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ORIGINAL ARTICLE

Abnormal development in larvae of *Sesamia nonagrioides* (Lepidoptera: Noctuidae) resulting from baculovirus-mediated overexpression of a JHE-related gene (*SnJHER*)

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Abstract. The Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae) has a unique and recently multiplied juvenile hormone esterase gene family (*SnJHER*) with particular transcriptional profiles and functional characteristics. Unlike conventional juvenile hormone esterase genes (*JHE*s), the *SnJHER* gene family seems to have been recently evolved from a common ancestral JHER gene. *SnJHER*s seem to be regulated by both ecdysone agonists and xenobiotics, while their real role in development remains to be exploited. In this study we transiently expressed the major *SnJHER* isoform in Bm5 and Hi5 cell lines. The JHER-expressing cell lines showed increased toxicity when treated with the juvenile hormone analog methoprene. Moreover baculovirus-mediated transient gene transduction of the *SnJHER* gene in larvae of *S. nonagrioides* resulted in moulting abnormalities. These were more marked after the additional application of the juvenile hormone analog methoprene. Our results indicate a potential mechanism by which SnJHER interferes with normal JHE.

INTRODUCTION

Juvenile hormones (JHs) belong to a group of structurally related sesquiterpenes, which either directly induce important developmental genes (e.g. juvenile hormone esterase gene) or indirectly deactivate genes that are induced by 20-hydroxyecdysone (20E) (Gullan & Cranston, 2010). The regulation of JH titers is critical in the development of insects. One key event is the clearing of JH that generally precedes the moult from the last larval to the pupal stage in holometabolous insects (Campbell et al., 2001).

In holometabolous insects juvenile hormone (JH) is thought to be the key endocrine regulator controlling their growth, development, metamorphosis, diapause and reproduction (Jones et al., 1982; Riddiford et al., 2003). Low JH titers cause a shift from isometric to anisometric development leading to the pupal and adult stages (Jones et al., 1982). 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 et al., 1990).

Degradation of JHs is an important mechanism by which insects control their JH titer, in which juvenile hormone esterases (JHEs) regulate this process (Hammock et al., 1985; Roe et al., 1990; Goodman et al., 2005). In parallel or subsequent to the action of JHEs, JH-specific epoxide hydrolases (JHEHs) are a second class of enzymes that inactivate JH by cleavage of the epoxide moiety (Hammock, 1985). JHEs belong to the α/β hydrolase fold superfamily of proteins, which degrade JHs with high selectivity even if they are present at 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 have seven highly conserved sequence motifs (RF, DQ, GQSAG, E, GxxHxxD, R/Kx$_{(6)}$R/KxxxR, and T) (Ward et al., 1992; Feng et al., 1999; Munyiri et al., 2007; Kamita et al., 2010, 2011).

To qualify as a bona fide JHE, Kamita & Hammock (2010) propose several biological and biochemical criteria: biologically, JHEs are esterases, which are essential for clearance of JH from an insect's body and whose titer correlates with the decline in JH; while biochemically JHE is defined as an esterase that is capable of rapidly hydrolyzing JH with a high k_{cat}/K_m ratio or low K_m, even in the presence of specific JH carrier protein. However, even if enzymes that stringently meet those criteria are identified,

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there are indications that other esterases contribute to the regulation of the JH titer (Gilbert et al., 2000; Tsubota et al., 2010; Gu et al., 2015).

The first JHE-encoding gene cloned was obtained from *Heliothis virescens* (Hanzlik et al., 1989). Phylogenetic analysis shows that lepidopteran JHEs form a clade that is distinct from that of other insect groups, such as Diptera and Coleoptera (Kamita & Hammock, 2010). In *Drosophila*, besides the canonical JHE gene, closely related JHE-like genes exist in the genome that seem to have acquired new functions, but have retained the capability of degrading JH, albeit with low efficiency (Crone et al., 2007). Since many insect esterases can metabolize JH, many templates exist for the evolution of a JH-specific esterase that is dedicated to the inactivation of this hormone (Crone et al., 2007).

JHEs (in the phylogenetic sense) therefore may not always be the major JH-degrading enzymes. Closely related enzymes, the juvenile hormone esterase related enzymes (JHERs), contain a cysteine residue immediately adjacent to the catalytic serine, in contrast to most other described esterases, including JHE, which have alanine at this position (Jones et al., 1994; Kontogiannatos et al., 2011). In *Trichoplusia ni* it is proposed that a JH-like compound could be the target of the JHER enzyme (Jones et al., 1994). *TniJHER* is not induced by the powerful JH analog, fenoxycarb, while it is highly expressed just before the metamorphic commitment to the pupal developmental program and away from the larval program (Jones et al., 1994). In this respect the expression of *JHER* appears similar to that reported for certain other genes that are highly expressed before, but not after, metamorphic commitment, such as the arylphorin gene that is controlled by ecdysteroids (Jones et al., 1994). It is suggested that, *TniJHE* and *TniJHER* are physically juxtaposed in *T. ni* (Jones et al., 1994).

In *S.nonagrioides* we characterized a *JHER* gene that has GQSCG instead of the normal GQSAG catalytic motif on its predicted protein (SnJHER) (Kontogiannatos et al., 2011). This gene is not responsive to the juvenile hormone analog (JHA) methoprene, but it is positively regulated by ecdysteroid analogs and the xenobiotic bisphenol A (BPA) (Kontogiannatos et al., 2011). Depletion of *SnJHER* by RNAi revealed its potential role in the regulation of the developmental programming in *S. nonagrioides*. *SnJHER* knock-down resulted in severe malformations including blockage of the larval-pupal-adult transition (Kontogiannatos et al., 2013).

