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ORIGINAL ARTICLE

Cloning and characterization of Methoprene-tolerant (Met) and Krüppel homolog 1 (Kr-h1) genes in the wheat blossom midge, *Sitodiplosis mosellana*

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Abstract Juvenile hormone (JH), a growth regulator, inhibits ecdysteroid-induced metamorphosis and controls insect development and diapause. Methoprene-tolerant (Met) and Krüppel homolog 1 (Kr-h1) are two proteins involved in JH action. To gain some insight into their function in development of *Sitodiplosis mosellana*, an insect pest undergoing obligatory larval diapause at the mature 3rd instar stage, we cloned full-length complementary DNAs of *Met* and *Kr-h1* from this species. SmMet encoded a putative protein, which contained three domains typical of the bHLH-PAS family and eight conserved amino acid residues important for JH binding. SmKr-h1 encoded a protein showing high sequence homology to its counterparts in other species, and contained all eight highly conserved Zn-finger motifs for DNA-binding. Expression patterns of *SmMet* and *SmKr-h1* were developmentally regulated and JH III responsive as well. Their mRNA abundance increased as larvae entered early 3rd instar, pre-diapause and maintenance stages, and peaked during post-diapause quiescence, a pattern correlated with JH titers in this species. Different from reduced expression of *SmMet*, *SmKr-h1* mRNA increased at mid-to-late period of post-diapause development. Topical application of JH III on diapausing larvae also induced the two genes in a dose-dependent manner. Expression of *SmMet* and *SmKr-h1* clearly declined in the pre-pupal phase, and was significantly higher in female adults than male adults. These results suggest that JH-responsive *SmMet* and *SmKr-h1* might play key roles in diapause induction and maintenance as well as in post-diapause quiescence and adult reproduction, whereas metamorphosis from larvae to pupae might be correlated with their reduced expression.

Key words diapause; juvenile hormone; *Kr-h1*; *Met*; *Sitodiplosis mosellana*

Introduction

Juvenile hormone (JH), a sesquiterpenoid produced by the insect corpora allata (CA), controls many key processes during the insect life cycle. JH prevents metamorphosis in larvae until they have attained an appropriate stage (Riddiford *et al.*, 2003; Jindra *et al.*, 2013), and stimulates oogenesis in adult females (Riddiford, 2012; Roy *et al.*, 2018). Besides its involvement in metamorphosis and reproduction, JH also regulates diapause, an adaptive strategy that insects use to survive seasonal adverse environmental

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conditions such as temperature extremes (Takaaki & Denlinger, 2002). The roles of JH in induction and maintenance of larval diapause were initially established by Yin and Chippendale (1973) in *Diatraea grandiosella*, and later further confirmed by studies in several other insect species, such as the Mediterranean corn borer, *Sesamia nonagrioides* (Eizaguirre *et al.*, 1998, 2005), the European corn borer, *Ostrinia nubilalis* (Yagi & Akaike, 1976), the yellow-spotted longicorn beetle, *Psacothea hilaris* (Munyiri & Ishikawa, 2004) and the beet webworm, *Loxostege sticticalis* (Jiang *et al.*, 2011). However, in the bamboo borer *Omphisa fucidentalis*, high JH causes termination of larval diapause (Singtripop *et al.*, 2000, 2008), suggesting functional divergence of JH in regulating this important physiological process. In addition, JH is known to terminate adult reproductive diapause and stimulate oogenesis in females of the linden bug, *Pyrrhocoris apterus* (Bajgar *et al.*, 2013; Smykal *et al.*, 2014a).

Understanding of JH action has been advanced by identification of the basic helix-loop helix (bHLH)/Per-Arnt-Sim (PAS) transcription factor Methoprene-tolerant (Met) (Ashok *et al.*, 1998) as an intracellular JH receptor (Charles *et al.*, 2011; Jindra *et al.*, 2015). Met was originally discovered in a *Drosophila melanogaster* mutant that is resistant to a JH analog, methoprene (Wilson & Fabian, 1986). Met-deficiency in *Tribolium castaneum* caused larvae to pupate prematurely (Konopova & Jindra, 2007). Met orthologs from *D. melanogaster*, *T. castaneum* and mosquito *Aedes aegypti* bind JH with high affinity (Miura *et al.*, 2005; Charles *et al.*, 2011; Li *et al.*, 2014; Jindra *et al.*, 2015). Met forms a transcriptionally active complex with another bHLH-PAS protein Taiman and directly activates downstream genes such as *Krüppel homolog 1* (*Kr-h1*) (Li *et al.*, 2011, 2014; Kayukawa *et al.*, 2012; Cui *et al.*, 2014). Kr-h1, a C_2H_2 zinc-finger type transcription factor, was found to transduce the JH signal downstream of Met. The respective functions of Met and Kr-h1 in JH signal perception and transduction to prevent metamorphosis have been demonstrated through RNA interference and gene editing experiments in holometabolous and hemimetabolous species (Parthasarathy *et al.*, 2008; Konopova *et al.*, 2011; Lozano & Belles, 2011; Smykal *et al.*, 2014b; Daimon *et al.*, 2015). In almost all cases, depletion of *Met* and *Kr-h1* mRNA levels in the juvenile stage results in precocious metamorphosis. Met also facilitates JH-induced ovarian development during adult reproduction in several species including the beetle *Colaphellus bowringi* (Liu *et al.*, 2016) and the linden bug *Pyrrhocoris apterus* (Smykal *et al.*, 2014a). However, the role of Met and Kr-h1 in larval diapause is yet to be determined.

