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An isoform of Taiman that contains a PRD-repeat motif is indispensable for transducing the vitellogenic juvenile hormone signal in *Locusta migratoria*

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ABSTRACT

Taiman (Tai) has been recently identified as the dimerizing partner of juvenile hormone (JH) receptor, Methoprene-tolerant (Met). However, the role of Tai isoforms in transducing vitellogenic signal of JH has not been determined. In this study, we show that the migratory locust *Locusta migratoria* has two Tai isoforms, which differ in an INDEL-1 domain with the PRD-repeat motif rich in histidine and proline at the C-terminus. *Tai-A* with the INDEL-1 is expressed at levels about 50-fold higher than *Tai-B* without the INDEL-1 in the fat body of vitellogenic adult females. Knockdown of *Tai-A* but not *Tai-B* results in a substantial reduction of *vitellogenin* expression in the fat body accompanied by the arrest of ovarian development and oocyte maturation, similar to that caused by depletion of both *Tai* isoforms. Either Tai-A or Tai-B combined with Met can induce target gene transcription in response to JH, but Tai-A appears to mediate a significantly higher transactivation. Our data suggest that the INDEL-1 domain plays a critical role in Tai function during reproduction as Tai-A appears be more active than Tai-B in transducing the vitellogenic JH signal in *L. migratoria*.

1. Introduction

Juvenile hormone (JH), a sesquiterpenoid secreted by the corpora allata, plays an essential role in insect metamorphosis and reproduction (Jindra et al., 2013, 2015a; Riddiford, 1994; Riddiford, 2012; Wyatt and Davey, 1996). In larval stages, JH works in coordination with 20-hydroxyecdysone (20 E) to maintain insects in their immature state by preventing metamorphosis. In the final molt, insects undergo pupation or eclosion because of the sharp decrease in JH titers (Riddiford, 1994; Wyatt and Davey, 1996). The molecular mechanisms of JH in the prevention of 20 E-induced metamorphosis have been subjected to in-depth studies since the identification of JH receptor, methoprene-tolerant (Met) (Ashok et al., 1998; Charles et al., 2011; Jindra et al., 2013, 2015b). However, the molecular basis of JH in regulating insect reproduction remains largely unknown. JH-dependent vitellogenesis and oocyte maturation have been reported in many insects, including the red flour beetle *Tribolium castaneum*, the linden bug *Pyrrhocoris apterus*, the German cockroach *Blattella germanica*, the brown planthopper *Nilaparvata lugens* as well as the migratory locust *Locusta migratoria* (Cruz et al., 2003; Lin et al., 2015; Parthasarathy et al., 2010; Raikhel et al., 2005; Smykal et al., 2014). *Met* depletion via RNA interference (RNAi) results in dramatically reduced *vitellogenin* (*Vg*) expression and arrested oocyte maturation in *T. castaneum*, *P. apterus*, *L. migratoria* and the bumblebee *Bombus ruderatus* (Parthasarathy et al., 2010; Shpigler et al., 2014; Smykal et al., 2014; Song et al., 2014). Met is a member of the basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of transcription factors. The proteins in this family perform functions by binding to another bHLH-PAS transcription factor to form a heterodimer (Partch and Gardner, 2010). In insects, JH induces the dimerization of Met with Taiman (Tai; also known as steroid receptor co-activator, SRC) to form a transcriptionally active complex to regulate the transcription of target genes (Charles et al., 2011; Guo et al., 2014; Kayukawa et al., 2012; Li et al., 2011; Song et al., 2014; Wu et al., 2016; Zhang et al., 2011).

Tai was originally identified through yeast two-hybrid assay and

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is required for the full transcriptional activity of its binding partners in vertebrates (Onate et al., 1995). In *Drosophila melanogaster,* Tai interacts with the ecdysone receptor (EcR) through the LxxLL motif and acts as EcR co-activator, engaging in the migration of specific follicle cells and border cells in ovaries (Bai et al., 2000). In *T. castaneum*, Tai is critical for lipid metabolism and growth during larval development. When *Tai* is depleted in *T. castaneum*, metamorphosis fails and beetles eventually die (Bitra et al., 2009). In *P. apterus*, *Tai* knockdown leads to the significantly declined transcription of *Vg* and the inhibition of oocyte development under the reproductive condition (Smykal et al., 2014). In the mosquito *Aedes aegypti*, knockdown of the Tai orthologue, p160/SRC coactivator of the ecdysone receptor (FISC) reduces the transcript levels of 20 E responsible genes including *Vg*, *vitellogenic carboxypeptidase* (*VCP*), *E75A* and *E74B* in the fat body of adult females, and consequently the number of eggs (Zhu et al., 2006). Further studies have revealed that JH, through Met/Tai heterodimer, triggers the transcription of gene coding for zinc finger transcription factor Krüppel-homolog 1 (Kr-h1) to repress metamorphosis and promote vitellogenesis (Konopova et al., 2011; Lozano and Belles, 2011; Minakuchi et al., 2008, 2009; Song et al., 2014). Loss of *Tai* function significantly decreases the *Kr-h1* expression (Kayukawa et al., 2012; Lozano et al., 2014; Zhang et al., 2011).

At least three isoforms of SRC have been identified in mammals due to the splicing variants at the 3′ end, which exhibit differential performances in hormone signaling (Dasgupta et al., 2014; Xu et al., 2009; York and O'Malley, 2010). In *B. germanica*, four isoforms of Tai, which are produced through the combination of two INDEL domains near the C-terminus, have been reported (Lozano et al., 2014). Two isoforms of *B. germanica* Tai with the INDEL-1 domain along with Met transmit the JH signal to prevent metamorphosis (Lozano et al., 2014). Previously, the Tai orthologue of *L. migratoria* was named SRC because of its amino acid sequence identity to the SRC/Tai of mammals and *T. castaneum* (Guo et al., 2014; Song et al., 2014). Based on the phylogenetic analysis and the proposal of nomenclature to insects by Lozano et al. (2014), we use Taiman in this report for the sake of the general evolutionary coherence. In the present study, two Tai isoforms, Tai-A with the INDEL-1 and Tai-B without the INDEL-1, were identified on the basis of locust transcriptome and genome (Wang et al., 2014). We found that *Tai-A* was expressed at significantly higher levels than *Tai-B* in the fat body. Knockdown of both *Tai* isoforms or *Tai-A* alone but not *Tai-B* resulted in the substantial reduction of *Vg* expression in the fat body as well as the arrest of oocyte maturation and ovarian growth. Though both Tai-A and Tai-B dimerizing with Met in the presence of JH facilitated the transcription of *Kr-h1*, Tai-A showed stronger capacity than Tai-B to induce *Kr-h1* transcription. Our data thus provide new insights into the JH signaling transduction during insect reproduction.

2. Materials and methods

2.1. Insects

The gregarious colony of *L. migratoria* was maintained under 14 L:10D photoperiods and at 30 ± 2 °C as previously described (Song et al., 2013). The locusts were fed with wheat bran supplied continuously and wheat seedlings provided once daily.

2.2. Sequence alignment and domain prediction

Protein sequences were aligned using ClustalW 2.0 software and visualized using BioEdit 7.2. The domains of Tai isoform A (Tai-A; GenBank: KU315327) and isoform B (Tai-B; GenBank: KU315328) were analyzed using NCBI CCD program.

