferrin mRNA and protein accumulation levels are up-regulated in response to cold shock (4 °C), sterile wounding and paraquat (10 mM) exposure (Kim et al., 2008b).

Transferrin is also known to function as an immune protein in insects; transferrin expression levels are up-regulated following exposure to bacteria, fungi, baculoviruses, pathogens and parasites in insects (Thompson et al., 2003; Ampasala et al., 2004; Valles & Pereira, 2005; Bergin et al., 2006; Lee et al., 2006; Guz et al., 2007; Paily et al., 2007; Wang et al., 2007, 2009; Magalhaes et al., 2008; Mowlds et al., 2008; Yun et al., 2009; Zhou et al., 2009). An enhanced Plutella xylostella larval susceptibility to infection with Bacillus thuringiensis was observed when transferrin was silenced (Kim & Kim, 2010). In Pr. brevitarsis, transferrin has been shown to function as an antioxidant under stress conditions (Kim et al., 2008a).

Interestingly, transferrin, as one of the identified proteins in Culex pipiens pallingis, is expressed more abundantly in a cypermethrin-resistant strain. In vitro experiments have indicated that transferrin-transfected cells also show enhanced cypermethrin resistance compared with null-transfected or plasmid vector-transfected cells, as determined by methyl labelled thymidine (3H-TdR) incorporation. These results indicate that the expression of transferrin could be induced by the xenobiotic cypermethrin in the resistant strains, and that transferrins might confer insecticide resistance in C. pipiens pallingis (Tan et al., 2012; Vezilier et al., 2013).

Some studies have shown that the expression of xenobiotic-response P450 genes/enzymes can be induced by gossypol, a secondary metabolite from cotton, and RNAI repression of the P450 gene results in a significant decrease in gossypol tolerance ability in H. armigera larvae (e.g. Mao et al., 2007). 2-tridecanone is a xenobiotic plant secondary chemical from the host plant Lycopersicon pennelli. In the present study, digital gene expression (DGE) libraries were used to screen sequences of genes that were differentially expressed between the control and the group treated by 2-tridecanone (data not shown). One of the identified segments that was significantly induced by 2-tridecanone treatment had high sequence homology with the transferrin gene. However transferrin's role in tolerance mechanisms to plant secondary chemicals is still unknown. In this study, a full-length cDNA encoding H. armigera transferrin (HaTrf) was isolated. The expression profiles of HaTrf in various tissues and developmental stages of H. armigera were analysed, and the effects of 2-tridecanone exposure on larvae were studied. Further, we investigated the effects of double-stranded RNA (dsRNA)-mediated depletion of HaTrf on the development, survival rates and tolerance to 2-tridecanone of larvae.

Results

Characterization of HaTrf cDNA

The full-length HaTrf cDNA consists of 2320 bp that spans an open reading frame (ORF) of 2052 nucleotides, a 31-bp 5′-untranslated region (5′UTR) and a 237-bp 3′UTR. The ORF encodes a 670 amino acid polypeptide with a molecular weight of approximately 76 kDa. Positions of potential iron-binding residues are indicated by rectangles, the conserved cysteine residues by '*' and conserved lysine residues by 'L' in Fig. 1, and '<' and '>' show the N-terminal lobe and C-terminal lobe, respectively. The 20 amino acid signal peptide sequence (MVSFIGIYLLVTLACVQA) is underlined in Fig. 1. The cDNA sequence has been deposited in GenBank under the accession number KJ641611. The predicted H. armigera transferrin protein has many characteristics common to the transferrin family, such as a typical transferrin domain (N-terminal lobe and C-terminal lobe, iron-binding residues, lysine residues). Alignment of the deduced amino acid sequences of HaTrf showed high identity with other transferrin family members from the Hymenoptera, Lepidoptera, Diptera and Coleoptera subclusters (Fig. 2). The molecular phylogeny of insect transferrins, which was examined using amino acid sequences from different insect species, is clearly grouped into different subclusters (Fig. 2). Phylogenetic analysis showed that HaTrf was clustered together with the Lepidoptera transferrins (Fig. 2).

