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Involvement of a putative allatostatin in regulation of juvenile hormone titer and the larval development in *Leptinotarsa decemlineata* (Say)

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ABSTRACT

Juvenile hormone III (JH III) plays primary roles in regulation of metamorphosis, reproduction and diapause in *Leptinotarsa decemlineata*, a notorious defoliator of potato. The neurosecretory cell-borne substance(s) negatively affects the final two steps in JH biosynthesis, catalyzed respectively by an epoxidase CYP15A1 and a juvenile hormone acid methyltransferase (JHAMT). In a few insect species other than *L. decemlineata*, the inhibitory substance is allatostatin (AS) neuropeptide. In this study, two putative *AS* genes encoding LdAS-C and LdAS-B precursors were cloned. Both *LdAS-C* and *LdAS-B* were expressed in the egg, larvae, pupae and adults, and highly expressed in the brain and the gut. Dietary introduction of double-stranded RNAs (dsRNAs) targeting *LdAS-C* and *LdAS-B* successfully knocked down respective target genes. Ingestion during 3 and 6 consecutive days of ds*LdAS-C* significantly increased the *LdJHAMT* mRNA levels by 3.8 and 9.9 fold respectively. In contrast, ingestion of ds*LdAS-B* only slightly increased the *LdJHAMT* expression level by 1.1 and 1.7 fold. Moreover, after one, two and three days' ingestion of ds*LdAS-C*, the relative JH levels in the hemolymph of treated larvae were 2.5, 4.2 and 1.9 fold higher than those in control beetles. Furthermore, ingestion of ds*LdAS-C* and ds*LdAS-B* significantly affected larval growth and delayed larval development. Thus, we provide a line of experimental evidence in *L. decemlineata* to support the concept that AS-C acts as an allatostatin and inhibit JH biosynthesis.

1. Introduction

Colorado potato beetle *Leptinotarsa decemlineata* (Say) is a notorious defoliator of potato, and often causes extremely large potato yield losses (Jiang et al., 2010, 2011, 2012; Lu et al., 2011; Shi et al., 2012). The beetle has a complicated and diverse life history (Alyokhin et al., 2008; Alyokhin, 2009). Juvenile hormone III (JH III), a member of sesquiterpenoids produced and secreted from a pair of small endocrine organs called corpora allata (CA), plays primary roles in regulation of several important events in *L. decemlineata* life cycle, such as metamorphosis (Vermunt et al., 1999), reproduction (De Loof and De Wilde, 1970; Dortland, 1979; Lefevere and De Wilde, 1984) and diapause (De Wilde and De Boer, 1969; De Loof and De Wilde, 1970; Schooneveld et al., 1977; Kramer, 1978; Lefevere and De Wilde, 1984).

The biosynthetic pathway of JH III is conserved in insects (Belles et al., 2005). The last two steps are the conversion of farnesoic acid to the active JH III by means of an epoxidation (C10, 11) and a methyl transfer, catalyzed respectively by an epoxidase CYP15A1 and a juvenile hormone acid methyltransferase (JHAMT) (Mayoral et al., 2009; Marchal et al., 2010). In *L. decemlineata*, the neural signal negatively affects the activities of CYP15A1 and JHAMT (Khan et al., 1982a,b). The CA inhibitory substances may come from a single group of about eight *L*-type lateral neurosecretory cells (Khan et al., 1984; Khan and Buma, 1985).

Further researches in other insect species reveal that the CA inhibitory substances are neuropeptides termed allatostatins (ASs) (Pratt et al., 1989; Woodhead et al., 1989; Stay, 2000). AS peptides have been classified into A, B and C types based on conserved structural characteristics (Stay and Tobe, 2007; Salma et al., 2012; Wang et al., 2012). Type A AS (FGLamides, AS-A) peptides have conserved pentapeptide sequence (Y/F)XFG(L/I)amide at the carboxy-terminus (Woodhead et al., 1989). Type B AS peptides contain common W(X_6)Wamide sequences (Lorenz et al., 1995; Williamson et al., 2001; Amare and Sweedler, 2007; Li et al., 2008; Abdel-latief and Hoffmann, 2014). The third peptide group AS-Cs have two subtypes, pQVRFRQCYFNPISCF (PISCF-AS) (Kramer et al., 1991) and SYWKQCAFNAVSCFamide (AVSCF-AS) (Hummon et al., 2006). Moreover, genomic analysis of *AS-C* genes identifies a paralog called *AS-CC* in several insect species (Veenstra, 2009). Genome-wide studies reveal that the three AS types exist in most insect species, with several exceptions (Stay and Tobe, 2007).