2.3. RNA extraction and qRT-PCR

The tissues from each locust were collected and frozen individually in liquid nitrogen and stored at −80 °C. Total RNA was extracted with TRNzol reagent (Tiangen). First-strand cDNA was reversely transcribed with 2 μg total RNA using FastQuant RT Kit with gDNase (Tiangen) according to the manufacturer's instructions. Relative transcript levels were measured by qRT-PCR performed in Mx3005 P detection system (Agilent) by utilizing RealMaster Mix SYBR Green Kit (Tiangen). The qRT-PCR program was initiated at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 58 °C for 20 s and 72 °C for 20 s. Melting curve analysis was conducted to confirm the specificity of amplification. The specificity of primers was confirmed by BLAST in the locust transcriptome database. The qRT-PCR products were sequenced for further confirmation of specificity. Relative gene expression levels were analyzed with the $2^{-\Delta\Delta Ct}$ method and normalized against levels of *β-actin* mRNA. For absolute quantification, *Tai-A* and *Tai-B* cDNAs fragments were amplified with respective qRT-PCR primers and ligated into pGM-T vectors (Tiangen) for linear regression analysis of Ct values and Log10 transformed cDNA copy numbers. The mRNA abundance was then quantified with the Ct values from qRT-PCR and the formula derived from the calibration experiments. The primers used for qRT-PCR are summarized in Table 1.

2.4. RNA interference

cDNA templates were amplified by PCR from the pool of fat body and ovary, cloned into pGEM-T Easy vector (Tiangen) and confirmed by sequencing. Double-stranded RNA (dsRNA) and small interfering RNA (siRNA) were synthesized by *in vitro* transcription with T7 RiboMAX Express RNAi system (Promega) following the manufacturer's instruction. In dsRNA interference experiments, each adult female was abdominally injected with 15 μg dsRNA (5 μg/μl in H_2O) within 12 h after adult emergence and boosted at the fifth day post adult emergence (5 P AE). In siRNA interference experiments, two siRNA sequences were synthesized for each target to obtain the efficient knockdown and a mixture of these two sequences at a ratio of 1:1 (total 40 μg; 5 μg/μl in H_2O) was injected into adult females using the same procedure to dsRNA treatment. dsRNA and siRNAs of *GFP* served as the respective mock control. RNAi efficiency and effects were examined at 8 PA E. For the effects of JH treatment on *Tai* or *Tai-A* RNAi, locusts that were injected with dsRNA were further treated with methoprene (150 μg in 5 μl acetone per locust) or acetone alone (solvent control) at 6 P AE, followed by the examination of *Vg* expression as well as the ovarian development and oocyte maturation at 8 PA E. Primers used for dsRNA synthesis are included in Table 1. The sequences of siRNAs are summarized in Table 2.

2.5. Tissue images, cell staining and confocal microscopy

The ovaries and fat bodies were dissected in PBS buffer and photographed with Nikon D7000 camera and Olympus CKX41 microscope. The tunica propria were removed and ovarioles were fixed with 4% paraformaldehyde for 0.5 h at room temperature. The fixed ovarioles were then permeabilized in 0.3% Triton X-100 in PBS. F-actin was stained with 0.165 μM Phalloidin-Alexa Fluor 488 (Invitrogen), and nuclei were stained with 5 μM Hoechst 33342 (Sigma-Aldrich). Images were obtained by ZEISS LSM 710 confocal microscope and processed with ZEN2010 software (Carl Zeiss).

2.6. Western blot and immunoprecipitation

As previously described, the fragments of *Met* (GenBank:

Table 1
Primers used for qRT-PCR and dsRNA synthesis.

	Gene or isoform	Forward primer	Reverse primer	Encompassed region (nt)[a]
qRT-PCR	*Tai-A*	ACGACGACAGGTTTCCAGGC	CGGCGACACGGGTTCACTC	4483–4659
	Tai-B	GCTTCCAGGTGGGGCAGGT	TGGCGCACGTATTCCGA	4569–4628
	Tai	GGGTGTTGATGTTGGTGGC	GATGAAACTCCCGATTCGTCT	2361–2453
	β-actin	AATTACCATTGGTAACGAGCGATT	TGCTTCCATACCCAGGAATGA	744–816
	VgA	CCCACAAGAAGCACAGAACG	TTGGTCGCCATCAACAGAAG	522–620
	VgB	AACGCCGACAGTGTTGGTATTC	ACCATCAGAAGTCGCTGGAAGT	487–620
dsRNA	*Tai*	TAACCCAGATACGCCGAAT	GTACTCATACTTGCTTATACGCTGT	284–714
	Tai-A	AGTTAACCATTCGCGGCATT	CCACCACCCGGCTGGTGA	4581–4961
	GFP	CACAAGTTCAGCGTGTCCG	GTTCACCTTGATGCCGTTC	76–495

[a] Nucleotide position indicates the coding sequence.

Table 2
Sequences of siRNA.

Gene or isoform	Forward sequence	Reverse sequence	Encompassed region (nt)[a]
Tai-A-1	GCGGCAUUAUGGAACGAAACUU	GUUUCGUUCCAUAAUGCCGCUU	4593–4612
Tai-A-2	GGGCCACCUCUGCCUGACCCGCUU	GCGGGUCAGGCAGAGGUGGCCCUU	4678–4699
Tai-B-1	CUCAGCUUCCAGGUGGGGCAGGUUU	ACCUGCCCCACCUGGAAGCUGAGUU	4565–4587
Tai-B-2	CUUCCAGGUGGGGCAGGUGGAUU	UCCACCUGCCCCACCUGGAAGUU	4570–4590
Tai-1	GAGUUGAUACUACAAAUAUCUU	GAUAUUUGUAGUAUCAACUCUU	902–921
Tai-2	GUCCUACUUCCCCAACUGUAUCUU	GAUACAGUUGGGGAAGUAGGACUU	3350–3371
GFP-1	ACGUAAACGGCCACAAGUUCAGCUU	GCUGAACUUGUGGCCGUUUACGUUU	65–87
GFP-2	CGGCAUCAAGGUGAACUUCAAGAUU	UCUUGAAGUUCACCUUGAUGCCGUU	480–502

[a] Nucleotide position indicates the coding sequence.

KF471131; nt 1–3108) and *Tai* cDNA coding sequences (GenBank: KF471132; nt 1–1785) were cloned into pAc5.1/Flag and pAc5.1/V5 vectors (Invitrogen), respectively (Wu et al., 2016). It must be noted that this previously described *Tai* cDNA fragment encompasses the nuclear localization sequence as well as the bHLH, PAS-A and PAS-B domains but lacks the INDEl-1 domain, which represents a truncated *Tai-B* isoform. Therefore, this constructed plasmid was renamed pAc5.1/V5-Tai-B here. The fragment of *Tai* cDNA coding sequence with nt 1–1785 plus nt 4581–4961 containing the INDEl-1 domain was also cloned into pAc5.1/V5 vector for the expression of truncated *Tai-A* isoform. This constructed plasmid was named pAc5.1/V5-Tai-A. All the clones above were confirmed by sequencing. The constructs of pAc5.1/V5-Tai-A or pAc5.1/V5-Tai-B together with pAc5.1/Flag-Met were transfected into *Drosophila* S2 cells using lipofectamine 2000 (Invitrogen). At 48 h after transfection, the cells were cultured with 10 μM JH III for another 6 h.