The orange wheat blossom midge *Sitodiplosis mosellana* (Géhin) is one of the most destructive pests of wheat in northern China and many other countries in northern Asia, Europe, and North America (Gaafar & Volkmar, 2010; Miao *et al.*, 2013; Jacquemin *et al.*, 2014). This species is univoltine and undergoes an obligatory larval diapause. Adults emerge from pupae in soil and oviposit in late April in most of northern China (Wang *et al.*, 2015). Hatched larvae of the initial two instars feed on developing kernels. Mature 3rd instar larvae drop to the ground during middle to late May, and burrow into the soil to spin round diapausing cocoons. They do not leave cocoons until the following year in mid-March. Therefore, the long period of larval diapause enables this species to not only cope with harsh seasons such as hot summers and cold winters, but also aligns its development with the phenology of the wheat.

Studies on *S. mosellana* have implied that JH plays a role in diapause regulation (Li *et al.*, 2006), but the molecular mechanism is not yet well understood. To begin to dissect the molecular action of JH here, we cloned cDNAs encoding Met and Kr-h1 from *S. mosellana* by reverse-transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques. Relative expression of Met and Kr-h1 was determined using quantitative PCR (qPCR) at various stages throughout the development. In addition, effects of JH treatments on *Met* and *Kr-h1* gene expression were examined. Our study has set a foundation for further exploration of their roles in regulation of *S. mosellana* development, potentially useful for effective control of this pest.

Materials and methods

Insect collection

S. mosellana at different developmental stages, including the 1st and 2nd instar larvae, the 3rd instar larvae in pre-diapause, diapause, post-diapause quiescent and developing stages, pre-pupae, pupae of early, middle and late stages, as well as adult males and females, were obtained according to methods previously described (Lamb *et al.*, 2000; Wu *et al.*, 2011; Cheng *et al.*, 2016). Specifically, during early to late May 2013, we gathered wheat spikes infested with *S. mosellana* from a wheat field at Zhouzhi County (34°09′ N, 108°13′ E), Shaanxi Province, China, at the grain filling stage, and collected larvae of the first two instars and pre-diapausing 3rd instar larvae by dissecting wheat spikes under stereomicroscopes. Larval stages were distinguished based on body size, color and shape. Subsequently, wheat ears containing mature pre-diapausing 3rd instar larvae were harvested and put on soil in a field insectary in Yangling, Shaanxi Province, China (34°16′

N, 108°4′ E). The soil was watered to maintain moisture for insects entering and breaking diapause. Cocooned larvae were collected from this field insectary monthly, from late June 2013 to late February 2014. We found that the vast majority of cocooned larvae collected in December or later could emerge as adults after being transferred to 25 °C, indicating that by December they have completed diapause and entered post-diapause quiescence (Cheng *et al.*, 2017). After mid-March, post-diapause developing larvae, pre-pupae, early to late pupal stages and adults were successively collected. All insect samples collected were frozen immediately in liquid nitrogen and stored at –80 °C until RNA extraction.

cDNA cloning of S. mosellana Met and Kr-h1

Total RNA was extracted from the pre-diapause 3rd instar larvae of *S. mosellana* using the RNAsimple Total RNA Kit (Tiangen, Beijing, China). The concentration and integrity of RNA were detected by a spectrophotometer Nanodrop2000c (Thermo Fisher Scientific, West Palm Beach, FL, USA) and 1% agarose gel electrophoresis, respectively. One microgram of genomic DNA-free total RNA was reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) according the manufacturer's protocol. The synthesized first-strand cDNA was stored at –20 °C until use.

To obtain cDNA fragments, primers of *Met* were designed based upon a transcriptome dataset we generated earlier from *S. mosellana* larvae, whereas degenerate forward and reverse primers for *Kr-h1* were designed based on the conserved amino-acid sequences from other dipterans including *Aedes aegypti* (GenBank accession no. EAT46451.1), *Anopheles darling* (ETN65255.1), *Anopheles sinensis* (KFB38362.1), *Bactrocera dorsalis* (XP_011213115.1) and *Ceratitis capitata* (XP_004520990.1) (Table S1). PCR reactions (in 20 μL) containing 10 μL of 2 × Taq MasterMix (CWBio, Beijing, China), 1 μL of cDNA, 1 μL of each primer (10 μmol/L) and 7 μL of RNase-Free dH_2O followed the procedure of 3 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 150 s at 72 °C for Met or 30 s at 72 °C for Kr-h1; 10 min at 72 °C. PCR fragments with the expected sizes were purified from 1% agarose gel using a DNA gel extraction kit (Tiangen Biotech, Beijing, China), ligated into the pMD™-19T vector (Takara, Dalian, China), and transformed into DH5α competent cells (CWBio, Beijing, China). Positive clones were selected using a blue-white screening, and cultured in liquid LB medium. Insert identities were verified by PCR and sequencing analyses (Invitrogen Company, Life Technologies, Shanghai, China).

Based on the cDNA fragment sequence information, gene-specific primers for nested 5′ and 3′-RACE were synthesized (Table S1). cDNAs used for RACE were synthesized from 1 μg of total RNA using a 3′-Full RACE Core Set with PrimeScript™ RTase and 5′-Full RACE Kit with TAP (TaKaRa, Dalian, China) according to the user's manual. The primary and secondary PCRs of 5′-RACE were carried out with the following protocol: the initial 3 min at 94 °C; followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C; and an extra extension of 10 min at 72 °C. For 3′-RACE, the same conditions were used except that the secondary PCR cycle number was 35. PCR products were examined by electrophoresis, recovered and sequenced. Finally the full-length *Met* and *Kr-h1* cDNAs were PCR amplified based on RACE-PCR sequencing results using primers shown in Table S1. The following amplification conditions were applied: 3 min at 95 °C; followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 150 s at 72 °C for *Met* or 60 s at 72 °C for *Kr-h1*; and 10 min at 72 °C. The PCR products were purified, cloned and sequenced as above for verification.