Three more protein isoforms of *SnJHER* (major isoform), which differ in point mutations, and several wide deletions throughout their ORFs (*SnJHER 2-4*) occur in *S. nonagroides*. Deletions are likely to have functional consequences since they result in the lack of several catalytic domains and modified N- and C-termini. Additional PCR and sequencing data, reveal the presence of at least three highly homologous *SnJHER* genes in the *S. nonagrioides* genome (*SnJHEgR*, *SnJHEgR1* and *SnJHEgR3*) suggesting that *SnJHERs* recently evolved from a common ancestral gene (Kontogiannatos et al., 2016).

*SnJHER*s have an uncommon physiological role that needs to be explored. In this study we transiently overexpressed the *SnJHER* gene (major isoform or isoform 1) (Kontogiannatos et al., 2016) in lepidopteran cell lines and larvae of *S. nonagrioides*. Our results reveal the possibility that, as is suggested for other *JHER* genes, *SnJHER* is an antagonist of the original *JHE* gene with distinct biochemical and biological functions.

MATERIAL AND METHODS

Insect rearing and determining the stage of development of the 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 16L : 8D conditions 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 moulting. At the 9th day of the last (6th) larval instar (L6d9), larvae transformed into prepupae and metamorphosis started.

Use of BmNPV as a gene transduction vector in *S. nonagrioides*

For functional studies, *Bombyx mori* nuclear polyhedrosis virus (BmNPV) was selected as described previously (Kontogiannatos et al., 2013). Recombinant GFP-expressing BmNPV (BmNPV-BmA::GFP) was shown to be able to infect efficiently cells and tissues of *S. nonagrioides* (Kontogiannatos et al., 2013). Viral GFP-expression is located mostly in the fat body, haemolymph, epidermal cells and tracheoles of infected larvae (Kontogiannatos et al., 2013). BmNPV-infected larvae are able to continue normal development in the absence of symptoms of polyhedrosis (Kontogiannatos et al., 2013). In contrast to larval infections, however, when insects are infected with BmNPV-BmA::GFP virus during the prepupal stage (6th instar d9), they are unable to complete the larval-pupal transition and most of them die as larval-pupal intermediates. Moreover, abnormal adults with fused pupal tissues and curly wings emerged from the surviving pupae (Kontogiannatos et al., 2013). BmNPV therefore can be used as a gene transduction vector during larval development but is not suitable for infections at the prepupal or pupal stage (Kontogiannatos et al., 2013).

Plasmids and virus constructs

PCR was performed with Phusion® High-Fidelity DNA polymerase using cDNA prepared from the fat body of larvae of *S. nonagrioides* as a template. PCR was performed using the JHER-HetEF/JHERHetER primer pair (Table 1) to amplify 1730 bp of the *SnJHER* cDNA and introduce flanking NotI recognition sequences and the consensus Kozak motif (GCCACC, light-grey shaded) just before the ATG codon (dark-grey shaded and in bold) (Table 1). The amplified PCR product was digested using NotI (New England Biolabs) and ligated into the NotI position of the pEIA-N-Flag expression vector (Douris et al., 2006) to generate the pEIA-N-Flag/SnJHER expression plasmid.

For the BmNPV-BmA::GFP/P10::N-Flag/SnJHER construction, the pEIA-N-Flag/SnJHER plasmid was digested using XhoI/SmaI (New England Biolabs) and the N-Flag/SnJHER ORF was ligated in the corresponding sites of the pFastBac™ Dual vector (Life Technologies), downstream of the P10 promoter. The recombinant plasmid was transformed into competent DH10Bac/BmNPV-BmA::GFP cells with helper plasmid as previously described (Kontogiannatos et al., 2013).

Table 1. Primers used in this study. Underlined: NotI sequence. ATG position is shown in bold.

Primer 5' → 3' used as	Name	Sequence	Tm°C
Forward	JHERHetEF	TTATGCGGCCGCTAGCCACC**ATG**TCGGCGAATAAGAACAACACTTTG	64
Reverse	JHERHetER	TTATGCGGCCGCGACACTAGGATGACGCACTCTTG	64
	Oligodt	GTCGACCTCGAG(T17)	

Transformed bacteria were selected on LB plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-α-gal and 40 µg/ml IPTG (Sigma) after O/N incubation at 37°C. Bacmid DNA derived from 5 bacterial clones was used to transfect Bm5 cells with Escort IV transfection Reagent (Sigma) and supernatants were collected 7 days after transfection as recombinant baculovirus stocks. After re-infection of Bm5 cells, baculoviral DNA was tested for the presence of the N-Flag/SnJHER expression cassette using PCR and the JHEWhof/JHE3RTr primer pair (Table 1). Infected BmNPV-BmA::GFP/P10::N-Flag/SnJHER Bm5 cells were analyzed in Western blots with anti-flag antibody in order to confirm correct protein expression. Titers of a BmNPV-BmA::GFP/P10::N-Flag/SnJHER clone with the highest expression level and a viral stock of BmNPV-BmA::GFP/BmA::dsLuciferase (Kontogiannatos et al., 2013) were adjusted to 10^7 pfu/ml before their use in in vivo experiments.