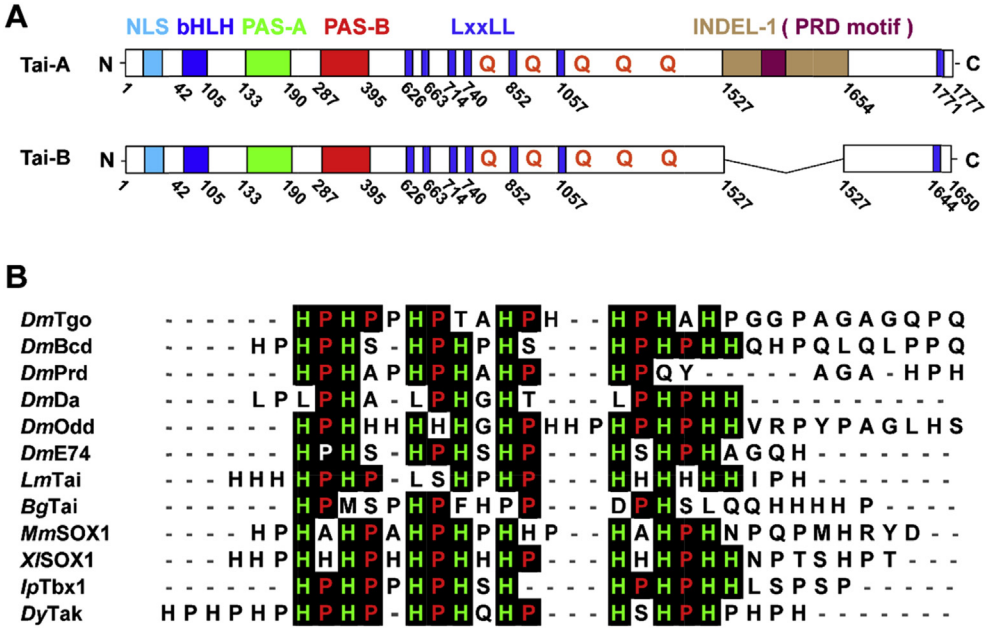

Fig. 1. Characteristics of two Tai isoforms of *Locusta migratoria*. (A) Both Tai-A and Tai-B have the identical sequences of nuclear localization sequence (NLS), bHLH, PAS-A and PAS-B domains as well as seven LxxLL motifs and five glutamine-rich (Poly Q) regions. An INDEL-1 domain containing the PRD-repeat motif is present in the C-terminus of Tai-A, but absent in Tai-B. (B) Amino acid sequence alignment of PRD-repeat sequences of Tango (Tgo), Bicoid (Bcd), Paired (Prd), Daughterless (Da), Odd, Eip74e (E74) of *Drosophila melanogaster* (*Dm*); Tai of *Locusta migratoria* (*Lm*Tai); Tai of *Blattella germanica* (*Bg*Tai); SOX-1 of *Mus musculus* (*Mm*SOX1) and *Xenopus laevis* (*Xl*SOX1), Tbx1 of *Ictalurus punctatus* (*Ip*Tbx1) and Tak1 of *Drosophila yakuba* (*Dy*Tak).

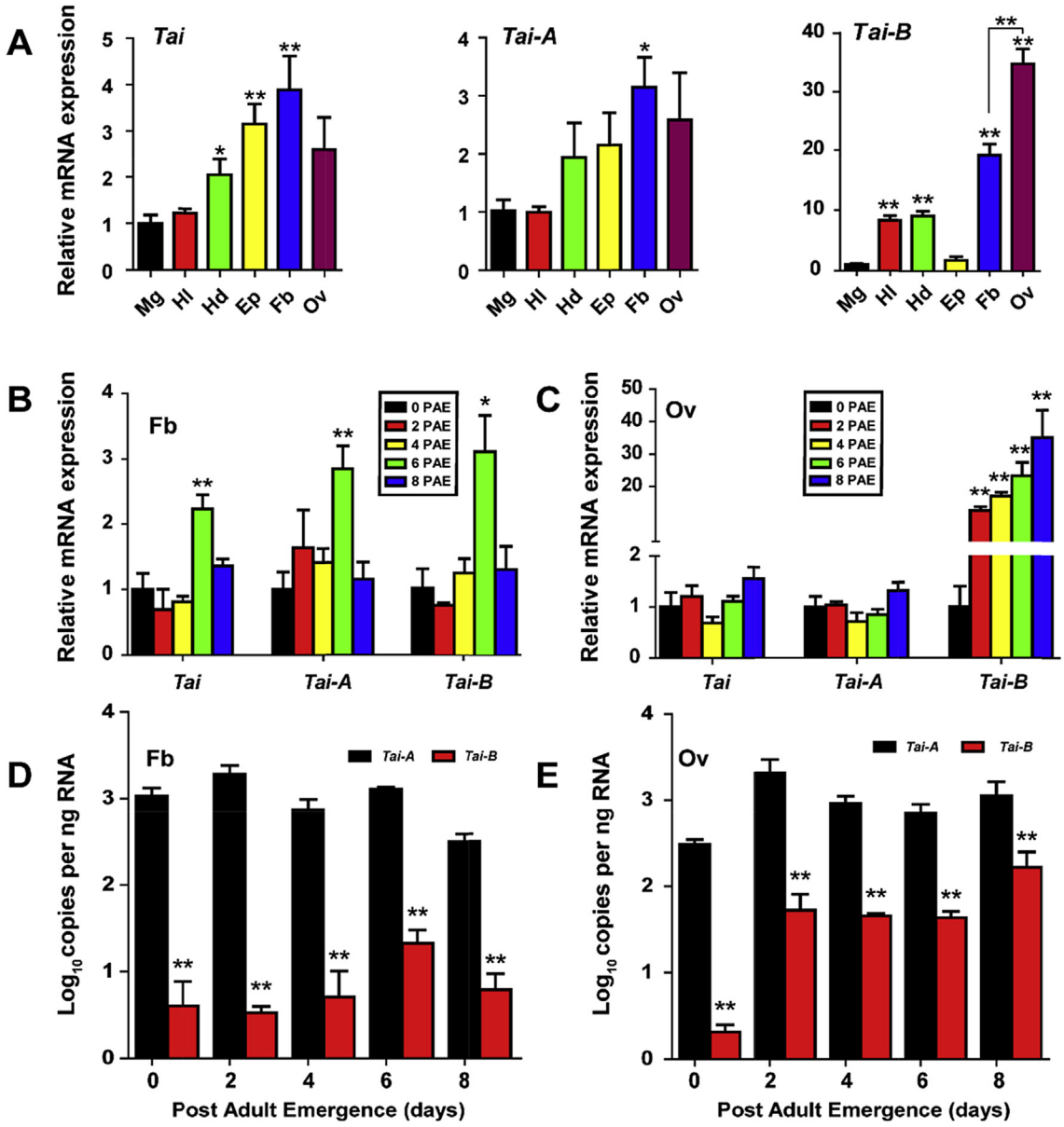

Fig. 2. Differential expression of *Tai-A* and *Tai-B*. (A) Relative mRNA abundance of *Tai*, *Tai-A* and *Tai-B* in midgut (Mg), hind leg (Hl), head (Hd), epidermis (Ep), fat body (Fb) and ovary (Ov) of adult female locusts at 8 PA E (the 8th day post adult emergence). *, $P < 0.05$ and **, $P < 0.01$ compared to the respective levels in Mg. n = 4. (B) and (C) Relative expression of *Tai*, *Tai-A* and *Tai-B* in fat body (B) and ovary (C) of adult females from 0 to 8 PA E. *, $P < 0.05$ and **, $P < 0.01$ compared to that on the day of adult emergence (0 P AE). n = 8. (D) and (E) Absolute quantification of *Tai-A* and *Tai-B* transcripts in the fat body (D) and ovary (E) during 0–8 PA E. Copy numbers are in 1 ng of total RNA. **, $P < 0.01$ compared to that at 0 PAE. n = 4.

