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Research paper

Functional characterization of ASTC (allatostatin C) and ASTCC (allatostatin double C) in *Clostera anastomosis* (Lepidoptera: Notodontidae)

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ABSTRACT

ASTC (allatostatin C) and ASTCC (allatostatin double C) are two neuropeptide genes that encode allatostatin C-like peptides. Whether these peptides inhibit or have other effects on juvenile hormone (JH) synthesis in the corpora allata remains in question. The juvenile hormone acid O-methyltransferase (JHAMT), a key gene in the JH biosynthesis pathway, was selected to study the effects of ASTC and ASTCC on juvenile hormones. In this study, we first characterized the expression patterns and correlations between ASTC and ASTCC in *Clostera anastomosis* under natural conditions, and then the functions of these two genes were investigated by RNAi. The results showed the expression of JHAMT was strongly positive correlated with ASTC and ASTCC in the heads of larvae after ASTC and ASTCC were knocked down simultaneously, while negative correlations were found in the heads of female adults. These results suggest that both ASTC and ASTCC may stimulate JH biosynthesis in larvae while inhibit in female adults of *C. anastomosis*. And after either ASTC or ASTCC was knocked down alone, the correlation between the two genes were positive, indicating ASTC and ASTCC may function dependently in female heads. In addition, simultaneous suppression of ASTC and ASTCC increased the mortalities of larvae and pupae.

1. Introduction

Juvenile hormone (JH) is secreted from the corpora allata (CA). It plays important roles in the regulation of several events during the insect life cycle, including growth, metamorphosis, molting, reproduction and diapause (De Wilde and De Boer, 1969; De Loof and De Wilde, 1970; Schooneveld et al., 1977; Kramer, 1978; Dortland, 1979; Vermunt et al., 1999). In insects, the biosynthesis of JH is rapidly and reversibly inhibited by a group of neuropeptides called allatostatins (ASTs), with a common C-terminal sequence (Stay and Tobe, 2007). The first members of this family of neuropeptides were first identified from the cockroach *Diploptera punctata* and called FGL amide, or the A-type family (FGLa/ASTs) (Woodhead et al., 1989). The second group, B-type ASTs, have a conserved carboxy-terminal W(X6)W amide and were first identified in the cricket (Lorenz et al., 1995). The C-type ASTs (ASTCs) were first identified in *Manduca sexta* and are characterized by the carboxy-terminal sequence PSFC (Kataoka et al., 1989; Janzen et al., 1991; Kramer et al., 1991). A global genome search revealed that ASTCs are not specific to Lepidoptera, they were also identified in *Tribolium castaneum* (Coleoptera) and *Apis mellifera* (Hymenoptera) (Audsley et al., 2013; Urlacher et al., 2016). ASTCs have been shown to inhibit JH biosynthesis in vitro in the CA in certain lepidopteran species, such as *Pseudaletia unipuncta*, *Galleria mellonella* (Bogus and Scheller, 1991; Bogus and Scheller, 1996), *Spodoptera littoralis* (Pfister Wilhelm and Lanzrein, 1996) and *Lacanobia oleracea* (Audsley et al., 1998; Audsley et al., 1999; Audsley et al., 2000). However, the roles of ASTCs in the regulation of JH have not been determined in other lepidopteran insects. Jansons et al. (1996) reported that Mas-ASTC had no inhibitory effect on JH biosynthesis in the sixth instar larvae or newly emerged adults of *P. unipuncta*, and the expression of ASTC was not associated with the titer of JH (Jansons et al., 1996). Oeh et al. (2000) showed that in female adult *Spodoptera frugiperda*, synthetic Mas-ASTC affected the rate of JH synthesis in the CA only when activated by an allatotropin (Oeh et al., 2000). And in *Tenebrio molitor*, the activity of Trica-ASTC in the CA depended on the age of the insect (Abdel Latief and Hoffmann, 2010). In addition, knocking down the expression of ASTC did not regulate JH biosynthesis in young virgin females of *S. frugiperda*, although reduce the amount of JH in the penultimate larvae and prepupae (Griebler et al., 2008).

