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Transcription factor E74A affects the ecdysone titer by regulating the expression of the *EO* gene in the silkworm, *Bomby mori*

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ABSTRACT

The formation of ecdysone pulse in insects is synergistically controlled by its biosynthesis and degradation. Previous studies have revealed the feedback regulation of the prothoracic gland (PG) activity to affect the hormone synthesis. However, the molecular regulatory mechanism of the ecdysone degradation is still unclear. In this study, we showed that ecdysone oxidase (EO) gene encoding a hormone metabolism enzyme was also induced by hormone itself in the domestic silkworm, *Bombyx mori*. Furthermore, luciferase reporter, chromatin immunoprecipitation and electrophoretic mobility shift assays showed that ecdysone inducible transcription factor E74A could bind to the *cis*-regulatory elements of the *EO* gene. Then, down-regulating the expression of the *E74A* by RNA interference (RNAi) decreased the expression of the *EO* gene and caused a higher ecdysone titer compared with the control. Thus, our results demonstrated a new feedback regulation degradation (EO) pathway controlled by ecdysone itself through transcription factor E74A, expanding the knowledge about the regulatory system that determines the formation of ecdysone pulse.

1. Introduction

Metamorphosis is a key stage in the developmental process of the holometabolous insects. This process is mainly triggered by a high level of steroid hormone ecdysone. In the whole life stages of insects, the ecdysone titer displays periodic and accurate fluctuations [39]. At the onset of the metamorphosis, ecdysone pulse cues to coordinate a complex pattern of behavioral, genetic and morphological changes of both larval and mature tissues [9]. Therefore, it is essential to understand the molecular mechanism of the ecdysone pulse in insects.

Like other steroid hormones, the accurate timing of ecdysone pulse in insects is also synergistically regulated by its biosynthesis and degradation. Any changes in these two processes are likely to affect the level and duration of the ecdysone pulse. During the larval stage, ecdysone is produced in prothoracic gland (PG) by a series of conversions from dietary cholesterol [44]. Previous studies have sequentially identified several cytochrome P450s and dehydrogenases/reductases belonging to Halloween gene family involved in the ecdysteroidogenesis pathway [2,19–21,24,42,43,46]. Once PG is stimulated by the prothoracicotropic hormone (PTTH) released from brain, the PTTH receptor Torso in PG could induce the mitogen-activated protein kinase (MAPK) cascade to increase the transcription of ecdysone biosynthetic enzymes, which may increase the ecdysone level [10,25]. Moreover, Halloween genes could also be regulated by ecdysone itself through several transcription factors. At the initial of the metamorphosis, ecdysone acts through ecdysone receptor (EcR) and Broad-complex Z4 (BrcZ4) to induce the expressions of the Halloween genes which further increase the ecdysone titer [18]. Similarly, some other transcription factors (Vvl, Kni, Antp and POU-M2) can directly stimulate the transcriptions of these steroidogenic enzyme genes [4,17]. Recently, several studies have proven that transcription factors also take part in the decline of ecdysone titer. At *Drosophila* metamorphosis onset, hormone receptors 3 and 4 (DHR3 and DHR4) repress the expressions of the Halloween genes, indicating that the two receptors can serve as a feedback controller on molting hormone biosynthesis [22,23]. Consequently, the expressions of the steroidogenic enzyme genes regulated by the transcription factors determine the level and duration of the ecdysone pulse.

Although the ecdysone concentration can be regulated by the turning on or off the Halloween genes as shown above, circulating active ecdysone must be inactivated to terminate the pulse [27]. Previous studies have demonstrated that some pathways including 3-epimerization and 26-hydroxylation can degrade the ecdysone, indicating their important roles in the regulation of hormone titer [12,26]. However, how insects regulate these pathways is still unknown. Ecdysone oxidase (EO) is a rate-limited-enzyme in the 3-epimerization pathway, a major route to degrade the ecdysone in Lepidoptera [12,35,37,38]. Our previous study found that a 77 base pairs (bp) fragment located on the promoter region of the *EO* gene is core regulatory region for the *EO* gene in the domestic silkworm, *Bombyx mori* [36]. In this study, we showed that the *EO* gene can also be induced by the ecdysone itself *in vivo*. Meanwhile, we demonstrated that the ecdysone inducible transcription factor E74A can

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directly bind to the *cis*-regulatory elements (CREs) of the *EO* gene [9]. In addition, RNA interference experiments further confirmed that E74A stimulated the expression of the *EO* gene to decline the ecdysone titer. Therefore, our results revealed the molecular mechanism of the ecdysone degradation in insects, expanding the knowledge to understand the regulatory system that determines the formation of the ecdysone pulse.

2. Materials and methods

2.1. Insects and cell culture

The strain *DaZao* of the domestic silkworm was reared on fresh mulberry leaves at 25 °C under a 12 h-light: 12 h-dark photoperiod. The silkworm ovarian cell line (*BmN*) was maintained in TC-100 insect cell culture medium (Invitrogen, USA) supplemented with 10% fetal calf serum (Gibico, USA) at 27 °C.