The expression profiles of HaTrf in various tissues and developmental stages

The qPCR results showed that HaTrf was expressed in egg, larva, pupa and adult samples. The transcription level

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Figure 1. Multiple alignment of transferrin amino acid sequences from various insects: Spodoptera litura transferrin (DQ470073.1), Bombyx mori transferrin (NM 001043549.1), Manduca sexta transferrin (M62802.1), Chilo suppressalis transferrin (AB158473.1), Plutella xylostella transferrin (AB262638.1), Choristoneura fumiferana transferrin (AY563106.1), Anagasta kuehniella transferrin (HM026347.1), Papilio xuthus transferrin 1 (AK402830.1). Positions of potential iron-binding residues are indicated by rectangles, conserved cysteine residues by '*' and conserved lysine residues by 'L'. '<' and '>' show the N-terminal lobe and C-terminal lobe, respectively. The 20 amino acid signal peptide sequence (MVSFIGIYLLVTLACVQA) is underlined. The similar and identical residues are shaded in gray and black.
A transferrin gene in *Helicoverpa armigera*

| Consensus | helicoverpa_armi | spodoptera_litur | bombyx_mori_tr | manduca_sesena_tr | chilo_suppressal | plutella_yxylote | chorisoneura_fu | anapasta_kuehni | papilio_xuthus |
|-----------|-----------------|-----------------|----------------|------------------|-----------------|-----------------|-----------------|----------------|--------------|
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of HaTrf was stable from the first to the fourth instar stages of larvae. However, HaTrf expression increased dramatically from the sixth instar to the pupa, and then decreased sharply in adults. The highest and lowest transcription levels for HaTrf were observed at the pupa stage and the egg stage, respectively (Fig. 3B).

As shown in Fig. 3A, HaTrf transcripts were much more abundant in the fat body and epidermis samples.
as compared with the haemolymph, head, gut and malpighian tubule samples.

**The induction of HaTrf transcription by 2-tridecanone**

Compared with the untreated group at each time point (0, 12, 24, 36 and 48 h), the expression of HaTrf increased dramatically 24 h after treatment with 2-tridecanone, and then decreased significantly 48 h after treatment, reaching the same level as the control (Fig. 3C).

**Effects of HaTrf RNAi on larval development, survival rates and tolerance to 2-tridecanone**

The HaTrf dsRNA-treated larvae showed a significant reduction of HaTrf expression compared with the larvae treated with only green fluorescent protein (GFP) dsRNA (Fig. 4). Compared with the control, 95% and 43% of HaTrf expression was suppressed at 24 and 36 h after feeding larvae an artificial diet containing 15 μg/g (w : w) HaTrf dsRNA, respectively. However, no significant inhibition of transcription was observed at 12 or 48 h after feeding. The transcription levels of HaTrf were almost completely suppressed (inhibition ratio 99%) at 12, 24 and 36 h after feeding with HaTrf dsRNA (35 μg/g), and reduced to 45% of the control levels at 48 h after feeding (Fig. 4).

The 5-carboxyfluorescein (5′FAM)-labelled dsRNA was used to observe the ingestion efficiency of the larvae feeding on the artificial diet. The fluorescence signal grew stronger during the period from 6 to 48 h after feeding the fluorescent-labelled dsRNA. At 48 h after feeding the labelled dsRNA, the strongest fluorescent signal was observed in nearly the whole body, except in the malpighian tubules (Fig. 5).

The efficacy of RNAi on larval growth was evaluated in second instar larvae by feeding an artificial diet with...
35 μg/g (w : w) HaTrf dsRNA for 1, 3 and 5 days. An apparent growth inhibition phenotype with a dramatic body weight decrease was observed in larvae after feeding for 5 days; there were obvious differences in larval development between HaTrf dsRNA and GFP dsRNA (Fig. 6A, B) individuals.

