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Final steps in juvenile hormone biosynthesis in the desert locust, *Schistocerca gregaria*

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ABSTRACT

Two genes coding for enzymes previously reported to be involved in the final steps of juvenile hormone (JH) biosynthesis in different insect species, were characterised in the desert locust, *Schistocerca gregaria*. Juvenile hormone acid *O*-methyltransferase (JHAMT) was previously described to catalyse the conversion of farnesoic acid (FA) and JH acid to their methyl esters, methyl farnesoate (MF) and JH respectively. A second gene, *CYP15A1* was reported to encode a cytochrome P450 enzyme responsible for the epoxidation of MF to JH. Additionally, a third gene, *FAMeT* (originally reported to encode a farnesoic acid methyltransferase) was included in this study. Using q-RT-PCR, all three genes (*JHAMT*, *CYP15A1* and *FAMeT*) were found to be primarily expressed in the CA of the desert locust, the main biosynthetic tissue of JH. An RNA interference approach was used to verify the orthologous function of these genes in *S. gregaria*. Knockdown of the three genes in adult animals followed by the radiochemical assay (RCA) for JH biosynthesis and release showed that SgJHAMT and SgCYP15A1 are responsible for synthesis of MF and JH respectively. Our experiments did not show any involvement of SgFAMeT in JH biosynthesis in the desert locust. Effective and selective inhibitors of SgJHAMT and SgCYP15A1 would likely represent selective biorational locust control agents.

1. Introduction

Juvenile hormones (JHs) represent a family of sesquiterpenoid hormones unique to insects. JHs are involved in several key processes during insect life. They play a central role in metamorphosis, moulting, ageing, diapause, reproduction, behaviour, polyphenism, as well as caste differentiation in social insects (Applebaum et al., 1997; Goodman and Granger, 2005; Hartfelder, 2000; Verma, 2007). JHs are synthesised and secreted from the corpora allata (CA), a pair of small specialised endocrine organs. Several JH homologues have been found – MF, JH acid, JH 0, JH I, 4-methyl-JH I, JH II, JH III, bisepoxy-JH III, skipped bisepoxide JH III and hydroxy JHs – of which JH III is the most widespread and predominant JH in insects (Darrouzet et al., 1997; Goodman and Granger, 2005; Kotaki et al., 2009). In *Schistocerca gregaria*, only JH III was reported as circulating in the haemolymph (Tawfik et al., 2000). The early steps in the biosynthetic pathway of JH III include the mevalonate pathway from acetyl-CoA to farnesyl pyrophosphate (FPP), a conserved pathway in both vertebrates and invertebrates. The late steps involve the hydrolysis of FPP to farnesol followed by oxidation to farnesal and farnesoic acid (FA). FA is finally converted to the active JH III by means of an epoxidation (C10,11) and a methyl transfer. The order in which these two final steps in JH III biosynthesis occurs, appears to be insect order dependent. In orthopteran and dictyopteran insects, FA is first methylated to methyl farnesoate (MF), which in turn undergoes a C10,C11 epoxidation to JH III. In Lepidoptera however, the converse appears to be the case: epoxidation precedes methylation (Fig. 1). Two genes (*JHAMT* and *CYP15A1*) involved in these last steps were recently identified in several insect species. *JHAMT* (juvenile hormone acid methyltransferase) was first functionally characterised in the silkworm, *Bombyx mori* (Kinjoh et al., 2007; Shinoda and Itoyama, 2003). *BmJHAMT* is specifically expressed in the CA. Its expression correlates well with the JH biosynthetic activity of the CA. BmJHAMT was shown to methylate the carboxyl group of JH I, II and III acids to produce the active JHs in the presence of *S*-adenosyl-L-methionine (AdoMet). Additionally, the enzyme is also able to catalyse the methylation of FA to MF (Shinoda and Itoyama, 2003).

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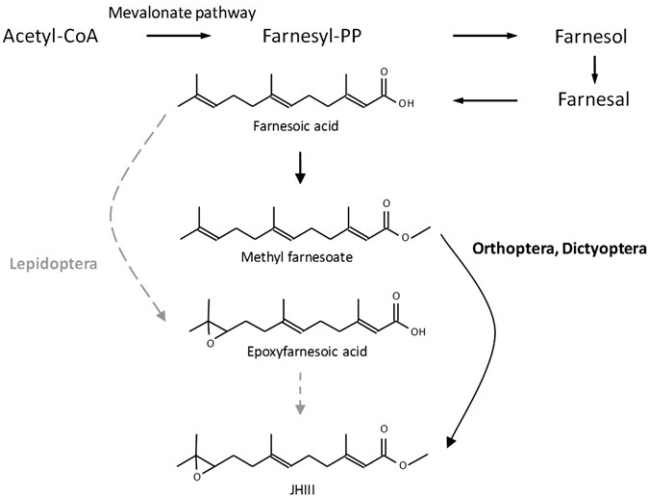

Fig. 1. Divergent final steps in JH III biosynthesis in different insect orders. The grey dashed arrows indicate the final two reactions as described previously in Lepidoptera. The final steps in Orthoptera and Dictyoptera are represented by black arrows.