2.2. Insect treatment

In order to survey whether 20E may affect the expression of the *EO* gene, 20E solution (1 μg/larva) (Sigma, USA) was orally injected into the 3rd-day fifth larvae through a 50 μL micrometer syringe. According to our previous study, *EO* gene was predominantly expressed in the silkworm midguts [35]. Therefore, in this study, after different time points, only the midguts from each treatment were dissected on ice, and immediately frozen and stored in liquid nitrogen, respectively. Every tissue sample was collected from five larvae. Dimethyl sulfoxide (DMSO) was used as the control. Thorax-abdomen (TA) ligation can shut off the secretion of ecdysone from PGs to abdomen [29,45]. In this study, the 7th day (one day before wandering stage) larvae of the last instar (5th instar) were abdomen-ligatured. Ligated larvae were kept at 25 °C. Twenty-four hours after ligation, the midguts were collected as shown above.

2.3. Plasmids construction

According to our previous study, the upstream regulatory regions (from −539 to +65) (E74A-wild) of the *EO* gene containing the 77 bp fragment were amplified [36]. Then the fragment was subjected to Genomatix MatInspector (http://www.genomatix.de) to predict the putative transcription factor binding sites. An E74A binding sites deletion fragment (from −508 to +65) (E74A-deletion) was obtained by PCR using the specific primers. In addition, two fragments with mutated E74A binding sites (E74A-mutant1 and E74A-mutant2) were also obtained. All fragments were then cloned into the PGL3-Basic vector (Promega, USA), and the sequence and orientation were confirmed by sequencing.

The open reading frame of the silkworm transcription factor E74A was downloaded from the National Center of Biotechnology Information (NCBI, Genebank no.: DQ471939). For the silkworm E74B, a predicted protein lacking N- terminus has been submitted to NCBI (Genebank no.: DQ471940) (Supplementary Fig. S1). To obtain the full-length E74B gene, we used the nucleotide sequence of this truncated gene as query to blast against the silkworm EST data, and finally found a candidate (NCBI, Genebank no.: KX881578). The new silkworm E74B protein contains a conserved N-terminus as shown in other Lepidopteran species (Supplementary Fig. S1). Then the two coding sequences with HA tag on their N-termini were amplified and cloned into the pIZ/V5-His vector (Invitrogen, USA), respectively.

All primers used in this study were listed in Supplemental Table S1.

2.4. Reporter gene assay

The recombinant plasmids (PGL3-Basic, PGL3-E74A-wild, PGL3-E74A-deletion, PGL3-E74A-mutant1 and PGL3-E74A-mutant2) were co-transfected with the reference plasmids (containing Renilla luciferase gene driven by ie1 promoter) into the silkworm cell line *BmN* using X-tremeGENE HP DNA Transfection Reagent (Roche, USA), respectively. The transfected method was used according to the manufacturer's instruction. At 12 h post transfection, the cells were treated with dimethyl sulphoxide (DMSO) or 20E (Sigma) at 1 μM. Twenty-four hours after 20E treatment, the cells were collected for luciferase assays. In order to further test whether the transcription factor E74 can directly regulate the *BmEO* gene, the recombinant reporter plasmids (PGL3-Basic, PGL3-E74A-wild, PGL3-E74A-mutant1 or PGL3-E74A-mutant2), pIZ-E74A or pIZ-E74B expression plasmids and reference plasmids were co-transfected as shown above. Forty-eight hours after transfection, the cells were collected for luciferase assays and RNA isolation.

2.5. Electrophoretic mobility shift assay (EMSA)

For EMSA, the complimentary double-stranded DNA probes (E74A-probe) were synthesized based on the putative E74A binding site of *BmEO* gene. In addition, probes with E74A binding site mutants were also synthesized (E74A-M1-probe) (Supplemental Table S1). The oligonucleotides were 5′-biotin-labeled (in Sangon Company, Shanghai, China). The non-labeled oligonucleotides of the E74A binding sites and mutated E74A binding sites were used as competitor of the biotin-labeled probes. The nuclear protein from cells transfected with pIZ-E74A or pIZ-E74B or pIZ vector was extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Nantong, China). The method was similar to the previous study [3]. The DNA binding reactions were performed in 20 μL solution containing 10 μg nuclear proteins with binding buffer (Beyotime, Nantong, China). The mixture was incubated for 20 min at 26 °C. Then 50 fmol labeled probe was added and incubated for an additional 20 min at 26 °C. For competition assays, a 50-fold amount of cold probe was pre-incubated with the nuclear protein for 10 min before the addition of the labeled probe. After treatment, the mixture was loaded on a 6.5% polyacrylamide gel and electrophoresed in 1 × TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.3). Then the gel was transferred onto a nylon membrane for 30–40 min. After crosslink at 120 °C for 30 min, the membrane was blocked for 1 h with blocking reagent (Beyotime, Nantong, China). Then the membrane was treated with streptavidin-HRP solution (at 1:1000 dilution) for 20 min. Finally, bands were detected by Pierce ECL plus Western blotting kit (Thermo Scientific, USA).

2.6. Chromatin immunoprecipitation (ChIP)

To confirm the binding of the transcription factors to the CREs, the ChIP assays were performed according to the manufacturer's instructions (Beyotime, Nantong, China). Cells transfected with pIZ-E74A or pIZ-E74B vector were used in this experiment. Firstly, the cells were fixed with 1% formaldehyde for 10 min at 37 °C. Then the cross-linked DNA-protein complexes were sheared into fragments with 200–1000 bp in length by sonication. Immunoprecipitation assays were performed using anti-HA and nonspecific rabbit IgG antibodies. The purified DNA from the immunoprecipitated chromatin was used as the template for PCR amplification. The used primers were listed in Supplementary Table S1.

