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Knockdown of ecdysis-triggering hormone gene with a binary *UAS/GAL4* RNA interference system leads to lethal ecdysis deficiency in silkworm

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Ecdysis-triggering hormone (ETH) is an integration factor in the ecdysis process of most insects, including *Bombyx mori* (silkworm). To understand the function of the *ETH* gene in silkworm, we developed an effective approach to knockdown the expression of *ETH in vivo* based on RNA interference (RNAi) and a binary *UAS/GAL4* expression system that has been successfully used in other insect species. Two kinds of transgenic silkworm were established with this method: the effector strain with the *ETH* RNAi sequence under the control of *UAS* and the activator strain with the *GAL4* coding sequence under the control of *Bombyx mori* cytoplasmic actin3. By crossing the two strains, double-positive transgenic silkworm was obtained, and their *ETH* expression was found to be dramatically lower than that of each single positive transgenic parent. Severe ecdysis deficiency proved lethal to the double-positive transgenic silkworm at the stage of pharate second instar larvae, while the single positive transgenic or wild-type silkworm had normal ecdysis. This *UAS/GAL4* RNAi approach provides a way to study the function of endogenous silkworm genes at different development stages.

Keywords ecdysis-triggering hormone; *UAS/GAL4* system; RNAi; transgenic silkworm

RNA interference (RNAi) has been developed as a powerful tool for gene-specific knockdown in many species including *Bombyx mori* (*B. mori*) (silkworm). Double-stranded RNA (dsRNA) molecules can be introduced into silkworm by direct RNA injection [1–4] or virus infection [5] to achieve efficient and transient inhibition of target gene expression. Transgenesis of an RNAi expression sequence against BmNPV in silkworm generated a heritable transgenic silkworm line with enhanced resistance to the virus [6]. We recently reported a heat shock inducible RNAi strategy in transgenic silkworm to inhibit the expression of the endogenous ecdysis-triggering hormone gene (*ETH*) [7].

The binary *UAS/GAL4* system was used widely to express genes in *Medaka* [8], *Drosophila* [9], and *Xenopus* [10]. In silkworm, the *UAS/GAL4* system has been used for tissue specific expression of target genes in photo-receptor cells and silk gland tissue [11]; more often, the system has been used to knockdown the expression of target genes [12,13]. However, until now, RNAi based on the binary *UAS/GAL4* system has not been reported in silkworm.

In this study, we developed in silkworm a new transgenic RNAi approach based on the binary *UAS/GAL4* system that successfully inhibited *ETH* gene, an important endogenous gene involved in regulating ecdysis behavior in insects [14–16]. In this method, piggyBac-mediated transgenesis was used to generate an effector transgenic silkworm line with an *UAS* element-driven DNA fragment coding for the RNAi sequences against the *ETH* gene and an activator transgenic silkworm line with *B. mori* cytoplasmic actin3 (*BmA3*) promoter-controlled *GAL4* sequence. Double-positive transgenic silkworms were produced by crossing the two lines, and they showed decreased *ETH* expression and ecdysis deficiency in pharate second instar larval stage.

Materials and Methods

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Plasmid construction

For generating the activator vector containing *BmA3-GAL4*, *BmA3* gene promoter sequence was polymerase chain reaction (PCR)-amplified from the plasmid pigA3 with the primers (up) 5′-CACTCGAGTGCGCGTTACCATATA-TGGTGA-3′ and (down) 5′-TAGCGGCCGCTTGAATTA-GTCTGCAAGAAAAG-3′, which contained *Xho*I and *Not*I sites respectively (underlined). The amplified fragment was treated with *Xho*I and *Not*I and then ligated into the vector pcDNA3.1 (Invitrogen, Shanghai, China) to form the construct p*BmA3*. The open reading frame of yeast transcriptional activator *GAL4* gene was obtained from the pChs-*Gal4* plasmid by *Hin*dIII digestion, and it was ligated into the multiple cloning site of the vector p*EGFP*-N1 (Takara, Dalian, China) to be the vector p-N1-*GAL4*. Then, the *GAL4*-SV40 polyA fragment was cut off from the p-N1-*GAL4* by *Eco*RI and *Afl*II and was inserted downstream of *BmA3* promoter into the vector p*BmA3*. From this vector, the *BmA3-GAL4*-SV40 polyA fragment was cut out and then inserted into the *Nhe*I and *Afl*II site of the vector pBac{3xp3-*EGFP*am} to generate the activator vector pBac{3xp3-*EGFP-BmA3-GAL4*af}.