Cell lines and Transfections

Bombyx mori Bm5 (Grace, 1967) and *Trichoplusia ni* Hi5 (BTI-TN-5B1-4) (Granados et al., 1994) insect cell lines were maintained in IPL-41 insect cell culture medium, supplemented with 10% fetal bovine serum (Life Technologies), at 28°C and sub cultured weekly. Bm5 and Hi5 cells were transfected following established protocols (Johnson et al., 1992). For transient expression studies, 0.7 µg of pEIA-N-Flag/SnJHER plasmid (N-Flag/SnJHER cloned in the NotI-site of the pEIA plasmid; Lu et al., 1997) was used. For construction of stable N-Flag/SnJHER -expressing cell lines, Hi5 cells were co-transfected with several concentrations of the puromycin resistance pEA-PAC plasmid (Douris et al., 2006) and subsequently selected at several concentrations of puromycin. Cell lines were transfected at 1 : 10 or 1 : 100 weight ratios between expression plasmid and antibiotic resistance plasmid (1.2 µg expression plasmid and either 120 ng or 12 ng of pEA-PAC). Stable cell lines were obtained after continuous selection of transfected cells for one month in 20 µg/ml or 50 µg/ml of puromycin. Three stable lines were obtained, designated 1/10–20, 1/10–50 and 1/100–50, based on the selection procedure.

Western blot analysis

Protein gel electrophoresis and Western blot analysis were carried out as previously described (Tsitoura et al., 2010). Transfected cells were collected by centrifugation and pellets were suspended in phosphate-buffered saline (PBS; 100 µl per 10^6 cells). After freezing for 15 min at –70°C, the cell suspension was subjected to high speed centrifugation (13000 rpm, 15 min) and both supernatants (soluble protein fraction) and cell pellets (insoluble protein fraction) were collected. Soluble protein fractions were diluted 1 : 1 (v/v) with cracking buffer and the cell pellets were solubilized in 200 µl cracking buffer (Georgomanolis et al., 2009).

Thirty µl of each fraction was loaded in individual lanes of protein gels. For Western blot analysis, proteins were immediately transferred to a PVDF membrane (Invitrogen). The membrane was blocked with 10% w/v non-fat milk. For detection of Flag-tagged SnJHER, the membranes were first incubated with rabbit anti-flag antibody (Sigma) at 1 : 1000 and subsequently with HRP-coupled anti-rabbit antibody (Chemicon) at 1 : 2000. Pierce SuperSignal West Pico chemiluminescent substrate (ThermoScientific) was used for detection.

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 a template for first strand cDNA synthesis using oligo-dT primer and Superscript™ II RNase H-Reverse Transcriptase (Invitrogen).

Bright field and UV field microscopy

All bright field and fluorescence observations were conducted directly on living cells or tissues using a Zeiss Axiovert 25 inverted microscope equipped with a HBO 50 illuminator for incident-light fluorescence excitation and a Zeiss filter set at 09 (450–490 nm excitation filter, 510 nm barrier filter).

Cytotoxicity assays

For determining the cytotoxicity of the JHAs, assays were performed using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Bornem, Belgium) as substrate. Before starting the experiment, Hi5 JHER-expressing and control cells were counted using a Bürker chamber and diluted in order to achieve concentrations of approximately 8×10^5 cells/ml. 200 µl of 8×10^5 of each cell line were placed in 96 well plates and incubated for 24 h at 28°C with 1 µL of DMSO and 1 µl of each of the JHAs (10 µM fenoxycarb, 20 µM kinoprene, 50 µM methoprene). Each treatment was replicated 6 times/ well plate and the experiment was repeated for a second time (12 replicates/ treatment). Numbers of viable cells were counted using the MTT technique according to (Decombel et al., 2004). To determine cytotoxicity, 20 µl of 10% FBS-IPL-41 medium containing 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) was added to 1/2 volume of the analyzed samples (100 µl), followed by incubation for 4 h at 28°C. After 4 h of incubation, the cells were attached to the bottom of the microplate, allowing the MTT mix to be removed using a multichannel micropipette. The cells were then incubated with 100 µl of isopropanol. The plate was shaken for 10 min, and the amount of formazan (index of viability) was determined by measuring absorbance at 450 nm with a FLUOStar Galaxy Unit microplate reader. The values for 12 replicates per treatment were normalized with the mean value of the DMSO treated control and data were expressed as % mean ± SD cell viability. Statistical analysis of absorbance results was performed on 12 replicates per treatment, using a One-way ANOVA with post-hoc Tukey honest significant difference (HSD) Test. Data were expressed as mean ± SD for 12 replicates. Differences were considered to be significant at the $p < 0.05$ level.

Native PAGE electrophoresis and general esterase activity assays

Native polyacrylamide gel electrophoresis was performed in a vertical unit using a 10% acrylamide gel in electrophoresis running buffer without SDS. Cell pellets, haemolymph and fat body tissue of larvae of *S. nonagrioides* were diluted in 200 µl of phosphate buffered saline (PBS), pH 7.4 (100 µl per 10^6 cells). After

