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Recent gene multiplication and evolution of a juvenile hormone esterase-related gene in a lepidopteran pest

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ABSTRACT

Juvenile hormone esterase (JHE) is a carboxylesterase that plays critical roles in regulating larval to adult transition by hydrolyzing the key developmental and reproductive hormone, juvenile hormone (JH). In the current work, we have cloned and sequenced a superfamily of juvenile hormone esterase related genes in *Sesamia nonagrioides* (*JHERs*). These seemed to have been recently multiplied from a common ancestral gene and consequently were inherited in the resulting populations as intron-less and intron-rich genes (loci). We sequenced three *JHER* genes (one intron-rich and two intron-less) and four cDNAs encoding for juvenile hormone esterase related sequences. Three cDNAs presented nucleotide deletions similar to alternative splicing events when compared with the introns of the intron-rich gene. The exons of the intron-rich gene were >98% identical with one of the intron-less gene and the homologous sequences of all the four cDNAs. Moreover, the second intron-less gene seemed to be almost identical with one of the four cDNAs. The fourth cDNA contained an extensive (in-frame) deletion inside its ORF. This mRNA seems to be encoded by another gene which's deletion was generated by homologous recombination. Interestingly, our data revealed differential expression patterns for the four cDNAs. This study provides an initial assessment of the diversity of *JHER* genes in a population of *Sesamia* and presents this species as an attractive model to study the diversification of JHE-like esterase genes and their functional consequences.

1. Introduction

Juvenile hormone (JH) is a key endocrine regulator for the control of growth, development, metamorphosis, diapause and reproduction in insects (Riddiford et al., 2003). JH belongs to a group of structurally related sesquiterpenes that regulate the transition of insects through their various developmental stages and determine their metamorphic transitions. JHs directly transcriptionally induce several genes, (e.g. juvenile hormone esterase, calmodulin and vitellogenin), while via indirect regulation, JHs deactivate genes that have been induced by 20-hydroxyecdysone (20E) (Gullan and Cranston, 2010). Important is also their role in the reproductive processes of insects (Hartfelder, 2000).

The regulation of JH titers is thus critical in the entire life of the insect. One key event is the clearing of JH that generally precedes the molt from the last larval stage to the pupal stage of holometabolous insects (Campbell et al., 2001). The very low JH titer at this time is generally achieved by the combined effect of reduced JH synthesis and the action of JH degrading enzymes (Roe and Benkatesh, 1990). Degradation of JHs is an important mechanism by which insects control the JH titer, while JH esterases (JHEs) and JH epoxide hydrolases (*JHEHs*) constitute regulating enzymes for this process (Roe and Benkatesh, 1990; Hammock, 1985; Goodman and Granger, 2005). JHEs belong to the α/β hydrolase fold superfamily of proteins which degrade JHs with high selectivity even if they are found in very low concentrations. They contain a well conserved active center with the characteristic GxSxG motif (Wogulis et al., 2006). At the primary amino acid sequence level JHEs possess seven highly conserved sequence motifs (RF, DQ, GQSAG, E, GxxHxxD, R/Kx$_{(6)}$R/KxxxR, and T) (Kamita and Hammock, 2010; Ward et al., 1992; Feng et al., 1999; Kamita et al., 2011; Munyiri and Ishikawa, 2007).

Six major forms of JH (JH 0, JH I, JH II, JH III, 4-methyl JH I and JH III bisepoxide) have been isolated from insects; all possess an α,β-unsaturated methyl ester at one end of the molecule and an epoxide at the other (Kamita et al., 2011). But these JH forms do not constitute all possible types of JH hormones that could be found in insects. Likewise JHE is not always the major JH-degrading enzyme. In *Trichoplusia ni* researchers revealed evidence for the existence of a JH-like compound that is catabolized by juvenile hormone esterase related (JHER) enzyme, which has a cysteine residue immediately adjacent to the catalytic

Abbreviations: JHE, juvenile hormone esterase; *JHER*, juvenile hormone esterase related; JH, juvenile hormone; 20E, 20-hydroxyecdysone; *JHEHs*, JH epoxide hydrolases; OTFP, 3-octylthio-1,1,1- trifluropropan-2-one.