Sequence analysis and phylogenetic tree construction

Open reading frames (ORF) of the putative *Met* and *Kr-h1* were determined by National Center for Biotechnology Information (NCBI) ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight (MW) and isoelectric point (pI) of deduced proteins were calculated by the Compute pI/Mw tool (http://web.expasy.org/protparam/). Functional domains were predicted by NCBI conserved domain search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Protein sequence similarity was determined using the Blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Alignment of deduced amino acid sequences was performed by the DNAMAN 6.0 software package (Lynnon Corporation, Pointe-Claire, Quebec, Canada). Phylogenetic trees were built using the neighbor-joining method (Saitou & Nei, 1987) with Molecular Evolutionary Genetics Analysis (MEGA version 6.0) software based on full-length protein sequences of 14 (for Met) and 13 (for Kr-h1) insect species from Diptera, Coleoptera and Lepidoptera (Fig. 3), and presented with bootstrap values based on 1000 replicates.

JH treatment

To study effects of JH on expression of *Met* and *Kr-h1* in diapausing larvae of *S. mosellana*, cocooned

larvae freshly collected in July were abdomen-injected with 23 nL JH III (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 0.1–0.3 pg/nL diluted in acetone using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). Injected larvae were placed in Petri dishes (with moist filter papers) and continuously incubated for 3, 6, 12 or 24 h in the 25 °C incubator prior to freezing in liquid nitrogen and stored in –80 °C for RNA isolation. Insects at this stage were chosen because they exhibited low expression of *Met* and *Kr-h1* (see Results). Injection doses were determined by physiological JH levels, that is the highest dose of JH III injected was comparable with the highest titer of native JH III reported in cocooned larvae of this species (Li *et al.*, 2006). Control insects were treated with equivalent volumes of acetone. All treatments were repeated three times with at least 20 individuals per replication.

Expression analysis using qPCR

To determine the developmental expression profiles of *S. mosellana Met* and *Kr-h1*, approximately 100 of 1st instar larvae, 40 of 2nd instar larvae, 20 of 3rd instar larvae at every diapausing stage, 20 pupae at each stage or adults, respectively, were used for RNA isolation. To analyze transcript abundance in JH-treated larvae, 20 individuals from each treatment were used for RNA isolation. Total RNA isolation and cDNA synthesis were performed as described above.

qPCR was carried out using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech) on the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Gene-specific primers (Table S1) were designed according to the full-length cDNAs obtained. The reference gene *gapdh* (GenBank number: KR733066) was used as the experimental control. qPCR reactions were performed in 20 μL volumes, which consisted of 10 μL of 2× SuperReal PreMix Plus, 1 μL of cDNA, 1 μL of each primer (10 μmol/L) and 7 μL of RNase-free H_2O. The following amplification conditions were applied: an initial denaturation at 95 °C for 15 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. A melting curve program, a temperature increasing from 55 °C to 95 °C at a rate of 0.5 °C/10 s, was performed to ensure qPCR amplification quality. In order to obtain a more precise quantification, we used the pooled cDNAs of different samples as the template to construct the standard curves for both target and reference genes. The cDNA templates were serially diluted. The results showed that the amplification efficiencies for primers of *Met*, *Kr-h1* and *gapdh* were 96.3%, 95.6% and 95.0%, respectively. Three biological replicates were prepared for each stage or treatment, and all samples were performed in triplicate.

The transcript abundance of each gene was estimated from the Ct (cycle threshold) values and relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). All data were presented as the mean ± standard error (SE). The difference among the groups was analyzed by one-way analysis of variance followed by Duncan's multiple range tests for pairwise comparison ($P < 0.05$). Correlation between the expression level of each gene and concentration of JH treatments was conducted using correlation analysis. All analyses were carried out using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

Results

Characterization of SmMet and SmKr-h1 cDNAs

The full-length cDNA of *S. mosellana Met* (*SmMet*) obtained by RACE-PCR was 5264 bp long (Fig. 1 A, GenBank accession number KY660529), consisting of a 2733 bp ORF, a 1 230 bp 5′-untranslated region (5′-UTR) and a 1301 bp 3′-UTR. The ORF encoded a protein of 910 amino acids with a predicted molecular weight of 102.3 kDa and a theoretical pI of 5.51. Four putative polyadenylation signals (aataaa) were identified at nucleotides 4248–4253, 4463–4468, 4527–4532 and 4854–4859, respectively. The full-length cDNA of *SmKr-h1* was 2327 bp (Fig. 1B, GenBank: KY646174), including an 1842 bp ORF, and 64 and 421 bp 5′- and 3′-UTRs, respectively. The encoded protein had 613 amino acid residues with a predicted molecular weight of approximately 67.7 kDa and a pI of 8.73. A putative polyadenylation signal was found at nucleotides 1934–1939.