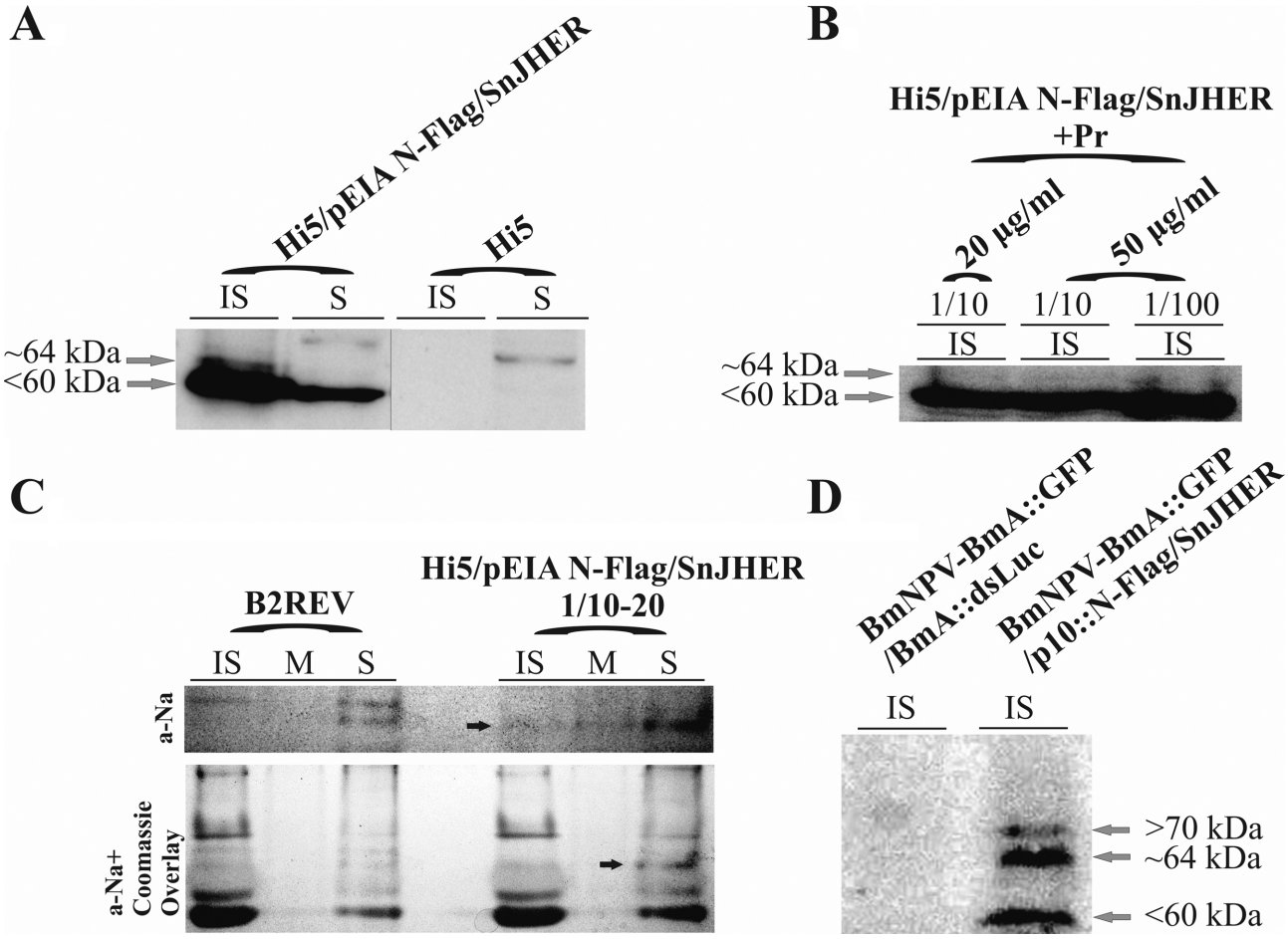

Fig. 1. Heterologous expression of SnJHER in insect cell lines. (A) Western blot analysis of the expression of SnJHER after transfection with pEIA-N-Flag/*SnJHER* plasmid. There is more SnJHER in the insoluble fraction (IS) of the cell extracts than the soluble fraction (S). As a control Hi5 cells that were not transfected were used. (B) Western blot analysis of SnJHER expression in stable cell lines with different concentrations of the puromycin resistance pEA-PAC plasmid (1/10 and 1/100) and several concentrations of the antibiotic puromycin (20 or 50 µg/ml). (C) General esterase activity gel assays of the JHER cell line using α-Napthyl acetate as a substrate (Up). As a control the B2REV cell line was used (which expresses antisense RNA of the B2 gene of Flock House virus; Swevers et al., 2016). IS – insoluble fraction, S – soluble fraction, M – growth medium. The same gel was re-stained with coomassie blue (Down). Arrows indicate JHER-specific esterase band. (D) Western blot analysis of BmNPV-BmA::GFP/P10::N-Flag/SnJHER infected Bm5 cells. BmNPV/BmA::GFP/BmA::dsLuciferase virus was used as a negative control. IS – insoluble fraction.

freezing for 15 min at –70°C, the cell suspension was subjected to high speed centrifugation (13000 rpm, 15 min). The freeze-thawing step was repeated one more time. Both supernatants (soluble protein fraction) and cell pellets (insoluble protein fraction) were collected. Supernatants were further diluted 1 : 1 (v/v) with PBS and the cell pellets solubilized in 400 µl of PBS. For growth media of cell lines the same procedure was performed by diluting directly the medium at 1 : 1 (v/v) with PBS. Samples were diluted 1 : 3 with 150 mM Tris-HCl (pH 6.8), 30% glycerol and 0.3% w/v bromopheno1 blue. Gels were run at 120 V constant voltage for 45 min. For detection of esterase activity the gels were first incubated for 15 min in 10 ml of 2.5 mg/ml Fast Blue RR (Sigma-Aldrich) diluted in 0.05 M phosphate buffer, pH 7.4. Subsequently 15 µl of 100 mM of α-Napthyl acetate (Sigma-Aldrich) substrate dissolved in acetone was added for visualization of esterase activity. The protein samples were quantified using the Bradford assay (Bradford, 1976) and gels were re-stained with Coomassie Brilliant Blue R-250 for protein visualization.