The cells were then harvested and lysed in the ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% Nonidet-P40, 1 mM PMSF, 1 mM NaF and a protease inhibitor cocktail (Roche). After incubation for 30 min on ice, the cell lysates were cleared by centrifugation at 14,000 × g for 10 min. Proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore). Western blot was carried out using the monoclonal Flag (MBL) or V5 antibody (Invitrogen). A monoclonal GAPDH antibody (MBL) was adopted as the loading control. For immunoprecipitation, lysates were incubated with anti-V5 antibody for 1 h at 4 °C followed by incubation with protein-A-Sepharose-conjugated beads at 4 °C overnight. The beads were collected by centrifugation. The beads and proteins were separated by boiling in Laemmli sample buffer. The proteins for western blot analysis were then processed as described above.

2.7. Luciferase reporter assay

The promoter region of locust *Kr-h1* (nt −237 to −6) containing a canonical E-box motif (CACGTG, nt −179 to −174) was cloned into pGL4.10 vector (Promega) (Song et al., 2014). S2 cells were transfected with pAc5.1/V5-Tai-A, pAc5.1/V5-Tai-B and/or pAc5.1/Flag-Met plus pGL4.10-*Kr-h1*$^{-237\ \text{to}\ -6}$ using Lipofectamine 2000 (Invitrogen) with the addition of 10 μM JH III. After 48 h, luciferase activity was measured using the Dual-Luciferase reporter assay system and analyzed with GloMax 96 Microplate Luminometer (Promega).

2.8. Statistical analysis

Statistical analyses were performed using Student's *t*-test by the

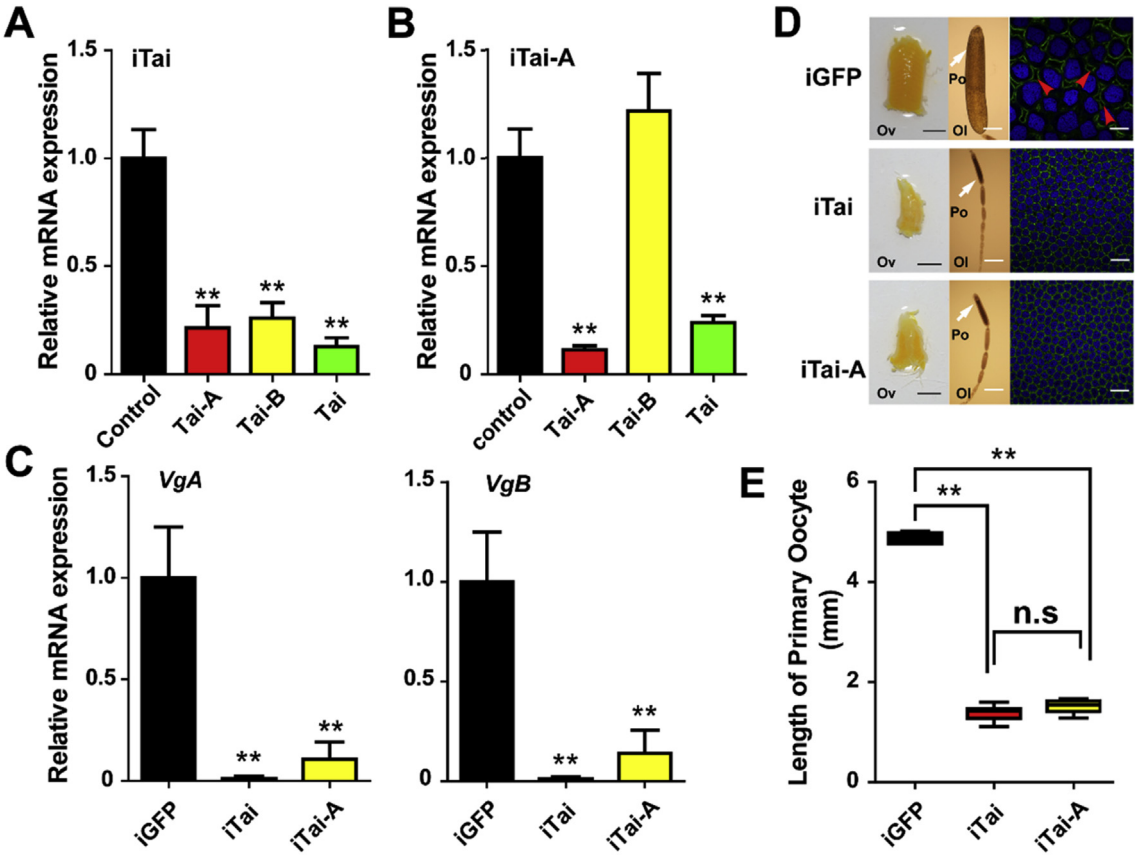

Fig. 3. Effects of *Tai* and *Tai-A* knockdown by dsRNA on vitellogenesis and oocyte maturation. (A–B) RNAi efficiency of *Tai* (iTai) and *Tai-A* (iTai-A) by dsRNA in the fat body at 8 PA E. **, $P < 0.01$ compared to the respective dsGFP controls. n = 8. (C) Relative expression of *VgA* and *VgB* in the fat body of 8-day-old adult female locusts subjected to iTai or iTai-A. **, $P < 0.01$ compared to the respective dsGFP controls (iGFP). n = 8. (D) Morphology of ovaries (Ov), ovarioles (Ol), primary oocytes (Po) and follicular epithelia of primary oocytes at 8 PA E after iTai and iTai-A. Cell nuclei were stained with Hoechst 33342 and F-actin was stained with Phalloidin Alexa Fluor 488. Red arrows indicate the patency. Scale bar: 5 mm for ovaries, 0.5 mm for ovarioles, 20 μm for follicular epithelia. (E) Statistical analysis of primary oocyte length of 8-day-old adult female locusts subjected to iTai and iTai-A. **, $P < 0.01$ compared to the dsGFP control; n.s, no significant difference; n = 16 for each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GraphPad Prism software. Significant differences are considered at $P < 0.05$. Values are represented by mean ± SE.