The effects of dsRNA-mediated depletion of HaTrf on larval tolerance to 2-tridecanone were investigated in second instar larvae by feeding an artificial diet containing both 35 μg/g (w : w) of HaTrf dsRNA and 0.1 mg/g of 2-tridecanone (w : w) (Fig. 7). The results showed that the survival rate dramatically decreased in larvae treated with HaTrf dsRNA. The survival rate was 64% for the treatment with HaTrf dsRNA and 83% for the GFP dsRNA control, through continuous feeding for 5 days. The survival rate was significantly lower (51%) in the group treated with the mixture of HaTrf dsRNA (35 μg/g) and 2-tridecanone (0.1 mg/g) compared with the other treatments (64% for mixture treatment of GFP dsRNA and 2-tridecanone, 79% for HaTrf dsRNA treatment and 83% for GFP dsRNA treatment). These results suggest that dsRNA-mediated depletion of HaTrf can significantly increase larval susceptibility to 2-tridecanone.
Figure 5. Systemic RNA interference effect in *Helicoverpa armigera* observed using fluorescent-labelled double-stranded RNA (dsRNA). Fifteen first-instar larvae were fed on a diet containing fluorescent-labelled dsRNA at 35 μg/g (w : w), and samples were collected at 6, 12, 24 and 48 h after feeding for fluorescence signal scanning. The left panels were observed using a stereo microscope (SM) and the right panels were observed at 492 nm (FAM) on an Olympus SZX8.

Figure 6. Effect of double-stranded RNA (dsRNA)-mediated depletion of *Helicoverpa armigera* transferrin (*HaTrf*) transcripts on larval development. (A) Photograph of the *HaTrf* dsRNA-mediated growth inhibition phenotype, as compared with the green fluorescent protein (GFP) dsRNA control. (B) *HaTrf* dsRNA-mediated body weight changes at 0 days (0 d), 1 day (1 d), 3 days (3 d) and 5 days (5 d) compared with the GFP dsRNA control, bars sharing the same letter for each group are not significantly different at $P > 0.05$.

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Discussion

With a few notable exceptions, each iron atom in insect transferrin proteins is coordinated to four conserved amino acid residues: an aspartic acid, two tyrosines and a histidine. Anion binding is associated with an arginine and a threonine in close proximity (Gomme et al., 2005; Takatsy et al., 2006). These six residues in each lobe were examined for their evolutionary conservation in the homologous N-lobe and C-lobe of 82 complete transferrin sequences from 61 different species (Gomme et al., 2005; Takatsy et al., 2006). Of the residues in the N-lobe, the ligand shows the most variability in sequence. Furthermore, of note, four of the 12 insect transferrins have glutamic acid substituted for aspartic acid in the N-lobe (as seen in the bacterial ferric-binding proteins; Gomme et al., 2005; Takatsy et al., 2006). In addition, there is a widespread substitution of lysine for the anion-binding arginine in the N-lobe in many organisms, including all fish, sea squirts, and many of the atypical transferrin family members (Gomme et al., 2005; Takatsy et al., 2006). The H. armiger transferrin protein sequence shares high homogeneity with other insects, possesses these structures and can be identified by these conserved residues (Fig. 1). These results strongly suggest that this protein is the transferrin of H. armigera.

HaTrf transcripts were found in various stages of development, from egg to adult; its expression level gradually increased from egg to pupa, then decreased dramatically from pupa to adult. The highest transcriptional level was observed at the pupa stage and the lowest level occurred at the egg stage (Fig. 3B). Nascimento et al. (2004) reported that the expression of transferrin may be regulated by juvenile hormone (JH) and 20-hydroxyecdysone (20E). mRNA expression of transferrin decreased in Apis mellifera after treatment with pharmacological levels of 20E. In Choristoneura fumiferana, transferrin was expressed at high levels during the processes of ecysis (Ampasala et al., 2004). It now appears that there is a possibility that transferrins function to deliver iron to eggs for storage in the form of ferritin. This may be regulated by JH. As few or no transcripts for transferrin were observed in eggs or early instar larvae stage, it is likely that the protein in eggs is endowed from the females. This phenomenon was also reported for Ephestia kuehniella (Guz et al., 2012). The highest transcriptional level for transferrin was observed in pupa (Fig. 3B); this may be related to the function to deliver iron or the titre of 20E and JH during this stage and may also reflect the ability of pupa to tolerate xenobiotic chemicals.