2.7. RNA interference

Based on the cDNA sequences of the silkworm E74A and E74B, we designed specific primers containing T7 promoter sequence. Meanwhile, we also designed primers to amplify the common region of E74A and E74B genes. The primers were listed in Supplemental Table S1. The PCR products were sequenced to confirm the specificity of the primers. The amplified fragments were then used as templates to generate double-strand RNAs (dsRNAs). The dsRNAs were

synthesized *in vitro* by Ribo-MAX Large Scale RNA Production systems-T7 (Promega, USA) using a manual method. Concentrations of the dsRNAs were quantified by UV spectrum absorbance. Ten microliter solutions containing 50 μg of ds-E74A, ds-E74B or ds-E74 were injected into the 7th day larvae of the last instar, respectively. The same concentration of ds-EGFP was used as control. Twenty-four hours later, the midguts were dissected and stored at −80 °C. In addition, 54 h later, 100 μL hemolymph was collected from the treated silkworm (3–5 individuals), and added with nine volumes of methanol.

2.8. Western blotting

The midguts collected from RNAi experiments were grinded in liquid nitrogen to powders. Then the powders were dissolved in 2% SDS solution at 37 °C for 2 h. Followed by centrifugation at 10,000 ×*g* for 10 min, the supernatant was collected for further analysis. BCA protein assay was used to quantify the proteins. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting experiment were performed as described in previous study [35]. Based on our previous study, the polyclonal antibody against the silkworm EO (at a dilution of 1:2500) shown above was used to do this assay [35].

2.9. Quantitative Real-time PCR (qRT-PCR)

For each treatment shown above, the stored tissues were grinded in liquid nitrogen to powders in liquid nitrogen. Total RNA was extracted by the Ultrapure RNA Kit (Beijing CoWin Biotech, China) and treated with DNase I (Takara, Japan) to remove the genomic DNA contamination. The RNA was quantified by the ultraviolet spectrophotometer, and then 1 μg RNA was reverse-transcribed to the first strand of cDNA by the EasyScript one-step gDNA removal and cDNA synthesis SuperMix kit (TransGen Biotech, China). The specific primers were designed and used in the quantitative real-time PCR (qRT-PCR) analysis (Table S1). The qRT-PCR was performed using real-time PCR Detection