To generate the effector vector containing cDNA coding for *ETH* RNAi, complete *ETH* code sequence was first cloned from silkworm. Total RNA was extracted from the epitracheal gland of the fifth instar larvae with RNeasy mini isolation kit (Qiagen, Shanghai, China) and reverse-transcribed by SuperScript II (Takara) with oligo(dT) primer in a reaction volume of 10 μl. As the template for PCR amplification of *ETH* cDNA, 2 μl of reverse transcription (RT) product was used with 30 cycles of 94 ºC for 30 s, 60 ºC for 40 s and 72 ºC for 30 s. The primer sequences were (up1) 5′-CTGTCGACATGACTTCAAAATTG-ACAATGATG-3′, (down1) 5′-GTCTGCAGTTTCTT-CATGCTTCCCATTTTTTT-3′, (up2) 5′-ACGGGCCCA-TGACTTCAAAATTGACAATGATG-3′, and (down2) 5′-GTCCGCGGTTTCTTCATGCTTCCCATTTTTTT-3′, which contained *Sal*I, *Pst*I, *Apa*I and *Sac*II sites, respectively (underlined). The two PCR fragments (with primer pair of up1/down1 or up2/down2) were treated with respective restriction enzymes and ligated tail to tail into multiple cloning site of the vector psiRNA to form the vector psi*ETH*. An intron from fibroin light chain gene was PCR-amplified from silk gland genomic DNA with primers, (up) 5′-CACCGCGGAGCCCACCTGGTGTT-AAGTGGTGA-3′ and (down) 5′-CACTGCAGTTACT-GGTGGTAGGACCTGTTGTG-3′ containing *Sac*II and *Pst*I sites, respectively (underlined). The amplified fragment was treated with *Sac*II and *Pst*I and ligated into the vector psi*ETH*. The double-stranded *ETH* (Ds*ETH*)-intron-SV40 polyA fragment from this construct was excised with *Sal*I and *Pst*I, and ligated into the vector p*UAS* to form the vector p*UAS*-Ds*ETH*. Then, the *UAS*-Ds*ETH*-SV40 polyA fragment was excised and inserted into the vector pcDNA3.1 to obtain the vector pcDNA-*UAS*-Ds*ETH*. Finally, the *UAS*-Ds*ETH*-SV40 polyA fragment was inserted into the vector pBac{3xp3-*EGFP*af} to generate the effector vector pBac{3xp3-*EGFP-UAS*-Ds*ETH*af}.

The sequence of the PCR products and resulting plasmids were confirmed by sequencing performed by a commercial service provider (Invitrogen).

***B. mori* strains and transgenic silkworm production**

Transgenic silkworm was constructed under piggyBac transposon introduction by way of microinjection the mixture of the helper pigA3 plasmid and the transgenic vector [17]. The effector or the activator construct and the transposase carrying the helper plasmid pigA3 were injected mid-ventrally into the preblastoderm eggs. Afterwards, the eggs were incubated until hatching in a humidified chamber at 25 ºC. Hatched larvae were transferred onto mulberry leaves. Positive G_1 larvae were selected and reared individually. The genotype of transgenic silkworm was analyzed by PCR. The sequence of primer pairs for *UAS*, *ETH* and *GAL4*, respectively, were (up*UAS*) 5′-GGTCGGAGTACTGTCCTCCG-3′; (dn*ETH*) 5′-TCG-AACGGCAAACTGTAGAC-3′, (up*GAL4*) 5′-AAGAT-GAAGCTACTGTCTTCTA-3′, and (dn*GAL4*) 5′-TTAC-GATACAGTCAACTGTCTTTGA-3′.

Real-time RT-PCR analysis

Total RNA was prepared from the 7 d embryos, first instar and pharate second instar larvae of effector [A(-)E(+)], activator/effector [A(+)E(+)], activator [A(+)E(-)] transgenic lines and wild-type silkworm using RNeasy Mini Isolation kit (Qiagen) and treated with RNase-free DNaseI (Promega, Madison, USA) [18]. Subsequently, cDNA were synthesized. The sequence of primer pairs for *ETH*, Ds*ETH* and *GAL4* were (up*ETH*) 5′-CGCTAAACACAG-CACCGTGAAC-3′, (dn*ETH*) 5′-TCGAACGGCAAACT-GTAGAC-3′, (upDs*ETH*) 5′-CGCGCGGACTCACAAC-AGGT-3′, (dnDs*ETH*) 5′-TATCCCATCACGTCCTCATC-3′, (upGAL4) 5′-AGTGCTCCAAAGAAAAACCGA-3′, and (dn*GAL4*) 5′-GGTCTTCTCGAGGAAAAATCAG-3′. Quantitative PCR measurement was performed with EvaGreen fluorescence dye (Biotium, Hayward, USA) on a Rotor-Gene 3000 Detection System (Biocompare, South San Francisco, USA). The RNA level for each group was measured in triplicates and normalized to an internal control