3. Results

3.1. Identification and characterization of two Tai isoforms in *L. migratoria*

Based on the transcriptome and genome (Wang et al., 2014), we cloned two Tai isoforms in *L. migratoria*, namely *Tai-A* with the open reading frame (ORF) of 5334 bp (GenBank: KU315327) and *Tai-B* with the ORF of 4954 bp (GenBank: KU315328). Both isoforms consist of a nuclear localization sequence near the N-terminus, followed by the bHLH, PAS-A and PAS-B domains as well as seven LxxLL motifs and five glutamine-rich (poly Q) regions (Fig. 1A). Sequence alignment analysis showed that locust Tai-A and Tai-B cDNAs have identical nucleotide sequence except the INDEL-1 domain (Lozano et al., 2014), suggesting the alternative splicing from the same gene. Tai-A has the INDEL-1 domain localized at nt 4581–4961, whereas Tai-B lacks this domain (Fig. 1A). In comparison with the Tai isoforms in *B. germanica* (Lozano et al., 2014), locust Tai-A appears to be the orthologue of *B. germanica* Tai-B, while locust Tai-B is the orthologue of *B. germanica* Tai-D. The INDEL-1 domain is also present in the published Tai sequences of *D. melanogaster*, *T. castaneum* and the honeybee *Apis mellifera* but not the silkworm *Bombyx mori* (Lozano et al., 2014). Further analysis showed that the INDEL-1 domain possesses a PRD-repeat motif rich in histidine and proline (Fig. 1B). In addition to Paired of *D. melanogaster* (Frigerio et al., 1986), the PRD-repeat motif has been reported in Tango, Bicoid, Daughterless, Odd and Eip74 E of *D. melanogaster*, SOX-1 of *Mus musculus* and *Xenopus laevis*, Tbx1 of *Ictalurus punctatus* and Tak1 of *D. yakuba* (Fig. 1B), which contributes to the transcriptional activation of target genes (Archer et al., 2011; Berleth et al., 1988; Dohrmann et al., 1990; Hoskins et al., 2007; Li et al., 2015; Liu et al., 2012; Ma et al., 2000; Oosterveen et al., 2013).

3.2. Differential expression of Tai-A and Tai-B

Using the total RNA isolated from adult female locusts at 8 PA E when the progress of vitellogenesis naturally reached the peak during the first gonadotrophic cycle, we initially measured the tissue expression profile of two Tai isoforms. As shown in Fig. 1A, Tai-A cDNA has the INDEL-1 domain localized at nt 4581–4961, whereas Tai-B lacks this domain. We thus designed the Tai-B specific primers for qRT-PCR by using the forward primer (GCTTCCAGGTGGGGCAGGT, nt 4569 to 4587) that crosses the lacking site of INDEL-1 (Table 1). Total *Tai* transcripts were

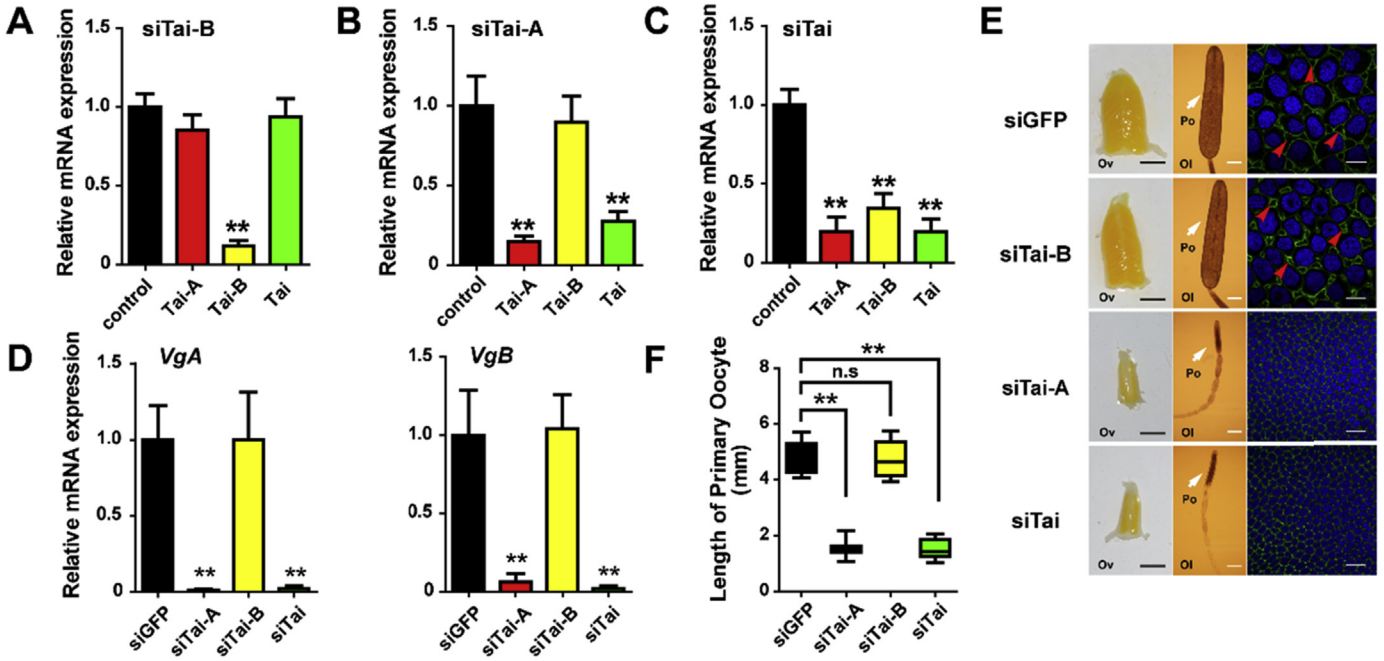

Fig. 4. Effects of *Tai-B*, *Tai-A* and *Tai* knockdown by siRNA on vitellogenesis and oocyte maturation. (A–C) RNAi efficiency of *Tai*-B (siTai-B), *Tai-A* (siTai-A) and *Tai* (siTai) by siRNAs in the fat body at 8 PA E. **, $P < 0.01$ compared to the respective GFP siRNA controls. n = 8. (D) Relative expression of *VgA* and *VgB* in the fat body of adult female locusts treated with siTai-A, siTai-B and siTai. **, $P < 0.01$ compared to the respective siGFP controls (siGFP); n = 8. (E) Morphology of ovaries (Ov), ovarioles (Ol), primary oocytes (Po) and follicular epithelia of primary oocytes after siTai-A, iTai-B and siTai. Cell nuclei were stained with Hoechst 33342 and F-actin was stained with Phalloidin Alexa Fluor 488. Red arrows indicate the patency. Scale bar: 5 mm for ovaries, 0.5 mm for ovarioles, 20 μm for follicular epithelia. (F) Statistical analysis of primary oocyte length of locusts subjected to siTai-A, siTai-B and siTai treatment. **, $P < 0.01$ compared to the siGFP control; n.s, no significant difference; n = 30 for each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly higher in the fat body, epidermis and head than that in the ovary, midgut and hind legs. Likewise, *Tai-A* mRNA was more abundant than *Tai-B* in the fat body than that in other tested tissues. By contrast, *Tai-B* expression exhibited drastic changes among the tissues, with higher levels in the fat body and ovary than in the midgut and epidermis (Fig. 2A). Interestingly, *Tai-B* mRNA levels were significantly lower in the fat body compared to that in the ovary (Fig. 2A). To reveal their expression dynamics during vitellogenesis and oocyte maturation, we next examined the relative levels of *Tai*, *Tai-A* and *Tai-B* mRNA in the fat body and ovary during the first gonadotrophic cycle. In the fat body, resembling the expression pattern of total *Tai* transcripts, *Tai-A* and *Tai-B* reached the peak at 6 PA E (Fig. 2B). In the ovary, the expression of *Tai* and *Tai-A* maintained stable after adult emergence, whereas the mRNA levels of *Tai-B* were increased approximately 12–16 fold at 2–4 PA E and further elevated to around 23–34 fold at 6–8 PA E (Fig. 2C). Knowing that *Tai-A* and *Tai-B* were differentially expressed, we further performed absolute quantitative real time RT-PCR to compare the amount of *Tai-A* and *Tai-B* in the fat body and ovary, two important organs in locust reproduction. As shown in Fig. 2D, the abundance of *Tai-A* was 43–51 fold more than that of *Tai-B* in fat body during the vitellogenic phase (Fig. 2D). Interestingly, the gap between the abundance of *Tai-A* and *Tai-B* in the ovary narrowed to 6–17 fold at 6–8 PA E (Fig. 2E).