HaTrf transcripts were abundant in fat bodies and in the epidermis, whereas the transcriptional level of HaTrf was quite low in the haemolymph, head and midgut, and especially low in malpighian tubules (Fig. 3A). Although transferrin transcripts are known to be expressed in various tissues in many insects, fat bodies are thought to be the most likely source of haemolymph transferrin. Many studies have found that fat bodies were the primary sites of transferrin expression. This was the case in Aedes aegypti (Harizanova et al., 2005), Drosophila melanogaster (Yoshiga et al., 1999; Levy et al., 2004a b; Dunkov & Georgieva, 2006), Bombus hortorum (Wang et al., 2009; Kim et al., 2006), Apr. germari (Lee et al., 2006), and Choristoneura humijerana (Ampasala et al., 2004). In Ae. aegypti, transferrin transcripts were also expressed in midgut and in ovaries, but the highest expression levels were found in fat bodies (Zhou et al., 2009). Transferrin transcripts were not detectable in the midgut of D. melanogaster (Harizanova et al., 2005), Bombus hortorum (Kim et al., 2006; Wang et al., 2009), C. bumijerana (Ampasala et al., 2004) or Apr. germari (Lee et al., 2006). Transferrin mRNA expression was not detected in the ovaries (Harizanova et al., 2005) or in the male reproductive accessory glands of D. melanogaster (Sirot et al., 2008). Although not expressed in the epidermis or muscle of Bombus hortorum (Kim et al., 2006; Wang et al., 2009), transferrin mRNA was detected in the epidermis of Apr. germari (Lee et al., 2006) and H. armigera.

Transferrin has been proposed to play several important physiological roles in insects (Geiser & Winzerling, 2012). RNAi-mediated reduction of transferrin transcript accumulation results in rapid induction of apoptotic cell death in fat bodies during exposure to stress (Lee et al., 2006; Kim et al., 2008b). 2-tridecanone, as a plant secondary chemical, induced the expression of HaTrf in sixth-instar larvae (Fig. 3C). There have been no reports of plant secondary...
The HaTrf transcripts, which were mainly expressed in fat bodies and the epidermis (Fig. 3A), might act in defence from 2-tridecanone damage in a direct or indirect way. Both the midgut and fat bodies are typically supposed to function as detoxification organs in insects. Many studies have shown that fat bodies contain a lot of detoxification enzymes that can play important roles in the detoxification of plant toxins (Liu et al., 2006; Schuler, 2011). It is well known that transferrin is a multifunctional protein. However, there have been no studies showing that transferrin can alter insect tolerance to plant secondary chemicals prior to this report. The potential role of HaTrf in defensive or other functions in H. armigera need further study. Our results show that RNAi of HaTrf affects the growth of insects; thus, HaTrf is a necessary gene for the growth and development of insects.

The expression level of HaTrf dropped at 24 h but then increased at 48 h when feeding with HaTrf dsRNA (15 μg/g, w : w) (Fig. 4). This may be because the lepidopteran gut is a hostile environment for nucleic acids as it has an alkaline pH (Terra et al., 1996) and presumably contains numerous RNases. Different insect midgut environments may require different concentrations of dsRNA to trigger gene silencing. Initiation of RNAi by dsRNA feeding may therefore require some level of optimization for various concentrations of dsRNA. Variation in the extent of silencing amongst different concentrations of dsRNA has also been observed when dsRNA is injected (Tomoyasu & Denell, 2004).