Abbreviations: JH, Juvenile hormone; CYP, cytochrome P450 monooxygenase; JHAMT, juvenile hormone acid methyltransferase; AS, allatostatin; RNAi, RNA interference; dsRNA, double-stranded RNA; LC–MS, liquid chromatography–mass spectroscopy; ANOVA, analysis of variance.

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Despite the structural differences, AS peptides share a common biological function, suppressing the production of JH in CA in a species-specific pattern. AS-A peptides act as true allatostatins in cockroaches, crickets, and termites (Yagi et al., 2008; Weaver and Audsley, 2009). AS-B peptides show allatostatin activity in only one cricket species (Lorenz et al., 1995). Even in this cricket, the activity is low relative to the AS-A family. Whether the AS-B peptides are true allatostatins need further researches to confirm. AS-C peptides exert allatostatic activity in several Lepidoptera and Diptera insect species (Kramer et al., 1991; Li et al., 2006).

In addition to their allatoregulatory activity, the peptides show other functions in insects and other invertebrates (Hoffmann et al., 1999; Stay, 2000; Weaver and Audsley, 2009). AS-A peptides inhibit muscle contraction in the foregut (Duve et al., 1995) and hindgut (Lange et al., 1993) of cockroaches, in the hindgut of *Rhodnius prolixus* (Zandawala and Orchard, 2013), in the ileum of blowflies (Duve and Thorpe, 1994), in the anterior midgut of *Drosophila melanogaster* (Vanderveken and O'Donnell, 2014), and in the oviduct of *Schistocerca gregaria* (Veelaert et al., 1996). They affect egg development in *Blattella germanica* (Martin et al., 1996). Moreover, the peptides stimulate the luminal activity of invertase and amylase in the *Diploptera* midgut (Fusé et al., 1999), regulate K^+ absorption in the midgut in *D. melanogaster* (Vanderveken and O'Donnell, 2014), and may indirectly affect post-feeding diuresis in *R. prolixus* (Zandawala and Orchard, 2013). AS-Bs inhibit spontaneous contractions of the hindgut and oviduct of *Locusta migratoria* (Schoofs et al., 1991) and significantly reduce the peristalsis rate of *Manduca sexta* (Blackburn et al., 1995). Bom-PTSP, another AS-B from *Bombyx mori* acts as a prothoracicostatic peptide (Hua et al., 1999). The AS-C from *D. melanogaster* appears to have an inhibitory effect on muscle contraction and decrease the heart rate (Price et al., 2002). These data indicate that all three types of ASs are pleiotropic peptides in insect species.

In coleopteran species, in vitro studies using corpora cardiaca (CC)/CA complexes of *Tenebrio molitor* reveal that *Tribolium castaneum* AS-B3 peptide inhibits CA activity, whereas TcaAS-C either inhibits or stimulates the JH biosynthesis depending on the intrinsic rate of hormone synthesis in the test animals (Abdel-latief and Hoffmann, 2010). Moreover, injection of double stranded RNAs (dsRNAs) targeting *TcAS-C* and *TcAS-B* into *T. castaneum* young pupae (4 hour-old) prolongs the pupal duration, and results in the production of about 50% deformed adults. Other 50% of the animals are intact in shape, but have incompletely developed ovaries and lay fewer eggs (Abdel-latief and Hoffmann, 2014). However, the in vivo allatostatic activity of AS-C and AS-B peptides in coleopteran species remains unproven.

In the present paper, two putative *AS* genes encoding LdAS-C and LdAS-B precursors were cloned in *L. decemlineata*. Dietary introduction of ds*LdAS-C* and ds*LdAS-B* successfully knocked down the respective target genes. Since the activities of CYP15A1 and JHAMT are lowered by neural signal (Khan et al., 1982a) in *L. decemlineata*, we tested whether *LdAS-C* or *LdAS-B* knockdown increases the mRNA level of *LdJHAMT* and the relative JH III levels in the hemolymph, and negatively affects the larval performance.

2. Methods and materials

2.1. Insect rearing

L. decemlineata larvae and adults were reared in an insectary according to a previously described method (Zhou et al., 2013).