3.3. The role of Tai isoforms in locust vitellogenesis and oocyte maturation

To determine the function of Tai isoforms in locust vitellogenesis and oocyte maturation, we designed two dsRNA. One dsRNA, dsTai is based on the common region of *Tai-A* and *Tai-B* to deplete both isoforms, whereas the other, dsTai-A recognizes the INDEL-1 domain for depleting *Tai-A* only. The characteristics of *Tai-B* cDNA sequence limited our efforts to carry out *Tai-B*-specific knockdown by dsRNA. As shown in Fig. 3A, dsRNA-mediated knockdown of *Tai* led to 79%, 74% and 87% reduction of *Tai-A*, *Tai-B* and *Tai* expression, respectively, in the fat body of adult females at 8 PA E. In the samples injected with dsTai-A, the mRNA levels of *Tai-A* and *Tai* dropped by 89% and 76% respectively, while *Tai-B* expression stayed unchanged (Fig. 3B), indicating the specificity of *Tai-A* knockdown.

We next examined the effects of *Tai* and *Tai-A* knockdown on locust vitellogenesis and oocyte maturation. The migratory locust *L. migratoria* has two coordinately expressed *Vg* genes, *VgA* (GenBank: KF171066) and *VgB* (GenBank: KX709496) (Dhadialla et al., 1987). In *Tai*-depleted fat bodies, the mRNA levels of both *VgA* and *VgB* sharply reduced to 1% of their normal levels at 8 PA E (Fig. 3C). When *Tai-A* was silenced, *VgA* and *VgB* transcripts decreased by 91% and 89%, respectively (Fig. 3C). As shown in Fig. 3D, either *Tai* or *Tai-A* knockdown resulted in the arrest of ovarian development and oocyte maturation. Statistical analysis of the average length of primary oocytes showed insignificant difference between *Tai*- and *Tai-A*-depleted groups (Fig. 3E), suggesting that *Tai-A* knockdown has the similar effects to *Tai* RNAi. To further distinguish the effects of *Tai* and *Tai-A* RNAi, the nuclei and cytoskeleton were stained to show the morphology of the follicle cells. Depletion of either *Tai* or *Tai-A* resulted in the size reduction of follicular cells and the shrink of cytoskeleton. The intercellular spaces (named patency) in the follicular epithelium were clearly observed in dsGFP-treated samples when the primary oocyte grew rapidly because of the uptake of Vg and other molecules. By comparison, no patency could be seen with *Tai*- or *Tai-A*-depleted locusts (Fig. 3D).

To perform *Tai-B* knockdown, we designed *Tai-B*-specific siRNAs that crosses the lacking site of INDEL-1 (Table 2). *Tai-A* and *Tai* siRNA knockdown were also conducted. Injection of *Tai-B* siRNAs resulted in 88% reduction of *Tai-B* mRNA levels in the fat body of

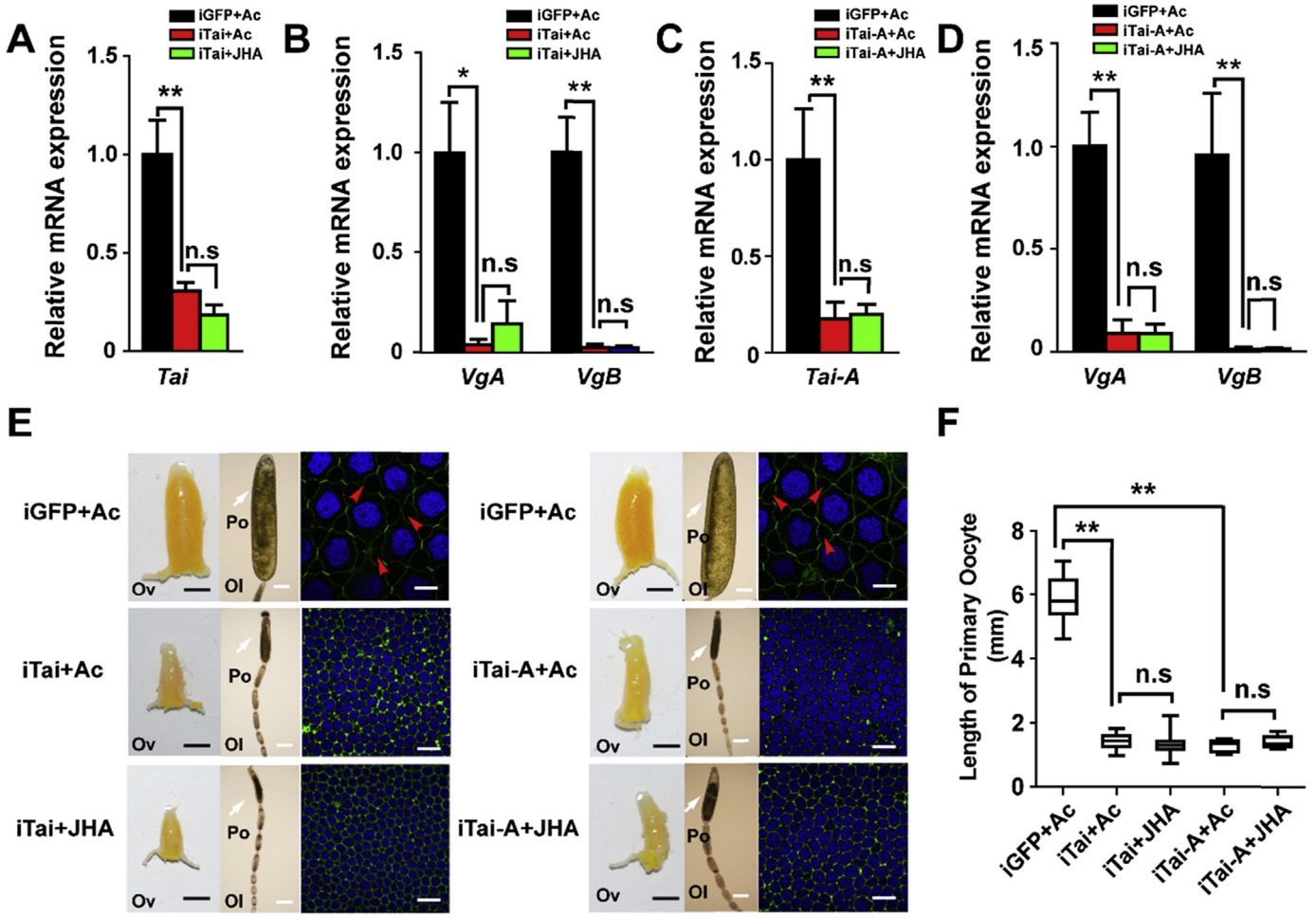

Fig. 5. *Tai* or *Tai-A* knockdown restrains the response of locusts to methoprene treatment. (A, B) Relative expression levels of *Tai*, *VgA* and *VgB* in the fat body of *L. migratoria* subjected to iTai further treated with acetone or methoprene (JHA). *, $P < 0.05$ and **, $P < 0.01$ compared to the respective iGFP controls; n.s, no significant difference. n = 8 for each group. (C, D) Relative expression levels of *Tai-A*, *VgA* and *VgB* in the fat body of *L. migratoria* subjected to iTai-A further treated with acetone or methoprene (JHA). **, $P < 0.01$ compared to the respective iGFP controls; n.s, no significant difference. n = 8 for each group. (E) Morphology of ovaries (Ov), ovarioles (Ol) and primary oocytes (Po) of *L. migratoria* subjected to iTai and iTai-A further treated with acetone or methoprene (JHA). Scale bars: ovaries, 5 mm; ovarioles, 0.5 mm. (F) Statistical analysis of primary oocyte length. **, $P < 0.01$ compared to the dsGFP control; n.s, no significant difference; n = 40 for each group.