There have been reports on orthologs of *JHAMT* in several other insect species, confirming its role in JH biosynthesis. Niwa et al. (2008) identified and functionally characterised *JHAMT* in the fruitfly, *Drosophila melanogaster*, and also in another dipteran, the mosquito *Aedes aegypti*, JHAMT was functionally characterised (Mayoral et al., 2009). Representatives of functional lepidopteran and coleopteran *JHAMT* were described in the eri silkworm, *Samia cynthia ricini* and the red flour beetle, *Tribolium castaneum* (Minakuchi et al., 2008; Sheng et al., 2008). Moreover, comparing gene expression and JH production in insects of wildtype, knockdown or overexpressed JHAMT, several of these studies have suggested that JHAMT is required for normal development (Kinjoh et al., 2007; Minakuchi et al., 2008; Niwa et al., 2008). *CYP15A1* was first functionally characterised in the cockroach *Diploptera punctata*. This gene was also found to be specifically expressed in the CA and was shown to encode a microsomal cytochrome P450 enzyme catalysing the epoxidation of MF to JH III (Helvig et al., 2004). To date, there have been no reports on the functionality of orthologs in any other insect species. But a partial *CYP15A1* was recently cloned from another cockroach species, the German cockroach, *Blattella germanica* (Maestro et al., 2010).

FAMeT (FA methyltransferase) – first reported in a crustacean, the shrimp *Metapenaeus ensis* (Gunawardene et al., 2001, 2002) – was initially suggested to be able to convert FA to MF, an active juvenoid end product in crustaceans. In this study, the production of MF was shown to increase in relation to increasing amounts of recombinant FAMeT in a radiochemical assay (Gunawardene et al., 2002). However, subsequent studies in other Crustacea (*Litopenaeus vannamei*, *Homarus americanus*, *Cancer pagurus*) and insect species (*Ceratitis capitata*, *Nilaparvata lugens* and *Melipona scutellaris*) did not verify this activity (Holford et al., 2004; Hui et al., 2008; Liu et al., 2010; Ruddell et al., 2003; Vannini et al., 2010; Vieira et al., 2008). Moreover, two recent studies in *D. melanogaster* have shown that DmFAMeT is not involved in JH biosynthesis (Burtenshaw et al., 2008; Zhang et al., 2010).

This report focuses on the cloning and characterisation of the orthologs of *JHAMT* and *CYP15A1* in a major pest insect, the desert locust, *S. gregaria*. Desert locust swarms can destroy agricultural production in some of the world's poorest countries and threaten the livelihood of a tenth of the world's population. These enzymes (SgJHAMT and SgCYP15A1) may constitute possible targets for selective pest control. Using q-RT-PCR, the tissue distribution and expression of these genes were examined throughout the 5th larval and adult development of *S. gregaria*. The conversion of FA to MF in Orthoptera is thought to occur through the action of a farnesoic acid methyltransferase. An RNAi-based approach was used to examine whether *SgJHAMT* encodes this enzyme and whether *SgCYP15A1* encodes a functional MF epoxidase. Additionally, the possible role of SgFAMeT in JH biosynthesis was studied using this same technique.

2. Material and methods

2.1. Animals

Desert locusts were reared under crowded conditions in large cages (38 × 38 × 38 cm), in which temperature (32 ± 1 °C), ambient relative humidity (40–60%) and light (13 h photoperiod) were kept constant. The animals were fed daily with dry oat flakes and fresh cabbage *ad libitum*. Following mating, mature females deposited their eggs in pots filled with damp sand. Each week, these pots were collected and set in empty cages, where eggs were allowed to hatch into first instar larvae. In the described experiments, 5th larval and adult locusts were collected at the time of ecdysis to obtain pools of synchronised animals.

2.2. Tissue collection

Tissues were dissected in locust ringer solution (1 L: 8.766 g NaCl; 0.188 g $CaCl_2$; 0.746 g KCl; 0.407 g $MgCl_2$; 0.336 g $NaHCO_3$; 30.807 g sucrose; 1.892 g trehalose; pH 7.2) under a binocular microscope and immediately transferred to liquid nitrogen to prevent RNA degradation. Tissues were stored at −80 °C until further processing.

2.3. Characterisation and phylogenetic analysis of SgJHAMT, SgCYP15A1 and SgFAMeT

Degenerate primers for *SgJHAMT* and *SgCYP15A1* were designed, based on conserved amino acid sequences found in a multiple sequence alignment of several Arthropod orthologs. (*SgCYP15A1* F: GTNYTNAAYWSNYMTNTGGGCNATG, based on VLNS/RLWAM; *SgCYP15A1* R: CCNGCCATRAANARRTCNARRCA, based on CLDL/FFMAG *SgJHAMT* F: TTYWSNTTYTAYTGYYTNCAYTGG, based on FSFYCLHW and *SgJHAMT* R: RTVRTGRTANGGNSWDATRWA, based on F/YISPYHH/D/Y). Partial sequences of *SgJHAMT* and *SgCYP15A1* were found using these primers in a T-gradient polymerase chain reaction (PCR) using REDTaq® DNA polymerase (Sigma–Aldrich Co.). CA cDNA was used in this amplification reaction with the following thermocycling profile: 3 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 2 min at 55 °C (with an 18 °C gradient) and 3 min at 72 °C. PCR products were loaded on a 1.2% agarose gel, separated during a 1 h gel electrophoresis and finally visualised using UV. Bands of the expected size were cut out and extracted with a GenElute™ Gel extraction Kit (Sigma–Aldrich Co.). Resulting DNA fragments were subcloned into a pCR4-TOPO vector using the TOPO® TA Cloning Kit (Invitrogen). DNA sequences were determined using the ABI PRISM 3130 Genetic Analyser (Applied Biosystems) following the protocol outlined in the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). At the same time, an in-house EST database of the central nervous system of *S. gregaria* became available and will soon become publicly accessible (Badisco et al., unpublished results). Hits were found for *CYP15A1* (Contig: LC.2139.C1.Contig2300) and *JHAMT* (singlet: LC01004X1B01) that allowed us to further complete their sequences. Finally, specific primers were designed to be used in a RACE-PCR (Rapid Amplification of cDNA Ends) using