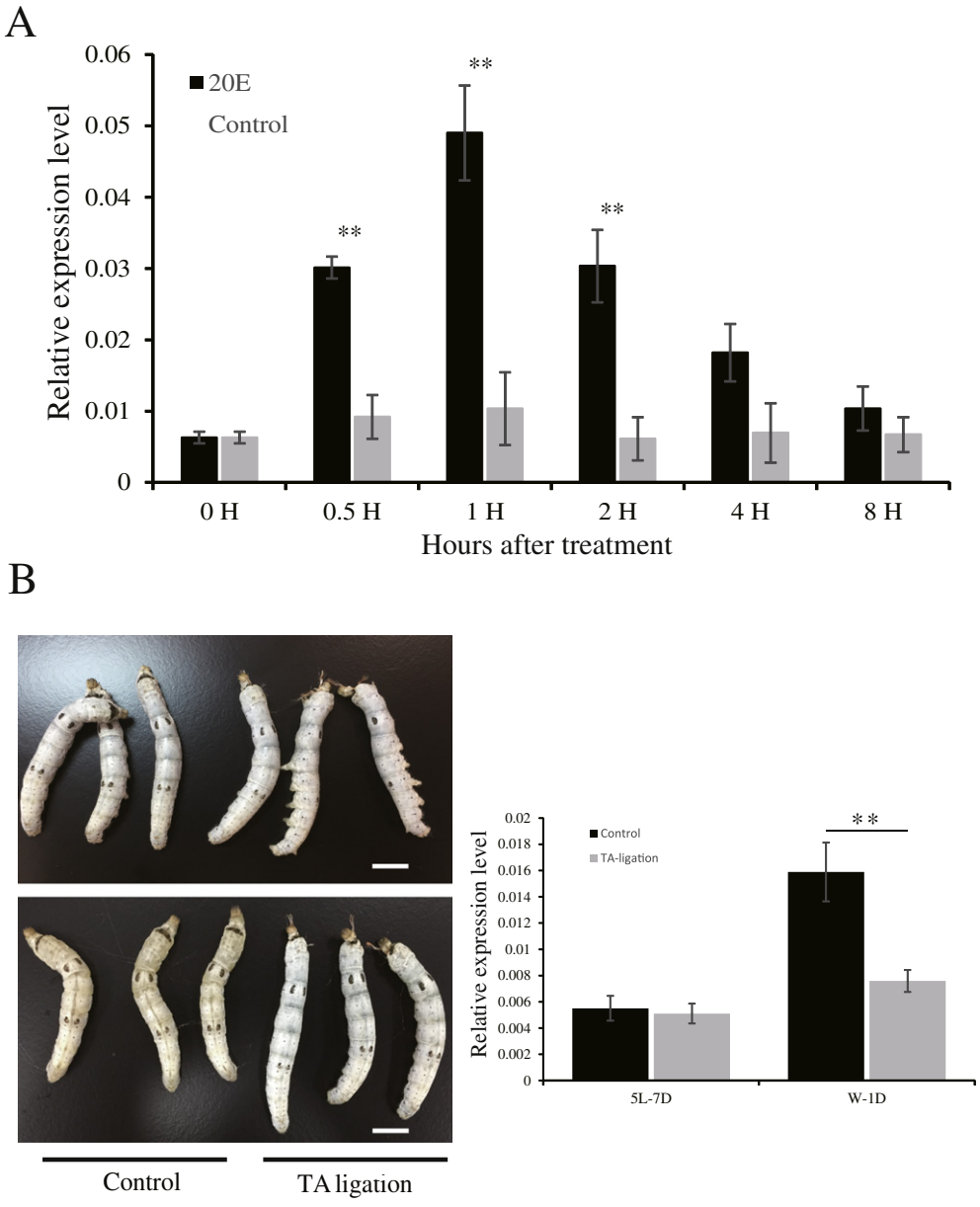

Fig. 1. 20E induced the expression of the *BmEO* gene. (A) The expression of the *BmEO* gene after orally injected 20E treatment; (B) The expression of the *BmEO* gene after TA ligation; For (A), 20E was orally injected into the 3rd-day fifth larvae. Different time points after injection, total RNA from midgut was extracted. For (B), the 7th day larvae of the last instar were abdomen-ligatured. Twenty-four hours after ligation, total RNA from midgut was extracted. The relative expression levels of the *BmEO* gene were normalized using the threshold cycle (Ct) values of the reference gene, ribosomal protein L3. Asterisks indicate statistical significance (*t*-test; $^{***}P < 0.001$).

System (CFX96, Bio-Rad, USA) with SsoAdvanced SYBR Green Supermix kit (Bio-Rad, USA). The silkworm ribosomal protein like gene 3 (RPL3) was used as the reference gene.

2.10. Ecdysteroid measurements

The hemolymph mixture with methanol was centrifuged at 12,000*g* for 10 min. An aliquot of supernatant was combined and dried at 70 °C, and then the dried extract was dissolved with 150 μL Enzyme Immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA, 0.1% BSA in 0.1 M phosphate buffer) (Sangon, China). Ecdysteroid levels were quantified via competitive EIA (Cayman Chemicals, USA) using anti-20E rabbit antiserum (Cayman Chemicals, USA), 20E acetylcholinesterase (AchE) tracer (Cayman Chemicals, USA) and standard 20E (Sigma, USA). The tracer is AchE-labeled 20E. This EIA is based on the competition between un-labeled 20E and AChE-labeled 20E for limited specific rabbit anti-20E antiserum sites. The AchE activity was quantified by Ellman's Reagent (Cayman Chemicals, USA), and the absorbance at 405 nm was detected with ELx800 absorbance microplate reader (Biotek, USA). All assays were performed in triplicate.

2.11. Statistical analysis

In this study, all the statistical analyses of the significance of the difference between the groups were performed by means of Student's *t*-test in the statistical R package.

3. Results

3.1. The responses of the BmEO gene to 20E

In order to investigate whether 20E could affect the expression of the silkworm *BmEO* gene *in vivo*, we firstly orally injected the hormone into the feeding larvae. The expression of the *BmEO* gene was elevated at 0.5 h after treatment, and reached a peak at 1 h after treatment, and then decreased to normal level at next time points (Fig. 1A). Thorax-abdomen (TA) ligation was widely used to survey the effect of the 20E on the insect development [29,45]. In this study, we performed this experiment to shut off the release of the hormone. Twenty four hours after treatment, the normal larvae initiated spinning, and their epidermis became transparent, and switched to the wandering stage. However, the ligated larvae almost had no change, indicating that molting hormone did not reach to the abdomen of the silkworm. We then found that the expression level of the *BmEO* gene in the ligated larvae was significantly lower than that in the control (Fig. 1B). These results indicated that ecdysone can positively regulate the expression of the *BmEO* gene as shown in other insects.

3.2. Identification of the CREs of the BmEO gene

Our previous study showed that a 77 bp fragment located on about 500 bp upstream of the transcription start site of the *BmEO* contains the core regulatory elements [36]. Having performed bioinformatics analysis, we found putative E74A binding sites (from −518 to −513) in this fragment. Multiple alignment showed that the predicted binding sequence of *BmEO* gene is the same as to the *Drosophila* binding sequence of E74A(CGGAAG) (Fig. 2A) [41]. As shown above, the *BmEO* gene can be regulated by the 20E, and the transcription factor E74A is also the 20E response element. Therefore, we focused on these binding sites. For the PGL3-E74A-wild plasmids, the luciferase activity showed an increase of about 3-fold after 20E treatment. However, when the E74A binding sites were deleted, 20E could not induce the change of the luciferase activity (Fig. 2B). Moreover, in the site mutagenesis experiment, when "GG" or "AA" were mutated, the luciferase activity also did not change. These indicated that the putative E74A binding sites are essential for the expression of the *BmEO* gene induced by 20E.