RESULTS

Heterologous expression of SnJHER

For biochemical characterization, SnJHER was heterologously expressed as a transgene in the Hi5 lepidopteran insect cell line or by genetically modified baculoviruses (see below). After transfection of Hi5 cells with pEIA-N-Flag/SnJHER plasmid, transfected cells were collected and insoluble and soluble protein fractions were prepared as described (Swevers et al., 2014).

In western blot using the anti-flag antibody, two cross-reacting proteins in the insoluble fraction were recorded, one weaker in density of approximately 64 kDa and one major band, which ran slightly faster of 60 kDa (Fig. 1A). In the soluble fraction, on the other hand, only the major band was recorded (Fig. 1A).

As shown in Fig. 1B, robust expression of SnJHER protein was also recorded in Hi5 transformed cell lines that over-express N-Flag/SnJHER (see M & M). The 1/10–50 cell line was further analyzed for general esterase activity

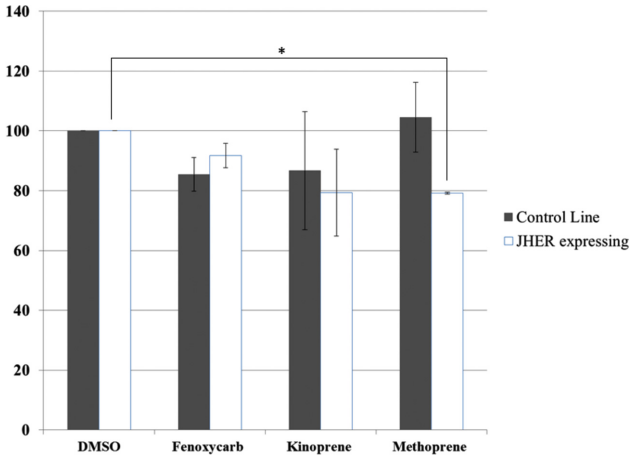

Fig. 2. Cytotoxicity of fenoxycarb, kinoprene and methoprene was recorded for the 1/10–50 (JHER expressing) and B2REV (control) cell lines using the MTT assay. Results are expressed as mean ± SD of 12 replicates. Asterisks indicate a significant result (One-way ANOVA with post-hoc Tukey HSD Test $p < 0.05$).

using gel assays and α-napthyl acetate as a substrate (α-Na). For detection of general esterase activity, three kinds of protein samples were analyzed: the growth medium and both insoluble and soluble fractions of the cell extracts. As a control we used a Hi5 cell line that was transformed following the same protocol but expressed as an irrelevant nucleotide sequence causing no biological or other consequences in the host cells (B2REV cell line; Swevers et al., 2016).

The three protein samples were quantified using Bradford assays and then electrophoresed in native PAGE (Fig. 1C). For general esterase activity assay the gels were first stained with fast blue RR (FBRR) and then incubated with the α-Na substrate. This assay revealed a completely different pattern of esterase activity in the Hi5-SnJHER cell line compared to the control; an esterase-specific electrophoretic band was recorded in all fractions tested, which was more intense in the medium and the soluble cellular fractions (Fig. 1C). The gel was then re-stained with coomassie blue in order to confirm equal protein quantities in the samples analyzed (Fig. 1C).

Cytotoxicity of JHAs in JHER expressing cell lines

The MTT method is a colorimetric method that measures the reduction of a component of tetrazolium (MTT) into formazan by viable cells (Mossman, 1983). Metabolism in viable cells produces "reducing equivalents" like NADH and NADPH. At death, cells rapidly lose the ability to reduce tetrazolium products. The production of the coloured formazan is therefore proportional to the number of cells in culture unless some metabolic processes in the cells are changed.

Previous studies (Soin et al., 2008) indicate that the order of cytotoxicity of JHAs to Bm5 and S2 cells at concentrations of > 5–10 μM was fenoxycarb > methoprene ~ kinoprene. To obtain comparable results we used concentrations at these orders of magnitude. Moreover in order to choose the appropriate dosage, we also performed bioassays at intermediate doses in order to determine the concentrations that do not have a toxicity greater than 50% and result in a more accurate statistical analysis (data not shown). For example we used concentrations of 10 μM for Fenoxycarb since it was more potent than kinoprene and methoprene, for which higher dosages were needed.

The SnJHER expressing 1/10–50 cell line and the control B2REV cell line were treated with the JHAs as described in M & M. Treatment with Fenoxycarb and Kinoprene did not result in significant differences in toxicity between the control and JHER expressing cell lines (Fig. 2). For methoprene, however, a significant reduction in cell viability was recorded for the JHER-expressing cell line, while the control lines were unaffected (Fig. 2).