Analysis of the deduced amino acid sequences by Signal P4.1 failed to detect signal peptides, suggesting that SmMet and SmKr-h1 were non-secretory proteins. Scanning for conserved domains showed that SmMet possessed three typical functional domains of bHLH-PAS protein family: bHLH (DNA binding and dimerization regions, a.a. 95–148), PAS-A (dimerization region, a.a. 174–246) and PAS-B (ligand binding and dimerization region, a.a. 360–467) (Figs. 1A and 2A) (Charles *et al.*, 2011; Li *et al.*, 2011, 2014). Further, eight conserved amino acid residues shown to be involved in JH binding in *Tribolium* Met (Charles *et al.*, 2011) were identified in the PAS-B domain (Fig. 2A). Likewise, SmKr-h1 contained all of the eight highly conserved C_2H_2 Zn-finger DNA-binding domains (Zn1–8), similar to previously identified homologues (Figs. 1B and 2B). Also, an adenosine monophosphate binding site motif HGKTNGTRA (a.a.116–124) (Tomkinson *et al.*, 1991) was well conserved (Fig. 1B).

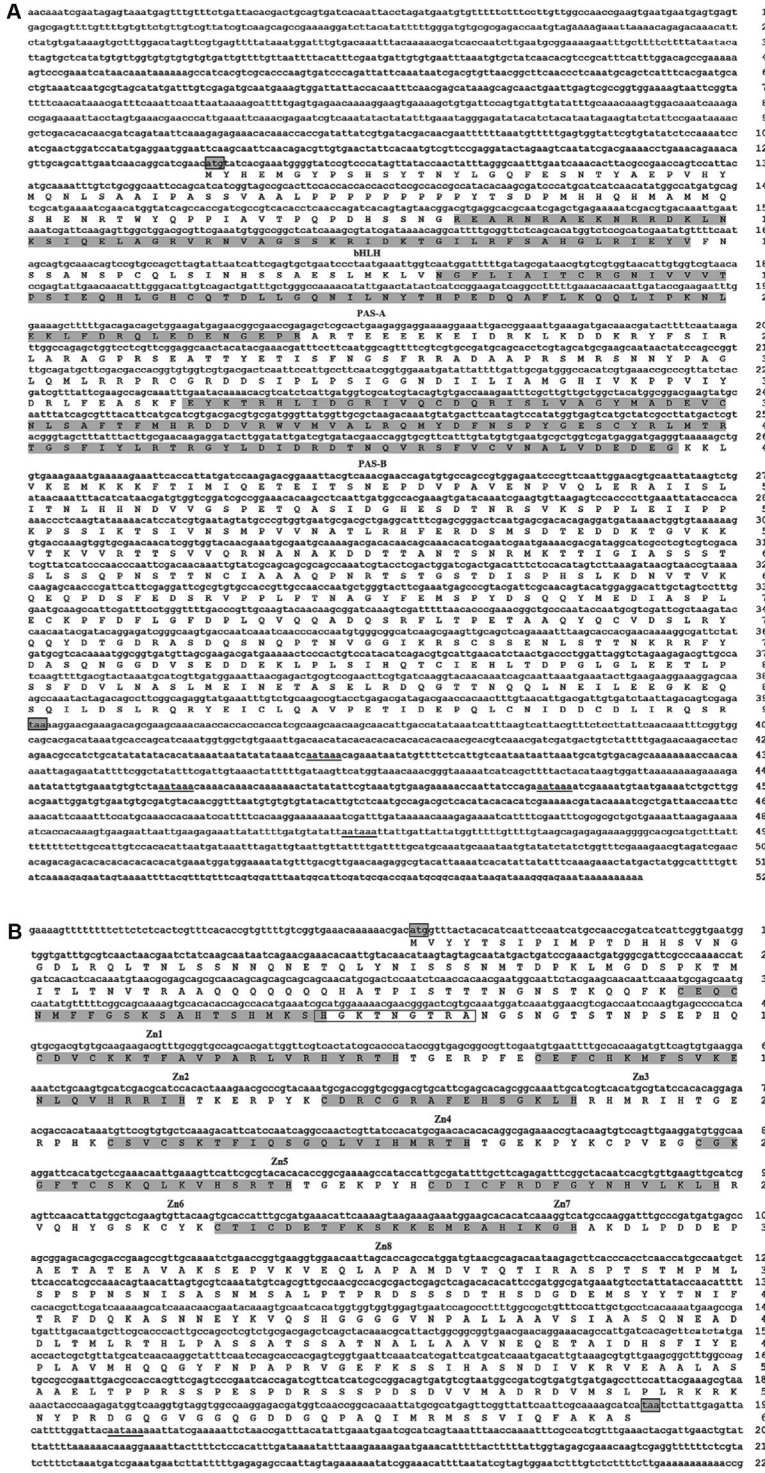

Fig. 1 Nucleotide and deduced amino acid sequences of Methoprene-tolerant (*Met*) (A) and Krüppel homolog 1 (*Kr-h1*) (B) in *Sitodiplosis mosellana.* Start codons (atg) and stop codons (taa) are shaded and boxed. The putative polyadenylation signals (aataaa) were underlined. Three typical domains of basic helix-loop helix (bHLH), PAS-A and PAS-B in Met are shaded and labeled; eight zinc-fingers in Kr-h1 were shaded and labeled from Zn1 to Zn8, respectively. An adenosine monophosphate binding site motif in Kr-h1 was boxed.