An analysis of insect genomes showed that most insect species actually have two genes that encode an ASTC-like peptide. The newly

Abbreviations: ASTC, allatostatin C; ASTCC, allatostatin double C; JHAMT, juvenile hormone acid O-methyltransferase; JH, juvenile hormone; RNAi, RNA interference.

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discovered paralogous gene was called ASTCC and was predicted to produce the peptide allatostatin double C (ASTCC) (Veenstra, 2009). There is now good evidence suggesting that both peptides act through the same receptor, as shown in *T. castaneum* and *A. mellifera* (Audsley et al., 2013; Urlacher et al., 2016). However, the function of ASTCC has not been characterized in Lepidoptera.

Many studies have shown that JH levels are controlled by the JH synthesis enzymes (Gilbert et al., 2000). The biosynthetic pathway of JH in insects involves 13 enzymes, including enzymes in the mevalonate pathway (MVAP) and the JH-branch steps. In most insect species, the last enzyme in the pathway is encoded by a single gene: juvenile hormone acid O-methyl-transferase (JHAMT). JHAMT is expressed predominantly in the CA (Sheng et al., 2007; Nouzova et al., 2011), and its expression level reflects the titer of JH in *Samia cynthia ricini* (Sheng et al., 2008). This relationship has also been confirmed in other lepidopteran species, including *M. sexta* (Hebda et al., 1994) and *Bombyx mori* (Kinjoh et al., 2007; Sheng et al., 2008).

In this study, we used RNA interference to characterize the functions of two genes, ASTC and ASTCC, in *Clostera anastomosis* (Lepidoptera: Notodontidae), which is an important pest of poplar. The expression levels of JHAMT in the animals whose ASTC and ASTCC genes were knocked down simultaneously were analyzed, and their effects on development were also investigated.

2. Materials and methods

2.1. Insects

Larvae of the black-back prominent moth *C. anastomosis* (L.) were collected from poplar stands near Harbin City, China and reared on fresh poplar leaves from the larva stage until the moths emerged and died naturally. At the appropriate time during the analysis, the animals were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. dsRNA synthesis

RT-PCR was performed to construct the templates used to synthesize dsRNA. The following T7 primers, ASTC-F; ASTC-R, ASTCC-F; ASTCC-R and dsJHAMT-F; dsJHAMT-R (Table 1) were used to amplify ASTC, ASTCC and JHAMT. The sequences of gene ASTCC, JHAMT and α-tubulin were obtained based on *C. anastomosis* transcriptome data (sequences in the Appendix 1).

Table 1
Primers in this study.

Primers	Sequences	Amplification efficiencies
α-Tubulin-R	5′-CATGAGGAGAGAGGTG-3′	102.5
α-Tubulin-F	5′-GCAAGGAAATCGTAGAC-3′	
rtASTCC-F	5′-TCCTCTATCGCTGTTGTT-3′	97.9
rtASTCC-R	5′-CTTCATGTAGTGCCTTCT-3′	
rtASTC-R	5′-CCATCCGTTCCTCAGCAT-3′	95.7
rtASTC-F	5′-GTCTCGCCGTACTCTTCGT-3′	
JHAMT-R	5′-TTATGTCTGTGAACCCAAT-3′	103.7
JHAMT-F	5′-GGTGACTGCCTGTTATCCA-3′	
ASTC-F	5′-GATCACTAATACGACTCACTATAGGG ATGAAGACCGCAGCGTATCAC-3′	108.9
ASTC-R	5′-GATCACTAATACGACTCACTATAGGG CTTGCGGAAGCAAGAGATGGG-3′	
ASTCC-F	5′-GATCACTAATACGACTCACTATAGGG ATGATGATGCGAAGTCCTCTATCG-3′	89.8
ASTCC-R	5′-GATCACTAATACGACTCACTATAGGGG AAACACGTCACCGCGTTGAA-3′	
dsJHAMT-F	5′-GATCACTAATACGACTCACTATAGGG ACATACCCTGCGACTACAA-3′	93.5
dsJHAMT-R	5′-GATCACTAATACGACTCACTATAGGG GGCGGATGACTTCAATAA-3′	