Baculovirus-mediated over-expression of SnJHER (major isoform) in larvae of *S. nonagrioides*

In order to assess the biological role of SnJHER over-expression in the larval tissues of *S. nonagrioides* the BmNPV-BmA::GFP virus (Kontogiannatos et al., 2013) was engineered with the N-Flag/SnJHER construct. As donor plasmid we used the pFastBac™ Dual vector in which N-Flag/SnJHER expression would be controlled by the late p10 gene promoter. The double recombinant virus was first used in Bm5 cell infections in order to identify the correct protein expression. Immunoblot analysis with the flag antibody in the insoluble fractions of the Bm5 infected cells revealed the presence of three electrophoretic bands [compared with the BmNPV/BmA::GFP/BmA::dsLuciferase control virus, which overexpresses a hairpin specific for firefly luciferase (Kontogiannatos et al., 2013)] (Fig. 1D). In addition, general esterase activity in the Bm5 cells infected with BmNPV-BmA::GFP/P10::N-Flag/SnJHER was higher than in Bm5 cells infected with control BmNPV/BmA::GFP/BmA::dsLuciferase (data not shown).

Fifth instar larvae on day 1 (low SnJHERs expression; Kontogiannatos et al., 2011, 2016) were injected with 50 μl of 10^7 pfu/ml of the BmNPV-BmA::GFP/P10::N-Flag/SnJHER virus or control BmNPV/BmA::GFP-BmA::dsLuciferase virus. There were three replicates of this experiment: n1 = 32, n2 = 33, n3 = 35 insects each (total n = 100). When randomly selected insects were analyzed for GFP expression 3 days post infection, GFP expression was recorded in all the tissues tested (Fig. 3A). Some of larvae infected with BmNPV-BmA::GFP/P10::N-Flag/SnJHER had difficulty moulting 5–8 days post infection (end of L5) (Fig. 3B). This was the case for 8 ± 4% of the larvae infected with BmNPV-BmA::GFP/P10::N-Flag/SnJHER but none of those injected with BmNPV/BmA::GFP-BmA::dsLuciferase (Fig. 3B). Abnormal animals were examined using fluorescence microscopy (2 per replicate) and the general esterase activity of samples of their haemolymph was determined using gel assays with α-Na as a substrate. Specific electrophoretic bands corresponding to ectopic esterase activity were recorded for BmNPV-BmA::GFP/P10::N-Flag/SnJHER infected animals, but not for the controls (data not shown).

The infected insects in the previous experiment that developed normally (day 8 post infection) (n1 = 28, n2 = 31, n3 = 33) were injected with 100 μM of methoprene. One