2.2. Molecular cloning and phylogenetic analysis

The expressed sequence tags (ESTs) of the putative *AS* and *JHAMT* genes were obtained from the *L. decemlineata* transcriptome data (Shi et al., 2013; Swevers et al., 2013; Wan et al., 2013; Kumar et al., 2014). To verify the correctness of the sequence, total RNA was extracted from the fourth instars and first-strand cDNA was synthesized using the reverse transcriptase (M-MLV RT) (Takara Bio., Dalian, China) and an oligo (dT)18 primer, and was used as a template to perform polymerase chain reaction (PCR) using primers in Table 1. The 5′- and 3′-RACE Ready cDNAs were synthesized from the fourth *L. decemlineata* instars following the manufacturer's instructions, primed by oligo (dT) primer and the SMARTer II A oligonucleotide using the SMARTer RACE cDNA amplification kit (Takara Bio., Dalian, China). Antisense and sense gene-specific primers (Table 1) corresponding to the 5′-end and 3′-end of the *AS* sequences, and the universal primer in the SMARTer RACE kit (Takara Bio.) were used to amplify the 5′- and 3′-ends, using the components and the thermal cycling conditions according to the manufacturer's protocols. After obtaining the full-length cDNAs, two pairs of primers (Table 1) were designed to verify the complete open reading frames (ORFs). The full-length cDNAs of *LdAS-C* and *LdAS-B*, and a fragment of *LdJHAMT* were submitted to GenBank (KJ939426, KJ939427 and KJ960227).

2.3. Bioassay using dsRNA

The same method as described recently (Zhou et al., 2013) was used to express ds*LdAS-C*, ds*LdAS-B* and ds*egfp* derived from a 206 bp fragment and a 228 bp fragment of respective genes, and a 414 bp fragment of enhanced green fluorescent protein gene with specific primers in Table 1, using *Escherichia coli* HT115 (DE3) competent cells lacking RNase III. Individual colonies of HT115 (DE3) were inoculated, and induced to express dsRNA by addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. The expressed dsRNA was extracted and confirmed by electrophoresis on 1% agarose gel (data not shown). Bacteria cells were centrifuged at 5000 ×*g* for 10 min, and resuspended in 0.05 M phosphate buffered saline (PBS, pH 7.4) at the ratio of 10:1 (concentration of 10×), and then used for bioassay. The concentration of dsRNA was about 0.5 μg/μl.

For bioassay, the suspension of the HT115 (DE3) clone expressed ds*LdAS-C*, ds*LdAS-B* or ds*egfp*, or PBS solution (control) was used to

Table 1
Primers used in RT-PCR, 5′ and 3′ RACE, ORF verification, dsRNA synthesis, and qRT-PCR.

Fragment name	Forward primer	Reverse primer
RT-PCR		
LdAS-C	GCAGGAAAAGACGAAAGA	GTGCCTTTGAATGCTTTAC
LdAS-B	ACCCTGCTGGCTTGTTAC	TGGAGGATAGGCTTGCTG
LdJHAMT	TCTGTTGATGAGGTGGAGTTC	CCCGCTCTAACAGTACTAATGA
RACE		
LdAS-C 5′-G	CGGGTTGAAGTAGCAGGCTCTGAA TCTA	
LdAS-C 5′-N	TGTGCGATCAGTTCCATCTGCCAC	
LdAS-C 3′-G		GGCAGATGGAACTGATCGCACA AA
LdAS-C 3′-N		CCGAACGAAAACGTAGATTCAG AGCC
LdAS-B 5′-G	GCCCAGCCTCCGTGAATGCTACTC	
LdAS-B 5′-N	GCCATCCACCTGTAGATTGTCATC CTGT	
LdAS-B 3′-G		TTCCCGAGGACAGGATGACAAT CTACAG
LdAS-B 3′-N		TGTGGGGAAAAAGAGGATGG AGTAGC
ORF verification		
LdAS-C	CAGCAGGAAAAGACGAAAGA	AAGAATGGGTGTGAAGAAAGTG
LdAS-B	ACCCTCCGATTGCCATAAA	GGTGATGTGTCAACGCCAGT
dsRNA synthesis		
ds*LdAS-C*	GGCATCTGGGAGAAAATAG	GAAGTAGCAGGCTCTGAATCT
ds*LdAS-B*	ACCCTGCTGGCTTGTTAC	TGGAGGATAGGCTTGCTG
ds*egfp*	AAGTTCAGCGTGTCCG	CTTGCCGTAGTTCCAC
qPCR		
LdAS-C	ATGCAAGGCTTTCTCCATCT	TTGATTGTTGTCCGGGTCTA
LdAS-B	TGTTGGAGCCATCAACTAGG	CCAACCATCAGTGAATTTGC
LdJHAMT	GGAAGTGGAGATGGCAAGTT	CTACCAACGAGTTTCCCGAT