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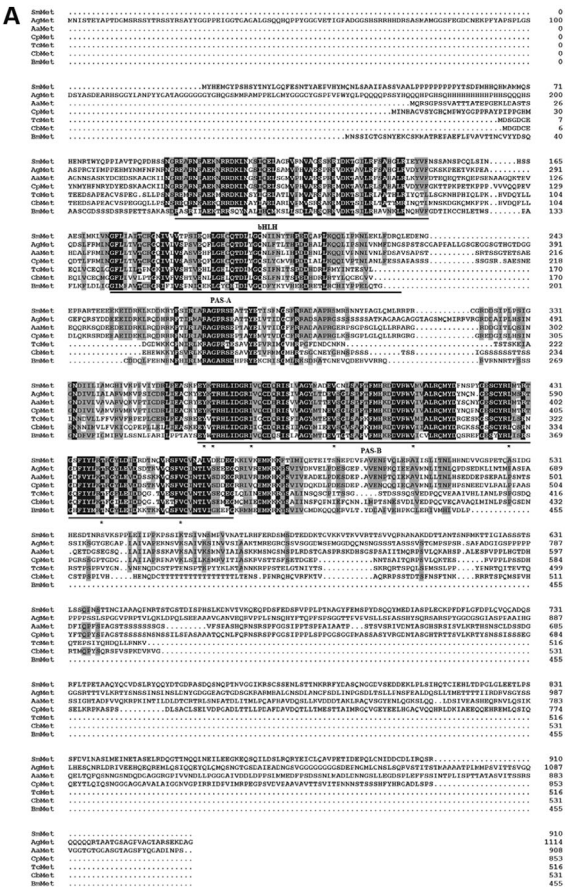

Fig. 2 Multiple sequence alignments of the deduced amino acid sequences of *Sitodiplosis mosellana* Methoprene-tolerant (Met) (A) and Krüppel homolog 1 (Kr-h1) (B) with other insect Mets and Kr-h1s. Identical or similar amino acids are shaded black or gray. Species abbreviations: Sm = *Sitodiplosis mosellana*, Ag = *Anopheles gambiae*, Aa = *Aedes aegypti*, Cp = *Culex pipiens*, Tc = *Tribolium castaneum*, Cb = *Colaphellus bowringi*, Bm = *Bombyx mori*, Dm = *Drosophila melanogaster*, Cc = *Ceratitis capitata*, Bd = *Bactrocera dorsalis*, Lc = *Lucilia cuprina*, Md = *Musca domestica*. The residue indicated by the asterisk (*) in the PAS-B domain (A) are the active sites that can form the ligand-binding pocket binding of juvenile hormone (JH).

Basic Local Alignment Search Tool analysis showed that SmMet shared the highest amino acid sequence identity (55%) to the homolog from *Anopheles gambiae* (ABC18327.1), 51%–52% to homologs from *Aedes aegypti* (AAW82472.1) and *Culex pipiens* (AAW81958.1), and 38%–45% to those from other insect orders including *T. castaneum* (NP_001092812.1), *Colaphellus bowringi* (AMK38169.1) and *Bombyx mori* (ACJ04052.1). Furthermore, sequences of bHLH and PAS-B domains were highly conserved whereas PAS-A was more variable (Fig. 2A). SmKr-h1 displayed 81% identity to *A. aegypti* Kr-h1 (EAT46451.1), and 72%–77% identity to Kr-h1s from *Drosophila melanogaster* (NP_477467.1), *Ceratitis capitata* (XP_004520990.1), *Bactrocera dorsalis* (XP_011213115.1), *Lucilia cuprina* (KNC32499.1) and *Musca domestica* (XP_011295274.1). As expected, phylogenetic analysis indicated that Mets and Kr-h1s from Dipteran, Coleopteran and Lepidopteran were separately grouped together (Fig. 3), consistent with the classical taxonomic divergence. Notably, SmMet and SmKr-h1 were more closely related to their homologs from the Nematocera (*A. gambiae*, *A. aegypti* and *C. pipiens* for Met and *A. aegypti* for Kr-h1).

Expression of SmMet and SmKr-h1 during diapause

To determine diapause-associated expression profiles of *SmMet* and *SmKr-h1*, we examined the relative mRNA levels of both genes using qPCR in four diapause-associated stages of 3rd instar larvae: pre-diapause (May), diapause (June–November), post-diapause quiescence (December–February of the following year) and post-diapause development (middle March–early April of the following year) (Fig. 4A). The transcript abundance of *SmMet* was slightly higher after entering diapause (June), maintained in stable levels from June to October, but rapidly increased from November, coinciding with the transition time from diapause to post-diapause quiescence, and reached the maximum in December and

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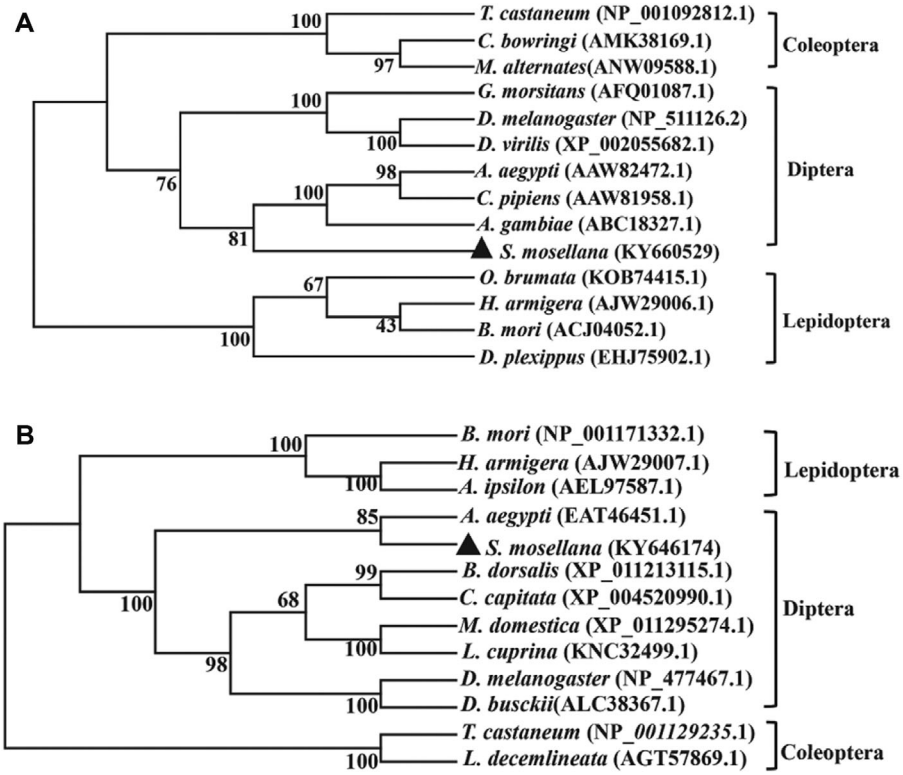