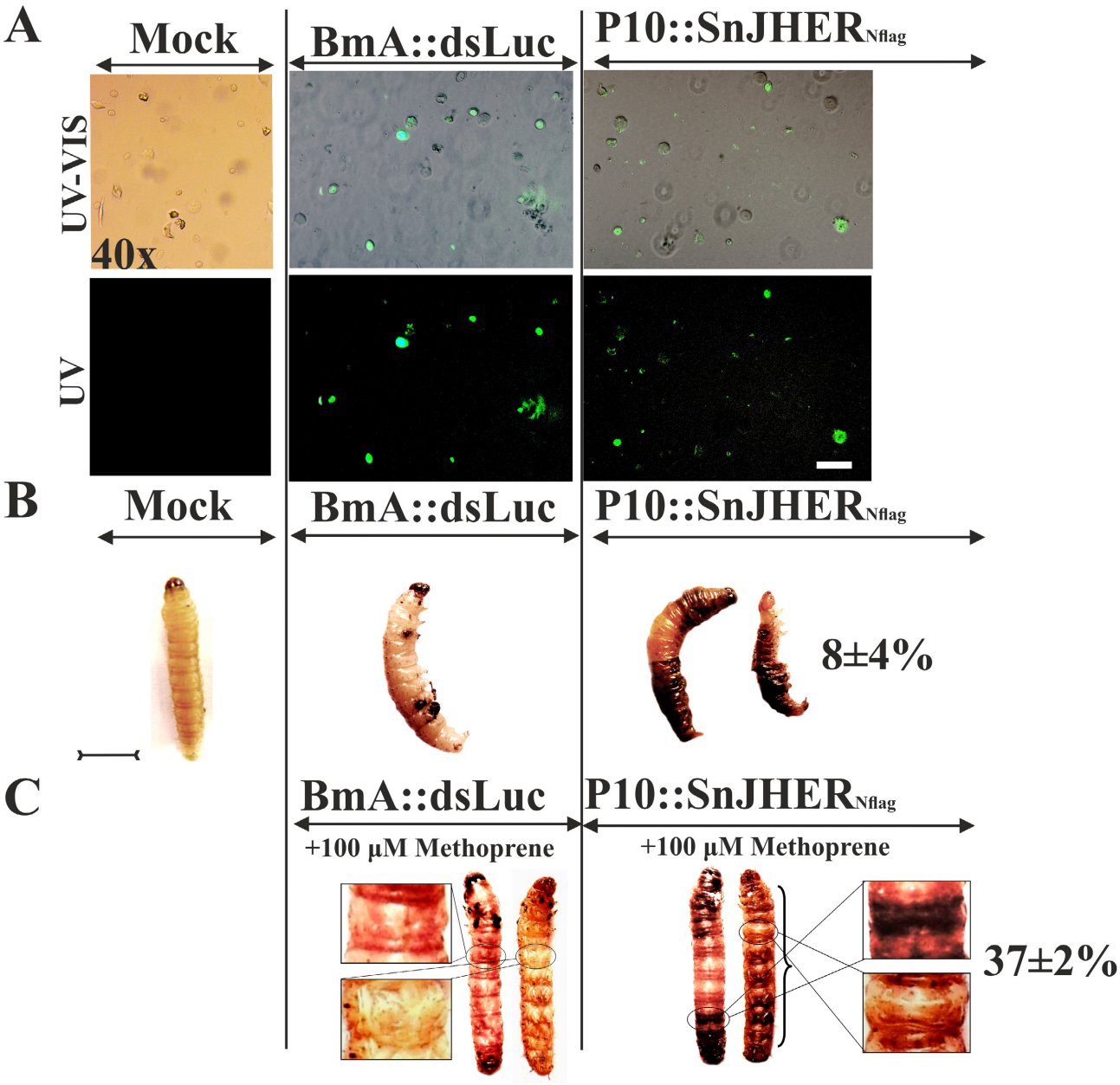

Fig. 3. Baculovirus-mediated over-expression of SnJHER in the larvae of *S. nonagrioides*. (A) Fluorescence microscope images of haemolymph cells from BmNPV-BmA::GFP/BmA::dsLuciferase and BmNPV-BmA::GFP/P10::N-Flag/SnJHER infected animals. "UV" corresponds to fluorescence only, while "UV-VIS" shows both fluorescent and bright field images. The length of the white bar corresponds to 200 µm. (B) Phenotypic analysis of larvae 8 days after being subjected to baculovirus-mediated N-Flag/SnJHER over-expression. The percentage of larvae (% ± SD) with a particular phenotype in the three experimental groups is indicated. (C) The abnormalities recorded in BmNPV-BmA::GFP/P10::N-Flag/SnJHER infected animals after treatment with 100 µM of the JHA methoprene. The percentage of larvae (% ± SD) with a particular phenotype in the three experimental groups is indicated. The vertical bar next to the mock-treated larva corresponds to a length of 1 cm.

to two days later (end of L5) these insects were examined for abnormalities. In the control BmNPV/BmA::GFP-BmA::dsLuciferase animals no abnormalities were recorded (Fig. 3C), but in contrast, the BmNPV-BmA::GFP/P10::N-Flag/SnJHER animals treated with 100 µM of methoprene developed severe malformations (blackening of cuticle at boundaries of segments, malformed segments). These abnormalities were recorded in 37 ± 2% of the injected larvae from three different batches (Fig. 3C).

At the end of the observation (10 days) a high percentage of the remaining methoprene-injected animals in both control and treated groups died as a result of the methoprene injection but none of them developed the abnormalities previously recorded.

DISCUSSION

Transient expression of the major isoform of SnJHER in Hi5 cells resulted in two bands in the insoluble protein fraction, (one weaker in density of approximately ~ 64 kDa and one major band running slower of 60 kDa); in the soluble fraction, on the other hand, only the major band was recorded. A similar pattern was recorded for stable lines and

when Bm5 cells were infected with BmNPV-BmA::GFP/P10::N-Flag/SnJHER virus (Fig. 1).

In cell lysates and supernatants of Sf21 cells that were infected with recombinant baculovirus over-expressing *Heliothis virescens* JHE, several protein bands of around 65 kDa were detected using western blot and specific antisera (Eldridge et al., 1992). Moreover, if cells were treated with tunicamycin (a strong inhibitor of N-linked glycosylation), the apparent size of the immunopositive bands decreased to 60 kDa. As is the case for all JHE and JHER proteins studied so far (Jones et al., 1994), SnJHER also contains glycosylation sites [NX(S/T)] in its predicted protein sequence (Kontogiannatos et al., 2011).

The electrophoretic diversity of SnJHER in cell lines can be explained by the post-translational modifications in the translated protein as is recorded for *H. virescens* JHE. However, little glycosylation seems to occur when SnJHER is expressed in Hi5 cells since the predicted unglycosylated form (60 kDa) is much more abundant than the glycosylated form (64 kDa) (Fig. 1). In baculovirus-infected Bm5 cells, on the other hand, more extensive glycosylation may have occurred (MW bands of 64 and 70 kDa; Fig. 1). Finally, a specific protein with esterase activity was present in cell extracts and media from stable Hi5 cell lines, indicating functional expression of SnJHER (Fig. 1).

JHER expressing cell lines seemed to respond in a more potent manner to the JHA methoprene than the control cell line. As occurred with the Sf9 cells (Giraudo et al., 2011), 50 μM of the JHA methoprene slightly reduced cell viability 24 h post treatment but only in the control B2REV cell line. In the JHER-expressing cell line, on the other hand, viability was reduced significantly by up to ~20%. In contrast, no significant alterations were recorded for the JHAs kinoprene and fenoxycarb in both JHER-expressing and control cell lines.

In *Culex quinquefasciatus* only hydroprene and kinoprene can compete for JHIII hydrolysis in in vitro bioassays. Methoprene and fenoxycarb seem to have no access to the active centre of the JHE enzyme (Kamita et al., 2011). However, it is reported that methoprene can be metabolized by esterases with a broad activity (Wright, 1976; Morello et al., 1980). In our study, SnJHER seems to enhance the toxic effects of methoprene, but not of fenoxycarb and kinoprene in the Hi5 cell lines. We therefore propose that in lepidopteran cells either methoprene is metabolized to a more toxic product by JHER, or JHER interferes with the protective/tolerance mechanisms against methoprene.

In order to assess the biological role of SnJHER, the larval tissues of *S. nonagrioides* were infected with a baculovirus that over-expresses SnJHER. The insects were infected at the beginning of the 5th instar, when *SnJHER* expression is low (Kontogiannatos et al., 2011, 2016). SnJHER over-expression resulted in moulting abnormalities 5–8 days post infection, which coincides with the moult to the 6th larval instar. These abnormalities were recorded for 8 ± 4% of the infected animals (Fig. 3B).