adult females at 8 PA E but had no significant effect on *Tai-A* expression (Fig. 4A), indicating the specificity of *Tai-B* siRNAs. The unchanged levels of *Tai* mRNA after *Tai-B* depletion (Fig. 4A) appeared to coincide with the low abundance of *Tai-B* in the fat body as shown in Fig. 2D. In the adult females injected with *Tai-A* siRNAs, the mRNA levels of *Tai-A* and *Tai* dropped by 85% and 73% respectively, but *Tai-B* expression was not affected (Fig. 4B). When *Tai* siRNAs were applied, the mRNA levels of *Tai-A*, *Tai-B* and *Tai* dropped to 20%, 34% and 19% of their normal levels, respectively (Fig. 4C). Interestingly, *Vg* expression was not affected by *Tai-B* knockdown (Fig. 4D). We observed normal oocyte maturation and ovarian development (Fig. 4E and F), and in two experiments slightly smaller ovaries and primary oocytes in *Tai-B*-depleted locusts. In the parallel experiments, knockdown of *Tai-A* or *Tai* by siRNA led to 94–99% reduction of *VgA* and *VgB* expression (Fig. 4D) as well as blocked ovarian development and oocyte maturation (Fig. 4E and F). The above results suggest that Tai-A, which is more abundant than Tai-B in the fat body, is prerequisite to high levels of Vg synthesis.

Previous studies have shown that exogenous treatment of methoprene mimics the JH function and restores the inhibited progression of vitellogenesis caused by chemical ablation of endogenous JH synthesis (Wyatt and Davey, 1996). We further treated *Tai* or *Tai-A* knockdown locusts with methoprene and investigating the effects on *Vg* expression as well as the ovarian development and oocyte maturation. As shown in Fig. 5A–B, additional methoprene treatment on *Tai*-depleted locusts failed to increase the transcript levels of *Vg* in the fat body. Similarly, knockdown of *Tai-A* precluded the methoprene-induced expression of *Vg* in the fat body (Fig. 5C–D). The capacity of methoprene to promote oocyte maturation and ovarian development was also blocked in *Tai*- or *Tai-A*-depleted adult female locusts (Fig. 5E and F). These data indicate the crucial role of Tai and Tai-A in transducing the vitellogenic signal of JH in *L. migratoria*.

3.4. *Tai-A is more potent than Tai-B in mediating induction of Kr-h1 transcription*

Our previous study has documented that Met and Tai (this previously described Tai is the truncated Tai-B isoform) form a heterodimer in the presence of JH III to activate the transcription of *Kr-h1* in *L. migratoria* (Song et al., 2014). Here we compared the capacity of two Tai isoforms interacting with Met to induce the transcription of *Kr-h1* in response to JH. As clarified in the section of Materials and Methods, the subclones used for the expression of Tai-A and Tai-B proteins in the immunoprecipitation and luciferase

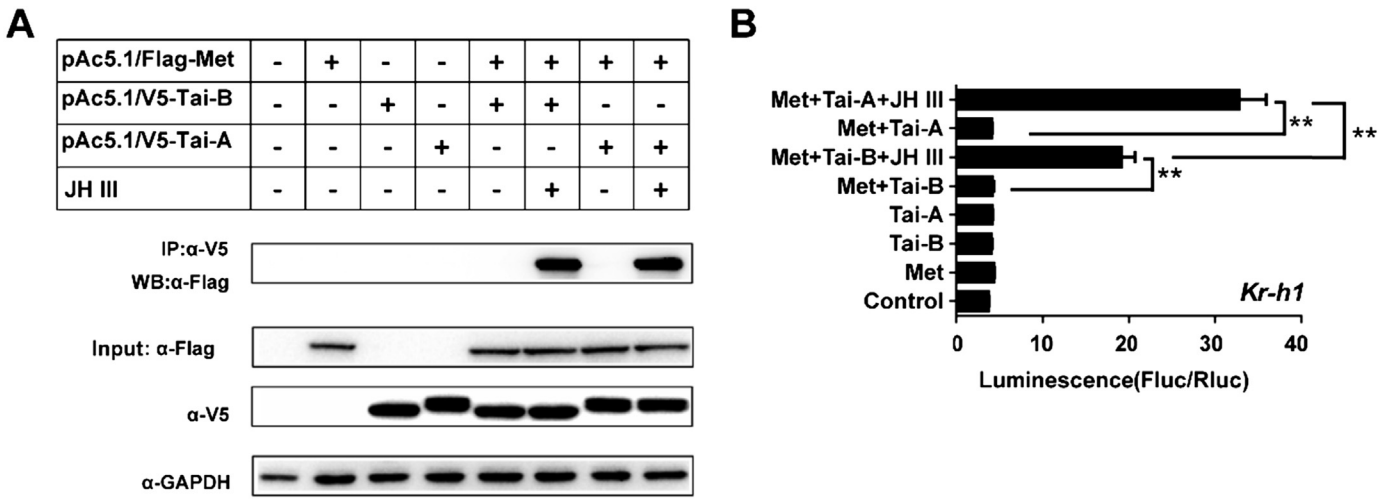

Fig. 6. Comparison of Tai-A and Tai-B in the induction of JH-dependent *Kr-h1* transcription. (A) Immunoprecipitation (IP) and western blot (WB) showing the expression of Flag-Met (second panel from the top), V5-Tai-A and V5-Tai-B (third panel from the top) in S2 cells, and the interaction of Flag-Met with V5-Tai-A and V5-Tai-B in the presence of JH III (upper panel). α-Flag, anti-Flag antibody; α-V5, anti-V5 antibody; α-GAPDH, anti-GAPDH antibody; Tai-A indicates the truncated Tai variant with the INDEL-1/PRD motif; Tai-B indicates the truncated Tai variant without the INDEL-1/PRD motif. (B) Luciferase reporter assays using S2 cells co-transfected with pAc5.1 empty vector + pGL4.10/*Kr-h1*$^{-237\ \text{to}\ -6}$ (control), pAc5.1/Flag-Met + pGL4.10/*Kr-h1*$^{-237\ \text{to}\ -6}$ (Met), pAc5.1/V5-Tai-A + PGL4.10/*Kr-h1*$^{-237\ \text{to}\ -6}$ (Tai-A), pAc5.1/V5-Tai-B + pGL4.10/*Kr-h1*$^{-237\ \text{to}\ -6}$ (Tai-B), pAc5.1/Flag-Met + pAc5.1/V5-Tai-A + pGL4.10/*Kr-h1*$^{-237\ \text{to}\ -6}$ (Met + Tai-A), pAc5.1/Flag-Met + pAc5.1/V5-Tai-B + pGL4.10/*Kr-h1*$^{-237\ \text{to}\ -6}$ (Met + Tai-B). JH III was used at 10 μM **, $P < 0.01$.