The transcript levels of HaTrf were suppressed by HaTrf dsRNA gradually, not instantaneously, after feeding. This may be because of the time required for the dsRNA to enter midgut cells and for dsRNA processing to mediate HaTrf transcript accumulation through the RNAi pathway. A fluorescent-labelled dsRNA control was used to observe the RNAi efficiency in larvae by feeding a diet containing dsRNA. Strong fluorescent signals could be observed at 12 h postfeeding. These stronger fluorescent signals spread to the midgut at 24 h postfeeding. The strongest fluorescent signals occurred in nearly the whole body, excluding the malpighian tubules, at 48 h postfeeding (Fig. 5).

We have cloned the first transferrin gene known from H. armigera. Based on the characteristics of the gene, it is a member of the transferrin family. The data from the present study suggest that transferrin plays an important role in larval development and in tolerance to 2-tridecanone in H. armigera. These results indicate the possible involvement of HaTrf in tolerance to plant secondary chemicals. The RNAi methods used here provide a base for further studies on transferrin gene function associated with the interaction between insects and plant secondary chemicals. Such studies will improve our understanding of the molecular basis of transferrin-mediated allelochemical resistance in H. armigera.

Experimental procedures

Insect culture

H. armigera population was collected from Handan in Hebei Province, China, in 1998, and reared on an artificial diet in a conditioned room maintained at 26 ± 1 °C, 70–80% relative humidity, with a photoperiod of 16:8 h (light : dark). The artificial diet consisted of the following: corn flour 300 g, soybean powder 100 g, yeast extract powder 100 g, citric acid 2.5 g, vitamin C 10 g, sorbic acid 1.5 g, vitamin B 1.5 g, erythromycin 0.05 g, propionic acid 5 mL, vitamin E 0.15 g, water 2.5 L. Adults were kept under the same conditions and supplied with a 10% sugar solution.

Cloning and sequence analysis of HaTrf

Total RNA was isolated from sixth-instar larvae of cotton bollworms using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. An additional DNaseI digestion was performed using RNase-Free DNaseI (Takara, Dalian, China). First-strand cDNA synthesis was carried out with a Reverse Transcription System (Takara) according to the manufacturer’s instructions. For amplification of a partial transferrin sequence, primers were designed according to the transferrin sequence from a DGE library of H. armigera. All primers are listed in Table 1. The 5′- and 3′-ends of the cDNA molecules were amplified by rapid amplification of cDNA ends (RACE) PCR using the GeneRACE Kit (Invitrogen). All PCR products were cloned into the pMD-18-SimpleT vector system (Takara) and sequenced. BLAST searches for homologous sequences and the prediction of conserved regions were performed by using on National Center for Biotechnology Information and EXPASY websites (http://www.expasy.org). CLUSTALW software (http://www.ebi.ac.uk/clustalw/) was used to perform multiple sequence alignments prior to phylogenetic analysis. The MEGA 5.0 program was used to construct the consensus phylogenetic tree using the neighbour-joining method (algorithm: Poisson correction, bootstrap values: 1000 replicates; Tamura et al., 2011).

qPCR analysis of HaTrf expression

The HaTrf expression profiles were determined by qPCR at various developmental stages (egg, larva, pupa, mated female and mated male), in various tissues (fat body, midgut, haemolymph, head, malpighian tubule and epidermis) and at different time points following 2-tridecanone treatment of newly moulted sixth-instar larvae. A bioassay method of mixing 2-tridecanone into the artificial diet was used to determine
the toxicity of 2-tridecanone to larvae. Larvae were exposed to the artificial diet containing 2-tridecanone (10 mg/g, w : w) and mortalities were then recorded at 12, 24 and 48 h after exposure. Approximately 500 mg of specimens was homogenized in liquid nitrogen with a mortar and pestle for the total RNA isolation. Three biological replicates were performed for all assays.