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serine, in contrast to most other described esterases, including JHE, which have alanine at this position (Jones et al., 1994). Moreover in *Sesamia nonagrioides*, it was found that the JH titer remained high in the presence of high JHE activity in diapausing larvae, indicating that JHE was not a major factor involved in the regulation of JH at this developmental stage (Schafellner et al., 2008). The authors (Schafellner et al., 2008) also discovered that the JHE-specific inhibitor 3-octylthio-1,1,1-trifluropropan-2-one (OTFP) did not provoke a developmental response in *S. nonagrioides* as observed in the sphingid *Manduca sexta* and some other insects, where JHE is required for metamorphosis and the enzyme's activities peak after the larvae reach a critical body mass for pupation (Schafellner et al., 2008). It was concluded that in *S. nonagrioides* the JH titer is regulated by several mechanisms, of which hydrolysis by the hemolymph JHE does not seem to be the major one (Schafellner et al., 2008). Similarly, Pérez-Hedo et al. (2010) discovered that in *S. nonagrioides* the molting process can occur in the absence of the brain. It was consequently concluded that an unknown head factor outside of the brain is needed for the pupal–adult molt (Pérez-Hedo et al., 2010).

Orthologs and paralogs are two fundamentally different types of homologous genes that evolved, respectively, by vertical descent from a single ancestral gene and by duplication (Koonin, 2005; Bratlie et al., 2010). For practical reasons, paralogs are defined as protein-coding sequences that have at least 30% sequence identity over more than 60% of their lengths (Bratlie et al., 2010; Mira et al., 2006; Blattner et al., 1997). According to Ohno (1970), there are three possible outcomes for a duplication event in which the gene duplicate is kept: neofunctionalization, sub-functionalization and conservation of function (Bratlie et al., 2010; Hahn, 2009). If there is no biological advantage in keeping the duplicated gene, then the gene may become inactivated by mutations (non-functionalization), reduced to a pseudogene and finally removed from the genome by deletion (Bratlie et al., 2010). Gene duplication can result from unequal crossing-over, retrotransposition, or chromosomal (or genome) duplication, the outcomes of which are quite different (Zhang, 2003). Unequal crossing-over usually generates tandem gene duplication (the duplicated genes are linked in the chromosome) (Zhang, 2003). In contrast retro-transposition occurs when a messenger RNA (mRNA) is retro-transcribed to complementary DNA (cDNA) and then inserted into the genome, while chromosomal or genome duplication occurs by a lack of disjunction among daughter chromosomes after DNA replication (Zhang, 2003). *JHE* genes are usually present as single copy genes in the genomes of insects belonging to different orders: for lepidopterans, this is the case for *Heliothis virescens* (Harshman et al., 1994), *Choristoneura fumiferana* (Feng et al., 1999), *M. sexta* (Hinton and Hammock, 2001) and *Bombyx mori* (Hirai et al., 2002). In *T. ni* the natural *JHE* and a *JHER* gene are physically juxtaposed suggesting that a gene duplication event has occurred (Jones et al., 1994). At that time, it represented the first reported evidence of a duplication event for a gene encoding a JH-inducible larval protein. The only other documented case of a duplication of a JH inducible gene was vitellogenin (Pearce and Yamamoto, 1993), but in that case both genes were still inducible by JH, while in the *T. ni*'s case only *JHE*, and not *JHER*, was JH-inducible (Jones et al., 1994).