Fig. 3 The neighbor-joining phylogenetic tree analysis built by MEGA 6.0 software for Methoprene-tolerant (Met) (A) and Krüppel homolog 1 (Kr-h1) (B) from *Sitodiplosis mosellana* and other insects.

January. Expression drastically declined thereafter, and returned to the pre-diapause level at the post-diapause development stage.

SmKr-h1 mRNA was also relatively low in pre-diapause and early period of post-diapause development (mid-March), and peaked during early-to-mid period of post-diapause quiescence (December and January). Different from *SmMet*, its expression distinctly increased again at the mid-to-late period of post-diapause development (Fig. 4B).

Effects of JH III on expression of SmMet and SmKr-h1 in diapausing larvae

SmMet and *SmKr-h1* exhibited similar expression patterns when larvae at diapause maintenance stage (collected in July) were subjected to JH III treatments. Gene expression was clearly induced at time points examined, that is 3–24 h after treatment, and showed significantly positive correlation with JH concentration, with correlation index of $r > 0.80$ ($P < 0.01$) except for *SmKr-h1* at 24 h ($r = 0.602$ [$P = 0.039$]). A concentration higher than 0.2 pg/nL did not further increase mRNA levels except for *SmMet* at 3 h after treatment (Fig. 5). The correlation was particularly strong at 6 h after treatment, with $r = 0.968$ for *SmMet* and 0.924 for *SmKr-h1*, followed by 3 and 12 h after treatment.

Expression of SmMet and SmKr-h1 at different developmental stages

In addition to diapausing larval stage, we also profiled expression of *SmMet* and *SmKr-h1* in various developmental phases throughout the entire life cycle of *S. mosellana*, including 1st and 2nd instar larvae, 3rd instar larvae in early and late stages, pre-pupae, pupae at all three stages, male and female adults (Fig. 6).

The lowest expression for both genes was detected in the 2nd instar larvae. Drastic increase occurred once insects became the 3rd instar larvae. At pre-pupal stage, *SmKr-h1* declined much more rapidly than *SmMet*. Furthermore, expression in adult males was significantly lower than that in adult females (Fig. 6).

Discussion

As key mediators of JH signals, Met and Kr-h1 are supposed to play a role in JH action in regulating larval

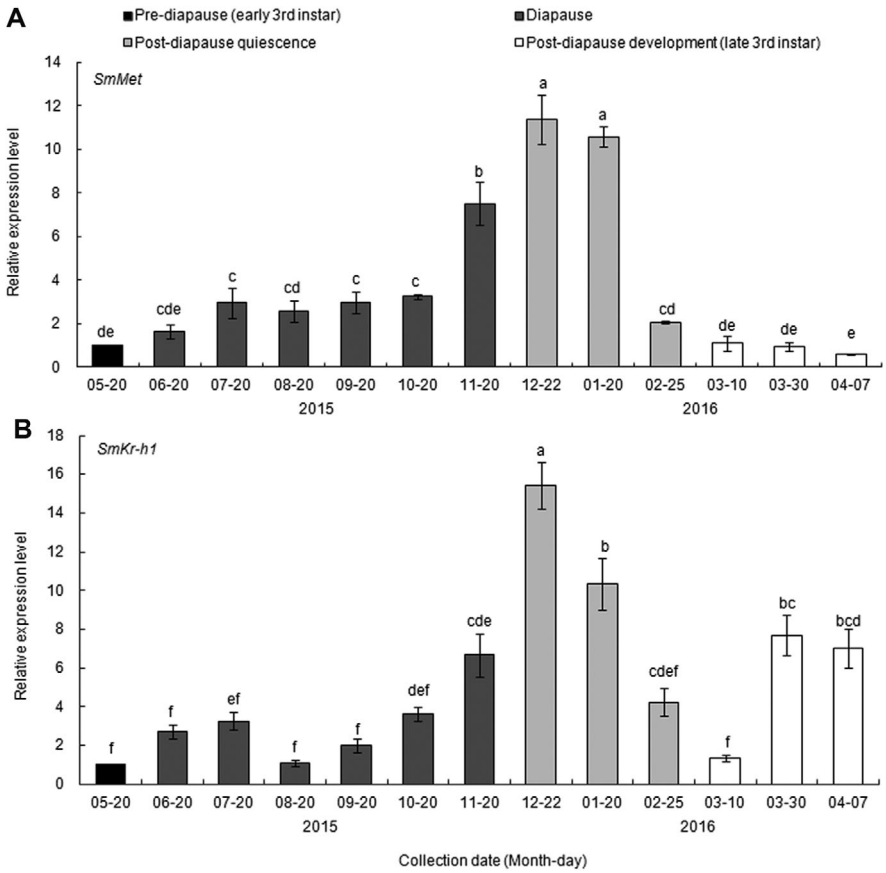

Fig. 4 Expression profiles of *Sitodiplosis mosellana* Methoprene-tolerant (*Met*) and Krüppel homolog 1 (*Kr-h1*) in pre-diapausing, diapausing and post-diapausing 3rd instar larvae. The expression level of each tested stage was relative to that of the pre-diapausing larvae, which was arbitrarily set at 1. Bars represent the means ± SE. Values followed by different letters were significantly different by Duncan's multiple range test ($P < 0.05$).