Previously, baculoviruses (based on *Autographa californica* multiple nuclear polyhedrosis virus or AcMNPV) were used to infect *H. virescens* larvae and to over-express modified forms of JHE, i.e. JHE-KK in which two lysine residues were replaced with arginine residues to reduce the efficiency of lysosomal targeting; JHE-SG in which the catalytic serine was replaced with glycine, which eliminated catalytic activity; JHE-KSK, which contained a combination of the above mutations; and JHE-KHK which is also based on JHE-KK but in which a catalytic histidine was converted to lysine (Bonning et al., 1995, 1997, 1999; van Meer et al., 2000). These experiments showed that JHE-KK is resistant to degradation in pericardial cells (Bonning et al., 1997), which resulted in 15% of the larvae showing symptoms of contraction paralysis (Bonning et al., 1999). In contrast, JHE-SG disrupted the moulting process; a considerable proportion of the larvae infected with AcJHE-SG died at the moult after developing extreme cuticular blackening (Bonning et al., 1995). Consequently biochemical analysis of JHE-KHK and JHE-KSK produced in insect cell cultures showed that mutation of the catalytic site serine in JHE-KSK or histidine in JHE-KHK removes all JHE catalytic activity (van Meer et al., 2000).

In this study, it was found that over-expression of SnJHER also results in moulting defects (Fig. 3B), similar to those produced by catalytically inactive JHE in *H. virescens*. This comparison suggests that SnJHER interferes with the action of conventional JHE in *S. nonagrioides*. While for further clarification of this hypothesis is needed the isolation of the conventional *JHE* in *S. nonagrioides*, it is noted that in *T. ni*, both JHER and conventional JHE hydrolyse JH at disproportionately higher rates at higher substrate concentrations and are similarly inhibited by an organophosphate (Kadono-Okuda et al., 2000). However, TniJHER is less sensitive to trifluoromethyl ketone transition state analogs (Kadono-Okuda et al., 2000) indicating that the two enzymes have different properties. Researchers therefore have proposed a model in which TniJHER is expressed just prior to metamorphosis in order to hydrolyse a JH-like substrate, which may differ from the JH substrates in other species (Kadono-Okuda et al., 2000).

There are indications that metamorphosis is regulated by different JHE-like enzymes and different JH-like substances in different species of Lepidoptera. In *S. nonagrioides*, the JHE-specific inhibitor OTFP does not provoke a developmental response as in the sphingid *M. sexta* and some other insects, in which JHE is required for metamorphosis (Schafellner et al., 2008). However, these experiments need to be interpreted with caution since it cannot be excluded that OTFP may inhibit other (unidentified) esterases that could result in the phenotypes observed.

That the enzymatic activity of SnJHER is distinctly different from that of JH is also illustrated by its interaction with methoprene (Figs 2 and 3C). In Hi5 cells, SnJHER increases the toxicity of methoprene and in larvae a greater incidence of abnormalities. By contrast, methoprene does not interfere with the enzyme activity of canonical JHE, at least in mosquitoes (Kamita et al., 2011) and it is suggested that methoprene interacts with other non-specific esterases in insects. Because SnJHER increases the toxic effects of

methoprene, it is possible that its overexpression interferes with the mechanisms in lepidopteran cells that protect them against JHAs. Given the complexity of expression of esterase enzymes in insects, it is possible that the major function of SnJHER is not related to developmental processes (moulting and metamorphosis) and that the abnormalities are caused by the overexpression of the enzyme at a sensitive period in development. Future studies should focus on the biochemical characterization of SnJHER, such as the identification of its preferential substrates. More specifically, its interaction with JH substrates (in terms of k_{cat} and K_m) should be determined and compared with canonical JHE enzymes.

Baculovirus infection can have a considerable effect on host endocrinology by interfering with JH and ecdysone regulatory pathways. Both AcMNPV and BmNPV encode for an ecdysteroid UDP-glucosyltransferase (EGT) gene, which inactivates ecdysone by conjugating the hydroxyl group at C-22 with a sugar (O'Reilly & Miller, 1989; Eldridge et al., 1992; O'Reilly et al., 1992; Kang et al., 1998). Insects infected with a virus containing the gene encoding EGT do not moult because of a lack of active ecdysone (O'Reilly & Miller, 1989; Eldridge et al., 1992; O'Reilly et al., 1992). Moreover the baculoviruses AdhoNPV and AdorNPV, which, respectively, kill *Adoxophyes honmai* (Lepidoptera: Tortricidae) slowly and quickly, decrease JHE but not JHEH activity levels (Saito et al., 2015). In *S. nonagrioides*, infections with both BmNPV and AcMNPV block larval-pupal and pupal-adult moults and AcMNPV arrests moulting in larvae (Kontogiannatos et al., 2013). On the other hand, larvae of *S. nonagrioides* infected with BmNPV were able to complete their development and show no symptoms of polyhedrosis (Kontogiannatos et al., 2013). We speculate that BmNPV is not as effective as AcMNPV in interfering with the hormonal pathways of *S. nonagrioides*, which is probably a consequence of its much more limited host range. BmNPV could be an appropriate gene transduction vector for functional studies on larvae of *S. nonagrioides*. But this method should not be used for functional analysis of genes implicated in larval-pupal transformation. For such studies, this method needs to be improved by genetically modifying BmNPV to have a smaller effect on the physiology of infected cells so that there is a clearer distinction between infection-related effects and those caused by transgene expression.

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