PCR was performed using the following program: 94 °C for 5 min; 35 cycles at 94 °C for 45 s, 49 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. ExTaq polymerase (TAKARA) was used to perform the reactions. The transcription reactions were performed using an in vitro transcription T7 kit (TAKARA) according to the manufacturer's instructions, The PCR products which containing the T7 promoter were used as a template for in vitro transcription combined with ATP, CTP, UTP and GTP. The reactions were allowed to proceed overnight at 42 °C and followed by both RNase and DNase digestion and purification step to obtain the dsRNAs. The dsRNAs were spectrophotometrically quantified before injection.

2.3. RNAi experiment

Synthesized dsRNAs (1 μl) were injected into the ventral abdomen of the larvae on the first day of the last instar or pupae using a 10-μl Hamilton syringe (Hamilton AG, Bonaduz, Switzerland). Both DEPC-treated water and the GFP (Green Fluorescent Protein) dsRNA were used as negative controls. Untreated animals were used as blank controls (CKs). This lets us verify the effect of mechanical damage by comparing water control and CKs, and investigate the off-target effects by comparing GFP control with treatment. In the study of RNAi effect, water-treated animals were used as control. To increase the knockdown effect, three concentrations of the dsASTC or dsASTCC (3 μg/μl, 1 μg/μl and 0.5 μg/μl) were injected. The results showed that the differences of interference effects among the three concentrations were not significant (data not shown). Therefore, only one concentration (3 μg/μl) was used in successive experiments (correlations between the expressions of JHAMT and ASTC/ASTCC, i.e., RNAi of JHAMT, RNAi of ASTC and ASTCC simultaneously).

Each animal was injected only once. For each dose, three of the treated larvae were randomly selected at 12 hpi (hours post-injection), 24 hpi, 36 hpi and 48 hpi (72 hpi in JHAMT RNAi) and stored at −80 °C for further experiments, including RNA extraction and real-time PCR. The whole bodies of insects were used for RNA extraction in the study of the correlation between the expressions of ASTC and ASTCC, while only the heads were used in the study of the correlation between the expressions of JHAMT and ASTC/ASTCC. The expression levels of ASTC/ASTCC were quantified first, and the expression of JHAMT was quantified only in animals which ASTC/ASTCC were successfully knocked down. A dose of 3 μg/μl was also applied to a group of at least 30 animals to investigate the effect of knockdown on mortality and developmental timing.

2.4. Real-time PCR

The head or the whole body of each animal was ground in liquid nitrogen, and total RNA was isolated using the STE method (Shui et al., 2008). Briefly, the samples were extracted with STE reagent (1.0 M NaCl, 0.5 M TRIS-HCL, 10 mM EDTA), then extracted with chloroform plus isoamyl alcohol and water saturated phenol. The extracted RNA was treated with RNase-free DNase I (TAKARA) to eliminate potential genomic DNA contamination prior to quantification by spectrophotometry. Then, 500 ng of the extracted total RNA was used to synthesize complementary DNA (cDNA) using a PrimeScript™ RT reagent Kit (Perfect Real Time) (TAKARA). The expression of the α-tubulin gene was used as internal control. Real-time PCR was performed in triplicate according to the manufacturer's instructions using TransStart Top Green qPCR SuperMix (TransGen Biotech) on an Agilent sequence detection system. The qPCR was performed using the following program: 94 °C for 5 min; 40 cycles at 94 °C for 30 s and 49 °C for 30 s (signal collected); and 72 °C for 30 s. A melting curve was added. The primers used for real-time PCR were shown in Table 1.

A melting curve analysis was performed to ensure that only a single product corresponding to the target sequence was amplified. All primer pairs were tested in advance to get similar amplification efficiencies.