Likewise, in *Drosophila*, three candidates for additional JHE esterases were reported, of which one candidate gene represented an adjacent duplication of the known, characterized *JHE* gene (Crone et al., 2007). However, functional analysis established that none of the three candidates could metabolize different JH substrates with sufficient sensitivity to suggest an important role in the degradation of JH during development (Crone et al., 2007). Also in the silkworm, *B. mori*, several additional genes were identified that encode members of the carboxyl/cholinesterase (CCE) family and that contain the GQSAG motif that is characteristic of JHE (Tsubota et al., 2010). Functional analysis however established also in this case that *Bombyx* JHE represents the canonical JH degrading enzyme and that the newly identified CCE enzymes with GQSAG motif may have other functions in animals, for instance in digestion or xenobiotic detoxification, although a role in the metabolism of JH-related compounds for some of them cannot be excluded (Tsubota et al., 2010). These studies illustrate that several genes encoding esterases that have structural similarities with JHE can exist in insect genomes, while their exact role in JH metabolism or development remains to be established.

In a previous study in order to identify and clone a *JHE* gene in *S. nonagrioides*, we performed RT-PCR using degenerate primers based on homologous sequences of *JHE* genes of other insect orders (Kontogiannatos et al., 2011). After 3′- and 5′-RACE PCR, we cloned the full length cDNA sequence of 1838 bp which seemed to be homologous to the sequences of the *JHE* genes of the other insect orders. Predicted amino acid sequence data however showed that this esterase had a unique GQSCG catalytic motif surrounding the catalytic serine of the predicted protein, which is identical to the motif found in the *JHER* gene of *T. ni*, but different from the characteristic QQSAG motif of canonical JHEs. Moreover semiquantitative RT-PCR data showed a unique gene expression pattern: the mRNA levels were not responsive to methoprene but were positively regulated by ecdysteroid analogs as well as by the xenobiotic bisphenol A (BPA). For all the reasons outlined above, we therefore characterized this cDNA as *JHE related* (*JHER*) rather than as *JHE* (Kontogiannatos et al., 2011). In a following paper we functionally characterized *SnJHER* by RNAi (Kontogiannatos et al., 2013): our data showed that *SnJHER* deficiency leads to larval–larval, larval–pupal and pupal–adult transition blockage, which indicates a function in the regulation of development, possibly through the metabolism of JH-like substances (Kontogiannatos et al., 2013). In the current work, the existence of different *JHER* genes that encode multiple mRNAs that may encode for additional JHER enzyme isoforms was investigated. Furthermore, we performed semi-quantitative PCR-analysis to evaluate the expression of different isoforms in order to clarify their potential biological role.

2. Methods

2.1. Insect rearing and staging of larvae

S. nonagrioides insects were maintained at 25 °C, 55 ± 5% relative humidity on an artificial diet (Kontogiannatos et al., 2011, 2013). Larvae reared under applied LD conditions (16:8, light:dark) completed their larval stage in 6 instars. The age of analyzed larvae within each instar was measured in days after the preceding ecdysis, using physiological markers such as body mass and head capsule width. Larvae were checked daily for molting. At the 9th day of the last (6th) larval instar (L6d9), larvae transformed into prepupae and entered metamorphosis.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from larvae and insect cells using TRIzol® reagent (Sigma) according to the supplier's instructions and stored at −80 °C. After treatment with RNase-free DNAse I (Promega), 1.5 μg of RNA was used as template for the first strand cDNA synthesis using oligo-dT primer and Superscript™ II RNase H-Reverse Transcriptase (Invitrogen). In all experiments the RNA was extracted from the whole body tissue of the analyzed animals.

2.3. PCR and semiquantitative RT-PCR

For semiquantitative RT-PCR analyses primers were selected which, because of the high homology of the 4 *SnJHER* cDNAs, could distinguish 2 or 3 isoforms each time. JHE3RTf/JHE3RTr primer set (Table 1) was used to amplify *SnJHER*, *SnJHER3* and *SnJHER4* cDNAs after 33 cycles (Fig. 2). The JHEMf/JHEMRTr primer pair was used to amplify both *SnJHER* and *SnJHER3* cDNAs after 36 cycles of polymerization, while JHEWhof/JHE5RTr was used for *SnJHER* and *SnJHER2* (39 cycles) (Table 1 and Fig. 2). As control, a part of the coding region of