diapause. However, very little is known of their functions during larval diapause. Herein, we took the initiative to clone the full-lengths of cDNAs of *Met* and *Kr-h1* from *S. mosellana*, an insect pest typically undergoing obligatory larval diapause, and performed gene/protein structure and phylogenetic analyses. We also explored developmental regulation of their expression throughout the insect life cycle, as well as their response to JH III treatments. Differing from many molecular studies on insect development, all insect samples used here were collected from the field, presumably more closely reflecting a natural situation.

Like other known Met proteins, SmMet contained all signature motifs of the bHLH-PAS protein family (Figs. 1A and 2A), underscoring a high structure conservation and function similarity of *Met* genes during insect evolution. *Tribolium* Met has been shown to specifically bind JH and mimics with high affinity through a well-conserved hydrophobic pocket within its PAS-B domain. Eight specific amino acid residues, Tyr252, Thr254, Ile262, Val280, Val297, Leu318, Thr330 and Cys347, predicted to form this ligand-binding pocket and proven to be necessary for JH binding (Charles *et al.*, 2011), were all identified in SmMet (Fig. 2A). A similar conservation of residues critical for JH binding has been found in Met from *A. aegypti* (Li *et al.*, 2014). The conserved nature of these residues further supported our hypothesis of SmMet being the JH receptor in *S. mosellana*. Likewise, high sequence similarity (81%) between SmKr-h1 and *A. aegypti* Kr-h1 (Figs. 2B and 3B) implies common functionality. The *AaKr-h1* gene contains JH response elements that mediate DNA binding of AaMet, which directly activates AaKr-h1 transcription in response to JH (Cui *et al.*, 2014; Li *et al.*, 2014). Thus, like in other insects, AaKr-h1 acts downstream of AaMet to play roles in JH signal transduction.

Relatively high JH is thought to be involved in the induction and maintenance of larval diapause and to prevent the secretion of ecdysteroids by suppressing

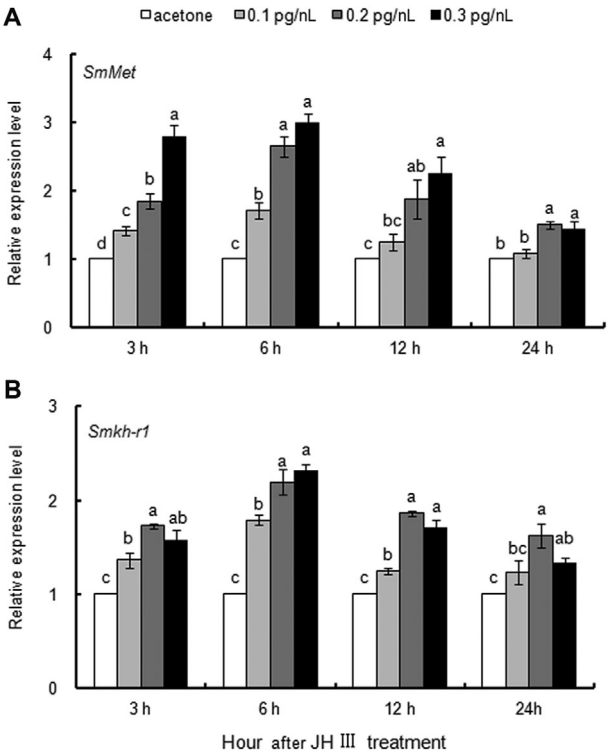

Fig. 5 Expression profiles of *Sitodiplosis mosellana* Methoprene-tolerant (*Met*) and Krüppel homolog 1 (*Kr-h1*) at 3, 6, 12 and 24 h time points after juvenile hormone (JH) III injection of diapausing larvae at concentrations ranging from 0 to 0.3 pg/nL. Expression level of each treatment was relative to that of acetone control, which was arbitrarily set at 1. Bars represent the means ± SE. Values followed by different letters within each group were significantly different by Duncan's multiple range test ($P < 0.05$).

activity of the brain-prothoracic gland axis, as reported in *D. grandiosella* (Yin & Chippendale, 1973), *Ostrinia nubilalis* (Yagi & Akaike, 1976), *Psacothea hilaris* (Munyiri & Ishikawa, 2004), *S. nonagrioides* (Eizaguirre *et al.*, 2005), *Loxostege sticticalis* (Jiang *et al.*, 2011) and other insects. Potential important roles for SmMet and SmKr-h1 in perceiving and transducing JH signal during larval diapause were reflected by coherent patterns between gene expression (Fig. 4) and JH titers (Li *et al.*, 2006), and by inducibility of these genes by JH III (Fig. 5). In contrast, ecdysteroid titers in *S. mosellana* decline dramatically after diapause initiation and remain low during diapause maintenance (Cheng *et al.*, 2009). Notably, the highest expression levels of *SmMet* and *SmKh-r1* were detected in the early post-diapause quiescence stage, a period morphologically indistinguishable from diapause (Cheng *et al.*, 2017). At this stage, *S. mosellana* increases production of both JH and ecdysteroid (Li *et al.*, 2006; Cheng *et al.*, 2009). Presumably, the larval quiescence state resulted from simultaneous increases in ecdysteroid that promotes diapause termination and growth, and in JH that counters such effect via SmMet and SmKr-h1. More studies related to quiescence stage are needed, particularly under natural developmental conditions, although such an approach is more time-consuming than indoor rearing. In addition, the reason for reduced expression in the 2nd instar is unclear, although such phenomenon was also observed in *Bactrocera dorsalis* larvae (Li, 2016) and *Tribolium castaneum* (Minakuchi *et al.*, 2009).