assays are not identical to the complete Tai-A and Tai-B isoforms but rather they are two truncated Tai variants with and without the INDEL-1/PRD motif. Western blot showed that Flag-Met, V5-Tai-A and V5-Tai-B were successfully expressed in S2 cells (Fig. 6A). Immunoprecipitation demonstrated that the expressed Flag-Met dimerized with both V5-Tai-A and V5-Tai-B in the presence of JH III (Fig. 6A), indicating the dependence of Met and Tai isoforms dimerization on JH. In the presence of JH III, the luciferase activity of cells co-transfected with pAc5.1/Flag-Met and pAc5.1/V5-Tai-B was elevated by 4.6-fold compared with that without JH treatment (Fig. 6B). By contrast, the co-expression of Flag-Met and V5-Tai-A with JH III treatment caused a 7.9-fold increase in *Kr-h1* reporter activity compared to that in the absence of JH III (Fig. 6B). Statistically, the combination of Flag-Met and V5-Tai-A was significantly more active in transactivation than Flag-Met + V5-Tai-B. These results indicate that though Tai-B in conjunction with Met and JH mediates target gene transcription, Tai-A dimerizing with Met in the presence of JH significantly enhances the transcription activity of JH-receptor.

4. Discussion

4.1. *Tai* isoforms in the transduction of the vitellogenic signal of JH

Distinct from four isoforms of Tai derived from the combination of INDEL-1 and INDEL-2 in the cockroach *B. germanica* (Lozano et al., 2014), two isoforms of Tai were identified in the migratory locust *L. migratoria*. These two isoforms of Tai in *L. migratoria* are distinguished by the INDEL-1 domain. Likewise, the INDEL-1 is present in Tai of *D. melanogaster*, *T. castaneum* and *A. mellifera*, whereas INDEL-2 is absent in these Tai sequences (Lozano et al., 2014). Nevertheless, neither INDEL-1 nor INDEL-2 is seen within the Tai of *B. mori* (Lozano et al., 2014). These results together suggest the variation of Tai isoforms in insects. Unlike Tai-B, locust Tai-A bears an INDEL-1 domain near the C-terminus. We therefore wondered whether *Tai-A* and *Tai-B* have differential expression patterns in the fat body and ovary during the first gonadotrophic cycle. Interestingly, *Tai-A* was much more abundant than *Tai-B* in the fat body. Given the marked differences of expression between these two isoforms, it might have been predicted that Tai-A and Tai-B function differently in locust vitellogenesis and egg production.

Previously, the function of Tai in oogenesis and its isoforms in metamorphosis has been investigated in the bug *P. apterus* and the cockroach *B. germanica*, respectively (Lozano et al., 2014; Smykal et al., 2014). However, direct evidence for the involvement of Tai isoforms in JH-stimulated vitellogenesis and oocyte maturation has been lacking. In *P. apterus*, loss of *Tai* function blocks Vg synthesis and oogenesis (Smykal et al., 2014). In *B. germanica*, depletion of all Tai isoforms in the nymphs results in 100% mortality, whereas knockdown of Tai isoforms with the INDEL-1 leads to precocious metamorphosis without the lethal effect (Lozano et al., 2014). We demonstrated in this study that the specific depletion of *Tai-A* in adult female locusts resulted in significantly reduced *Vg* expression in the fat body as well as the arrested oocyte maturation, blocked ovarian growth and shrunk follicular epithelium, similar to that caused by knockdown of both two Tai isoforms. Moreover, further application of JH analog on *Tai-A*-depleted locusts was unable to restore the defective phenotypes to the normal levels, resembling the failure of JH treatment on *Tai* knockdown. These data therefore address the importance of Tai-A with the INDEL-1 domain in JH-mediated vitellogenesis and egg production in *L. migratoria*. Considering the much higher levels of Tai-A transcripts in the fat body, Tai-A likely contributes more to Vg synthesis. As locust ovaries are panoistic, the yolk protein precursors are synthesized in the fat body, released into hemolymph and transported to maturing oocytes through the patency in the follicular epithelium (Wyatt and Davey, 1996). The reduced *Vg* expression from *Tai-A*-depleted fat bodies might consequently result in blocked follicular epithelium development, oocyte maturation and ovarian growth. Interestingly, knockdown of *Tai-B* had no significant effect on *Vg* expression. Consequently, normal ovarian development and oocyte maturation were seen with *Tai-B*-depleted locusts. The data suggest that compared to Tai-A, Tai-B that lacks the INDEL-1 domain and is less abundant in the fat body is unlikely a major player in transducing vitellogenic signal of JH in *L. migratoria*. While the specific function of Tai-B remains to be investigated, the relative high expression levels of *Tai-B* in the ovary suggest that the function of Tai-B may be more related to ovary.

4.2. Tai isoforms in the regulation of gene transcription

The function of Tai as a coactivator for Met to form an active JH-receptor complex has been established in several insect models (Charles et al., 2011; Guo et al., 2014; Kayukawa et al., 2012; Li et al., 2011; Wu et al., 2016; Zhang et al., 2011). However, the performance of Tai isoforms in the regulation of target gene transcription has not been elucidated excepted for that of *T. castaneum* (Lozano et al., 2014). Interestingly, both isoforms of *T. castaneum* Tai have relatively similar activity in transducing the JH signal and activating the *Kr-h1* reporter, though the isoform without the INDEL-1 appears to mediate a slightly higher activation than the other isoform with the INDEL-1 in the presence of JH and Met (Lozano et al., 2014). In the present study, our luciferase assays using the *Kr-h1* promoter combined with Met in the presence of JH demonstrated that locust Tai-A with the INDEL-1 mediates a higher activation of target gene transcription than Tai-B without the INDEL-1. Our observations thus suggest that the INDEL-1 domain plays a role in the enhanced activity of Tai for JH-mediated gene expression in *L. migratoria*.

The INDEL-1 domain of Tai encompasses a PRD-repeat motif, which was originally identified in *Drosophila* Paired (Frigerio et al., 1986). When the PRD-repeat motif was truncated, the ability of Paired to drive the transcription of downstream genes *engrailed* and *gooseerry* is diminished (Cai et al., 1994; Han et al., 1989). It has also been reported that Tango with the deletion of PRD-repeat motif significantly reduces its activity in stimulating the transcription of *breathless* (Sonnenfeld et al., 2005). Moreover, mutation in the PRD-repeat motif of Paired-box-gene 3 (Pax3) alters the transactivation activity of Pax3 on downstream genes with the complex e5 sequence in the promoter regions (Cao and Wang, 2000). The PRD-repeat motif help constitute an extended conformation and present arrays of histidine chains, which recruit metal irons to form a histidine-metal zipper and promote the formation of dimers (Chakrabarti, 1990; Janknecht et al., 1991; Thummel, 1990). The presence of INDEL-1 in locust Tai-A that is more abundant than Tai-B in the fat body is likely to help interact with its binding partners like Met and satisfies the synthesis of Vg and other molecules, which may typically represent the adaption of gene function during the evolution of insect reproduction.

Acknowledgments

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