3.3. Transcription factor E74A can directly bind to the CREs of the BmEO gene

In order to further survey whether 20E inducible E74A could directly bind to the core regulatory elements of the *BmEO* gene, *E74A*-pIZ expression plasmids and reporter vectors were co-transfected into the silkworm cell line without the 20E treatment (Fig. 3A). In the pIZ transfected cells, the expression signal of the *BmE74A* was not observed (Fig. 3B), and the luciferase activity driven by E74A-wild promoters of the *BmEO* gene was similar to the control vector (PGL3-Basic). However, in the presence of overexpression of *E74A*, E74A-wild promoters could significantly increase the luciferase activity (Fig. 3A). For the E74A mutant promoters, both of them could not enhance the luciferase expression with or without E74A protein. Furthermore, EMSA using biotin labeled probes containing the E74A binding site was performed and demonstrated a shifted band that was specifically blocked by competitive probes but not by mutant probes (Fig. 3C). In addition, mutant E74A probes with biotin also could not produce shifted band. Our EMSA results indicated that transcription factor E74A specifically

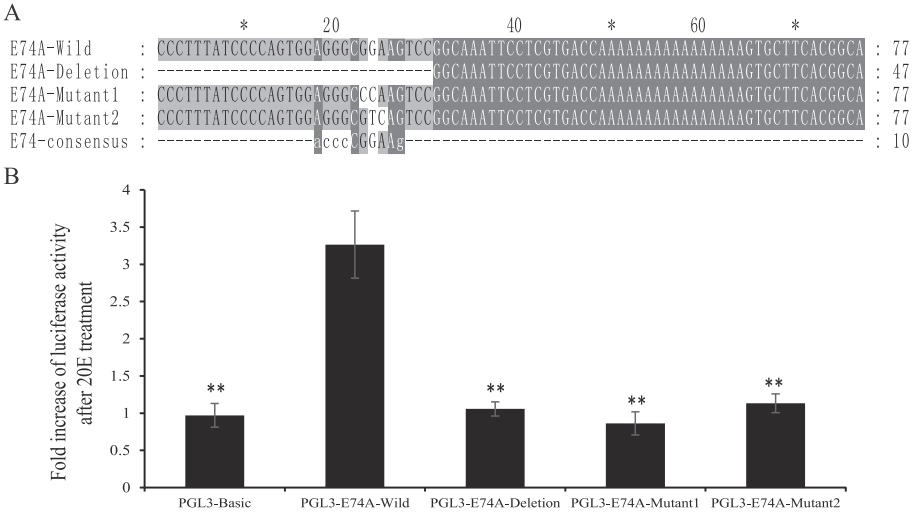

Fig. 2. Identification of the CREs of the *BmEO* gene. (A) Multiple sequences alignment of E74A binding sites and the corresponding sequences used for the luciferase reporter assay. (B) The fold increase of luciferase activity for the fragments containing wild or deleted or mutated E74A binding sites after 20E treatment. Asterisks indicate statistical significance (*t*-test; ** $P < 0.01$).