First-strand cDNA synthesis was performed with 1 μg total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Takara). qPCR was conducted on an Applied Biosystems 7500 qPCR System (Applied Biosystems, Foster City, USA) following the manufacturer’s recommendations using a Real Master Mix SYBR Green PCR kit (Invitrogen). The thermal cycling protocol was as follows: initial denaturation was 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s. The fluorescence signal was measured at the end of each extension step at 60 °C. The melting curves were measured by taking continuous fluorescence readings while increasing the temperature from 58 to 95 °C at 0.5 °C increments for 10 s. A standard curve was generated for each set of primers. The primers’ (RTHaTrf F and RTHaTrf R, RTEF-F and RTEF-R) efficiency was 98%. HaTrf gene expression analysis was performed using the primer pair RTHaTrf F and RTHaTrf R (Table 1). Each sample was analysed in triplicate and normalized to the internal control, primer pair RTHaTrf F and RTHaTrf R (Table 1). The primers’ curve was generated for each set of primers. The primers’ efficiency was determined by qPCR by using the primers qHaTrf-F and qHaTrf-R (Table 1).

Effects of HaTrf RNAi on larval development, survival rates and tolerance to 2-tridecanone

Based on the HaTrf sequences and predicted possible interference sites obtained using the online prediction software (http://www.dkfz.de/signaling/e-rnai3/), we designed specific primers using DNAMAN 6.0 software (Lynnon Corp., Quebec, Canada; http://www.lynnon.com). A 667-bp fragment of HaTrf (position 204–870) was amplified and cloned into pMD-18simple-T (Takara), using the primer pair dsRNAi-Tf1 and dsRNAi-Tf2 (Table 1) containing the additional T7 promoter sequences. Purified plasmids served as templates for RNA syntheses using a MEGAscript T7 transcription kit (Ambion, Austin, TX, USA). GFP dsRNA, which was used as the control, was synthesized using the same procedures with the primers dsGFP-F and dsGFP-R (Table 1). dsRNA from GFP and HaTrf were derived by using the MEGAscript T7 transcription kit with an extended transcription time of 5 h at 37 °C. The resulting dsRNA was digested by DNase I and RNase to remove DNA and any single-stranded RNA, and finally dissolved in diethy pyrocarbonate (DEPC) water. A 5’ FAM-labelled dsRNA control (Genema, Shanghai, China) was used to observe the RNAi efficiency of larvae during feeding.

Second-instar larvae, after being starved for 12 h, were exposed to the artificial diet containing HaTrf dsRNA (15 or 35 μg/g, w : w) for 12, 24 and 36 h; GFP dsRNA was used as a control and DEPC water was used as a negative control. Thirty larvae were used in each treatment and three replications were performed. The dsRNA-mediated depletion of HaTrf transcripts was determined by qPCR by using the primers qHaTrf-F and qHaTrf-R (Table 1). The following experiments were carried out in order to elucidate the effect of HaTrf silencing on the development of larvae. Artificial diet coated with dsRNA (35 μg/g, w : w) was fed to second-instar larvae for 1, 3 or 5 days. During the treatment period, the dsRNA-containing artificial diet was replaced every 24 h. Each treatment contained 25 larvae and had four replicates. After the treatments, the larvae in each treatment were collected and weighed.

In order to investigate the effect of RNAi HaTrf on the survival rate of larvae and their tolerance to 2-tridecanone, 25 second-instar larvae were fed on artificial food that contained 2-tridecanone (0.1 mg/g, w : w) and dsRNA (HaTrf dsRNA or GFP dsRNA, 35 μg/g, w : w), with four replicates per treatment group. Larvae fed on artificial food mixed with dsRNA without 2-tridecanone were used as a control. The larvae were fed...
continuously with the mixed diet and the diet was replaced every 24 h. Larval survival rates were recorded at the following time points: day 0 (start of experiment), day 1, day 3 and day 5. The larvae were considered to be alive if they moved in response to touching.

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