It has been well-established that a marked reduction in JH titer accompanied with increased ecdysteroid levels at the end of the final larval instar of holometabolous insects permits larval–pupal transition (Jindra *et al.*, 2013; Ureña *et al.*, 2016; Kayukawa *et al.*, 2017). Declined *S. mosellana Met* and *Kr-h1* expression levels in the prepupae stage (Fig. 6A) coupled with the dramatic rise in ecdysteroid titers (Cheng *et al.*, 2009) support their involvement in larval–pupal metamorphosis. Interestingly, expression of *SmKr-h1* increased somewhat at late 3rd larval stage (Fig. 6B). While reduced Kr-h1 expression is necessary to permit pupal development informed by the action of the Broad-complex gene (Konopova & Jindra, 2008; Suzuki *et al.*, 2008; Minakuchi *et al.*, 2009; Kayukawa *et al.*, 2014, 2016), the transient up-regulation of SmKr-h1 mRNA could be associated with suppression of precocious adult development as previously shown in *T. castaneum* (Ureña *et al.*, 2016). At this time, Kr-h1 is up-regulated by factors in addition to JH, namely 20E (Kayukawa *et al.*, 2014).

Recent studies indicated that Met and Kr-h1 also participate in insect reproduction. For example, knock-down of *Met* or *Kr-h1* in the adult female cockroach *Diploptera punctata* and the migratory locust *Locusta migratoria* resulted in an obvious reduction of vitellogenin transcripts, accompanied by blocked oocyte maturation and ovarian growth (Marchal *et al.*, 2014; Song *et al.*, 2014). Disruption of *Met* or *Kr-h1* in the adult female brown plant hopper *Nilaparvata lugens* caused a delay of the preoviposition period and a significant reduction in the number of eggs laid (Lin *et al.*, 2015). Higher expression of *S. mosellana Met* and *Kr-h1* in female adults than male adults (Fig. 6) implies that they may also play crucial roles during reproduction.

In summary, this study provides molecular characterization of *S. mosellana Met* and *Kr-h1*, encoding JH-regulated transcription factor homologs that are most likely involved in JH-mediated larval diapause as well as adult reproduction. Further research into their regulatory mechanisms may contribute to novel effective ways of controlling this insect pest.

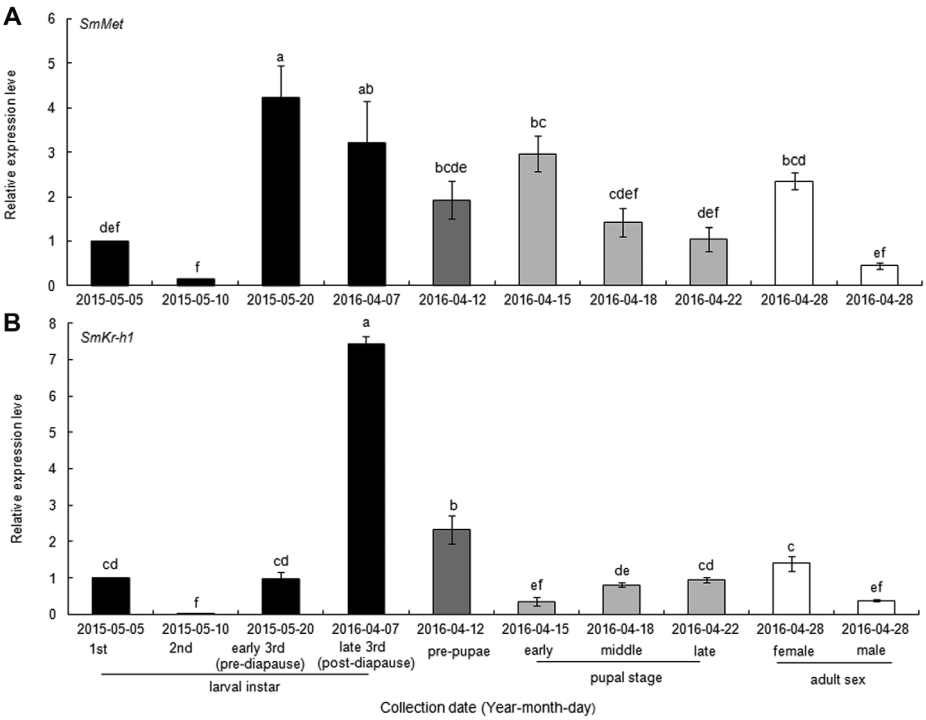

Fig. 6 Developmental expression profiles of *Sitodiplosis mosellana Met* and Krüppel homolog 1 (*Kr-h1*). Expression level of each stage was relative to that of 1st instar larvae, which was arbitrarily set at 1. Bars represent the means ± SE. Values followed by different small letters were significantly different by Duncan's multiple range test ($P < 0.05$).

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Disclosure

The authors declared that there is no conflict of interest of any kind regarding the content of the manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primer sequences used in this study.