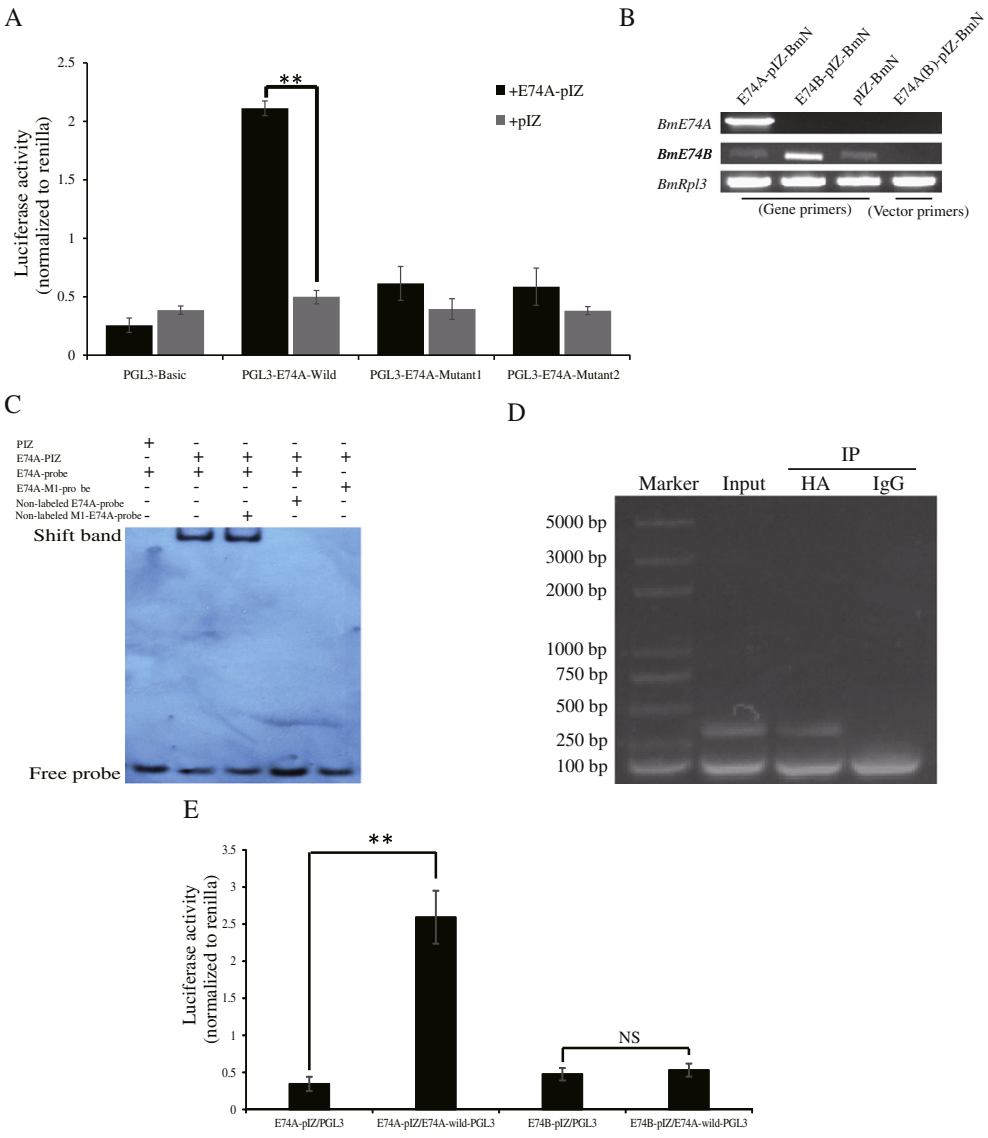

Fig. 3. Transcription factor E74A directly binds to the CREs of the *BmEO* gene. (A) Effect of overexpressed silkworm E74A protein on the luciferase activity for the fragments containing wild or deleted or mutated E74A binding sites; (B) BmE74A and BmE74B expressed in the transfected cells *BmN*; (C) EMSA analysis of the E74A CREs of the silkworm *EO* gene binding to nuclear proteins isolated from the *BmN* cells transfected with pIZ-E74A or pIZ-vector. (D) ChIP assays of the binding of E74A to the overlapping CREs in the *BmEO* promoter *in vivo*. (E) Effect of overexpressed silkworm E74B protein on the luciferase activity for the fragments containing wildE74A binding sites. For B, "isoform primers" means the isoform specific primers and is used to test whether E74A or E74B is expressed in the transfected cells; "vector primers" means the universal primer for vector and is used to avoid plasmid pollution for the E74A or E74B amplification. For D, the cells were collected 48 h after transfection for ChIP assays. IP means Immunoprecipitation. Asterisks indicate statistical significance (*t*-test; ** $P < 0.01$).

bound to the promoter region of the *BmEO* gene. To further examine whether or not E74A could bind to the predicted CREs *in vivo*, chromatin immunoprecipitation (ChIP) assays were performed in the HA-tagged E74A overexpression cells. Compared with the IgG treatment, we found that a positive band corresponding to the CREs in the promoter of *BmEO* gene was detected by PCR when we used the anti-HA antibody (Fig. 3D).

In insects, *E74* gene encodes two protein isoforms A and B, which share a common C-terminal Ets DNA-binding domain, and have a unique N-terminal sequence [33] (Supplementary Figure S1). Both E74 proteins are critical for the insect development [5,6]. E74A can bind to the CREs of the *BmEO* gene. Thus, we want to know whether or not E74B has the same function. We firstly surveyed the expression of the *BmE74B* in the transfected cells, and found that the expression signals were detected in all cells including pIZ-transfected cells by the *BmE74B* specific primers (Fig. 3B). As shown above (Fig. 3A), the promoters containing E74A-wild region did not drive the luciferase activity in the pIZ-transfected cell in which the *BmE74B* was also expressed. This suggested that BmE74B could not bind to the CREs of the *BmEO* gene. In the *BmE74B* overexpressed cell, the luciferase activity was similar to the control, further confirming that E74B did not affect the expression of the *BmEO* gene (Fig. 3E). Furthermore, we also confirmed this result by EMSA and Chip assay (Supplementary Fig. S2).

Then we performed RNA interference for the silkworm *E74A* or *E74B* to investigate their functions in the expression regulation of the *BmEO* gene and ecdysone titer *in vivo*. Firstly, we surveyed the expression patterns of *BmE74A* and *BmE74B* at different developmental stages. From Fig. 4A, the two isoforms showed distinct expression profiles in midgut at different developmental stages. The expression of the *BmE74B* increased during the late feeding stage, followed by a sharp elevation at early wandering stage (0–12 H). Then the expression of the *BmE74B* declined rapidly to a low level until pupation. By contrast, the expression level of the *BmE74A* increased following the *BmE74B* at different developmental stages, and reached a peak at 48 h after initiation of

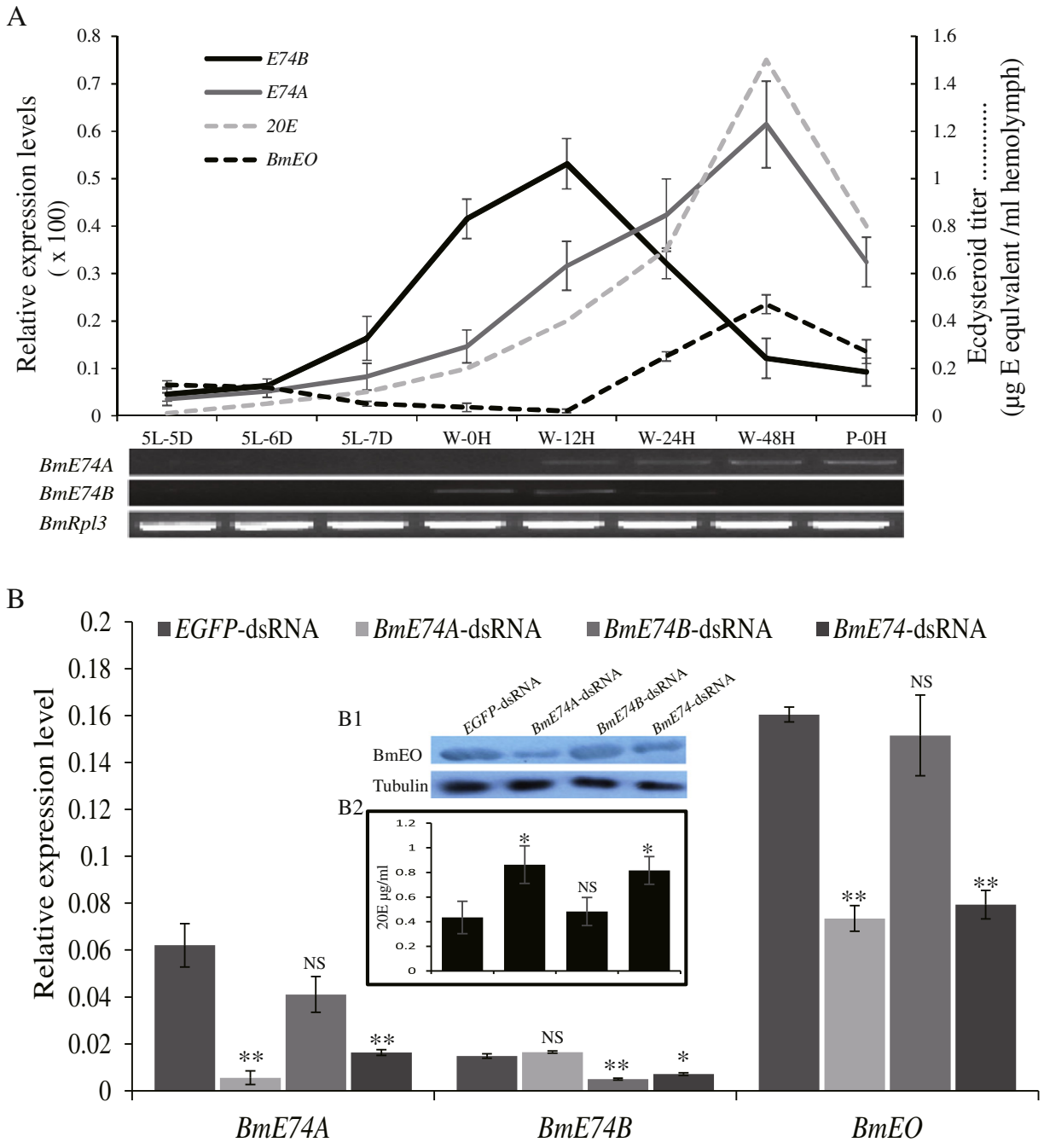

Fig. 4. E74A regulated the expression of the *BmEO* in vivo. (A) The expression profiles of the silkworm *E74A*, *E74B* and *BmEO* gene in the midgut at different developmental stages; (B) RNAi experiments examine the effect of E74 on the expression of the *BmEO* gene. For A, 5L represents the 5th instar larvae. W represents the wandering stage. The gray dot line represents the developmental changes in hemolymph ecdysteroid titer based on the data as previously described [11]. The black dot line means the expression pattern of the *BmEO* gene. The expression data of the *BmEO* gene are 10-fold of the actual values; For inner figure in B, B1: Western blotting analysis for the BmEO protein in RNAi larvae; B2: 20E concentration in RNAi larvae. Asterisks indicate statistical significance (*t*-test; ** $P < 0.01$; * $P < 0.05$).

wandering stage (Fig. 4A). The expression patterns of the *BmE74A* and *BmE74B* in midgut were similar to the previous observation in silk gland [31]. The expression data of *BmEO* gene collected from our previous study was also shown in Fig. 4A [35]. Interestingly, we found that the expression profile of *BmEO* gene was consistent with *BmE74A* gene and ecdysone titer. We then knocked down the expressions of *BmE74A* and *BmE74B* using the specific dsRNAs, respectively. Besides, we also synthesized a common dsRNA for the shared region of the *BmE74A* and *BmE74B*. We performed the experiments in the silkworm larvae at the 7th day of the last instar. Compared with the control, isoform specific dsRNA can significantly decrease the expression level of the corresponding isoform (Fig. 4B). The common E74 dsRNA blocked the two isoforms. Interestingly, in the *BmE74B* RNAi larvae, the expression of the other isoform *BmE74A* was similar to the control. The same result was also obtained for the *BmE74B* in the *BmE74A* RNAi larvae. Therefore, the expressions of the two E74 isoforms have no effect to each other in the silkworm.

The expression level of the *BmEO* gene decreased about 50% in both the *BmE74A* RNAi and *BmE74* RNAi larvae. However, knocked down *BmE74B* did not affect the expression of the *BmEO* gene (Fig. 4B). Western blotting experiment also confirmed these results (Fig. 4B, inner Fig. B1). EO is the rate-limited enzyme of the 3-epimerization

pathway to degrade ecdysone titer in Lepidoptera [44]. In the domesticated silkworm, the knockout of *EO* gene could significantly elevate ecdysone concentration at the metamorphosis stage [16]. Thus, we further examined the ecdysone titer of the RNAi larvae. Fifty-four hours after dsRNA injection when the normal silkworm complete pupal ecdysis and reduce the ecdysone titers, the *BmE74A* RNAi and *BmE74* RNAi larvae retained significantly higher ecdysone concentration compared with the control (Fig. 4B, inner Fig. B2). Similar to the qRT-PCR and Western blotting experiments, the ecdysone titer did not change in the *BmE74B* RNAi larvae. Taken together, our results suggested that 20E inducible E74A can affect the ecdysone titer by regulating the expression of the *EO* gene in the domestic silkworm.

4. Discussion

The periodic and accurate fluctuations of ecdysone titer are essential for the insect molting and metamorphosis [39]. Ecdysone titer is controlled by its biosynthesis and degradation. Previous studies have demonstrated that the synthesis of ecdysone could be controlled by the feedforward and feedback regulation [4,17,18,22,23]. In addition to correctly turning on or off the ecdysone synthesis pathway, insects must degrade the active ecdysone from target tissues to terminate the ecdysone pulses at some critical time points. Several enzymes involved in degradation of ecdysone have been identified [26,35]. However, how insects regulate these enzymes to control the ecdysone titer is still obscure.

In this study, we found that the *BmEO* gene was rapidly induced at thirty minutes after oral injection of 20E. Interestingly, *EO* gene in *Spodoptera littoralis* could not be stimulated by hemolymph injected 20E. However, ecdysteroid agonist (RH-5992) could significantly elevate the expression of *EO* gene in the species [37]. The different responses of *EO* gene to the 20E may be correlated with the mode of injection. A previous study suggested that the ingested (orally injected) exogenous ecdysone could be predominantly transformed into 3-epiecdysone, the final product of ecdysone epimerization, in *B. mori* larvae [47]. Conversely, only trace 3-epiecdysone was detected in the larvae treated by hemolymph injection of ecdysone. Nevertheless, our data showed that the silkworm ecdysone oxidase, as an ecdysone metabolism enzyme, was also positively regulated by the ecdysone itself. For other ecdysone metabolic enzymes, this characteristic may be common. *CYP18A1* gene encoding a cytochrome P450 enzyme with ecdysone 26-hydroxylase activity could also be induced by 20E [15]. These results suggested that insects may utilize elegant feedback mechanisms to stimulate ecdysone degraded enzyme to rapidly decline the active ecdysone following a peak.

Ecdysone firstly binds to the EcR/USP complex and subsequently induces several primary and late response transcription factors, including Broad complex (Br-C), E75, hormone receptor 3 and fushi tarazu (ftz) [7,8,28,30]. These factors transduct the ecdysone signal and stimulate the target genes at special developmental stages. Furthermore, we tested the molecular mechanisms how ecdysone affects *EO* gene by luciferase reporter assay, EMSA and ChIP. Our results demonstrated that ecdysone inducible transcription factor E74A directly regulated the expression of the *EO* gene in the silkworm. E74A belongs to Ets transcription protein involving in a variety of important biological processes [32]. Previous studies have proven that E74A is required for the insect metamorphosis [5,6]. Insect E74A has another isoforms, E74B. They share the same C-terminal containing DNA binding domain, and have the completely different N-termini. In mosquito, both the E74 isoforms could bind to an E74 consensus motif CGGAA [33]. However, our experiments in the silkworm cells showed that only E74A bound to the putative CREs(CGGAA) of the *BmEO* gene. Why silkworm E74B cannot bind to the same motif as E74A is an interesting question. One possible reason is that E74B needs to interact with a cofactor to regulate the expression of target genes. A previous study demonstrated that E74B acts synergistically with EcR/USP dimer to activate the expression of the vitellogenin (*Vg*) in *Aedes aegypti* [34]. By contrast, E74A did not affect the promoter activity of *Vg in vivo* though they share the same DNA binding domain [34]. In addition, some Ets proteins were found to interact with other cofactors to play their roles [1,13,40]. Therefore, we will further test whether the silkworm E74B needs cofactors in the future.

Both the *E74* isoforms are activated by ecdysone, but display different responses. *E74B* is expressed in response to low 20E titer and repressed at a significantly higher 20E concentration, whereas *E74A* is induced by the high levels of hormone [9]. This differentiation may make them regulate different kinds of target genes. In *Drosophila*, E74A protein regulates a subset of ecdysone response late genes and does not affect the transcription of most ecdysone primary-response genes [5]. In the silkworm midgut, the expression pattern of the *E74A* is consistent with the fluctuation of the ecdysone titer and correlated with that of the *BmEO* gene, suggesting its possible role in governing the expression of the later genes *in vivo* (Fig. 4A). Furthermore, RNAi confirmed these results. In total, we demonstrated that *EO* gene could be up-regulated by ecdysone mediated by E74A. Our results expand the knowledge about the regulatory system that determines the ecdysone titer. At the beginning of the metamorphosis, low 20E concentration triggers the primary response genes, such as BR-C, E75B, E74A and HR3 [14]. Meanwhile, a feed forward loop through EcR further increases the hormone titer until it reaches the peak [18]. When insects do not need high level of 20E, the ecdysone degradation gene *EO* was induced by ecdysone itself mediated by the E74A transcription factor to decline the high hormone titer which may assure the termination of the ecdysone pulse.

As shown above, the shape and duration of the ecdysone pulse are regulated by several mechanisms including positive and negative feedback controls of the PG activity [4,17,18,22,23]. Besides, ecdysone degradation is also a critical route to maintain circulating hormone at a proper level. In this study, we revealed the molecular regulatory mechanism of the ecdysone degradation enzyme EO in the silkworm, and demonstrated that it is a new feedback regulation degradation pathway mediated by ecdysone inducible factor E74A to regulate the ecdysone pulse. Combined with feedback controls of the PG activity in previous studies, feedback regulation degradation pathway constitutes an autonomous regulatory system that determines the formation of the ecdysone pulse.

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Conflicts of interest

No potential conflict of interest was reported by the authors.

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