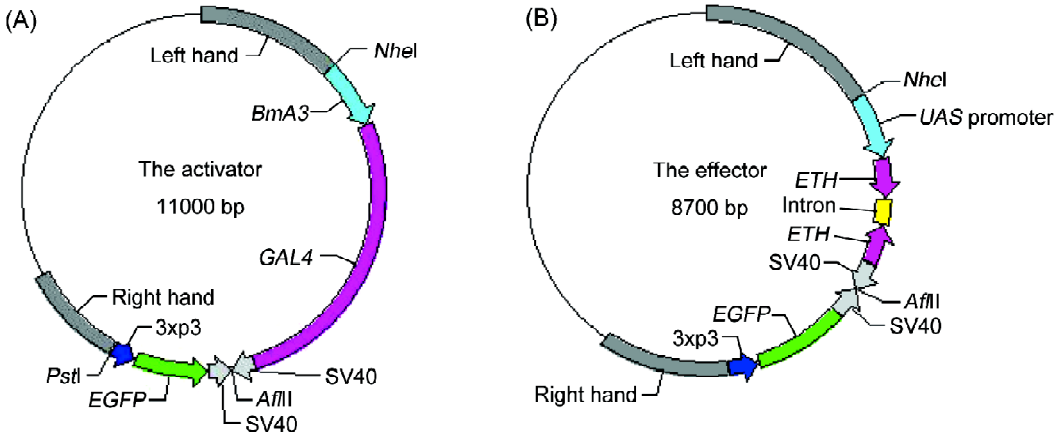

Fig. 1 Physical map of activator and effector transgenic vectors (A) The activator transgenic vector pBac{3xp3-*EGFP-BmA3-GAL4*af} consists of the full open reading frame of the yeast transcriptional factor *GAL4* under the control of *Bombyx mori* cytoplasmic actin3 (*BmA3*) promoter and, in the opposite transcription orientation the enhanced green fluorescent protein gene (*EGFP*) under an eye-specific expression promoter 3xp3 as the transgenic screening marker. (B) The effector vector pBac{3xp3-*EGFP-UAS*-Ds*ETH*af} consists of the DNA-binding motif (*UAS*) for *GAL4* linked to the tandem sequence of sense and antisense complementary DNA from the targeted gene ecdysis-triggering hormone (*ETH*) and the 3xp3 driven *EGFP* gene in the opposite transcription orientation. Gray bars represent left arm and right arm of the piggyBac transposon.

of *B. mori GAPDH*.

Statistical analysis

Mean values and standard error were calculated for each group, and groups were compared using Student's *t*-test. $P<0.05$ denotes a statistically significant difference. $P<0.01$ denotes a statistically very significant difference.

Results

Production of the binary *UAS/GAL4* RNAi expression system in transgenic silkworm

The physical maps of the plasmids of the *UAS/GAL4*-mediated RNAi system are illustrated in **Fig. 1**. The *GAL4* open reading frame was placed downstream of the *BmA3* gene promoter to achieve a stable expression of *GAL4* protein in silkworm [**Fig. 1(A)**]. The *ETH* sense and antisense cDNA sequences were joined tail to tail and located downstream of the *UAS* promoter in order to transcribe the Ds*ETH* RNA activated by the *GAL4* protein [**Fig. 1(B)**].

Both plasmids contained the enhanced green fluorescent protein gene (*EGFP*) driven by eye-specific expression promoter 3xp3, which served as a screening marker for transgenic silkworm. Both plasmids also contained the left and right arm of the piggyBac transposon.

Following piggyBac-mediated transgenesis, hatched larvae (G_0) were kept to develop into moths. The resulting G_0 moths were allowed to intercross to produce the G_1 silkworm. The insertion of a foreign gene into the silkworm genome was confirmed in the effector transformed lines by inverse PCR using genomic DNA extracted from the silk glands of G_1 larvae. The genomic junction sequences of 390, 235 and 58 bp, which flanked the 5′ piggyBac inverted terminal repeat in three transgenic lines, were analyzed (**Table 1**). The search in Silkworm Knowledgebase (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=insects) confirmed that these junction

Table 1 Identification of the genomic insertion of the *UAS*-Ds*ETH* vectors in G_1 transformed lines by inverse PCR

UAS-Ds*ETH* No.	G1 line length (bp)	5′-genomic sequence
1	390	GGCC*TTAA*ACAATAACAATCGACGCACG (*Hae*III site)
2	235	GGCC*TTAA*AATATAATTGATACTGTACA (*Hae*III site)
3	58	GGCC*TTAA*TGATGCCCGTTTACGATAGT (*Hae*III site)

Ds*ETH*, double-stranded ecdysis-triggering hormone.

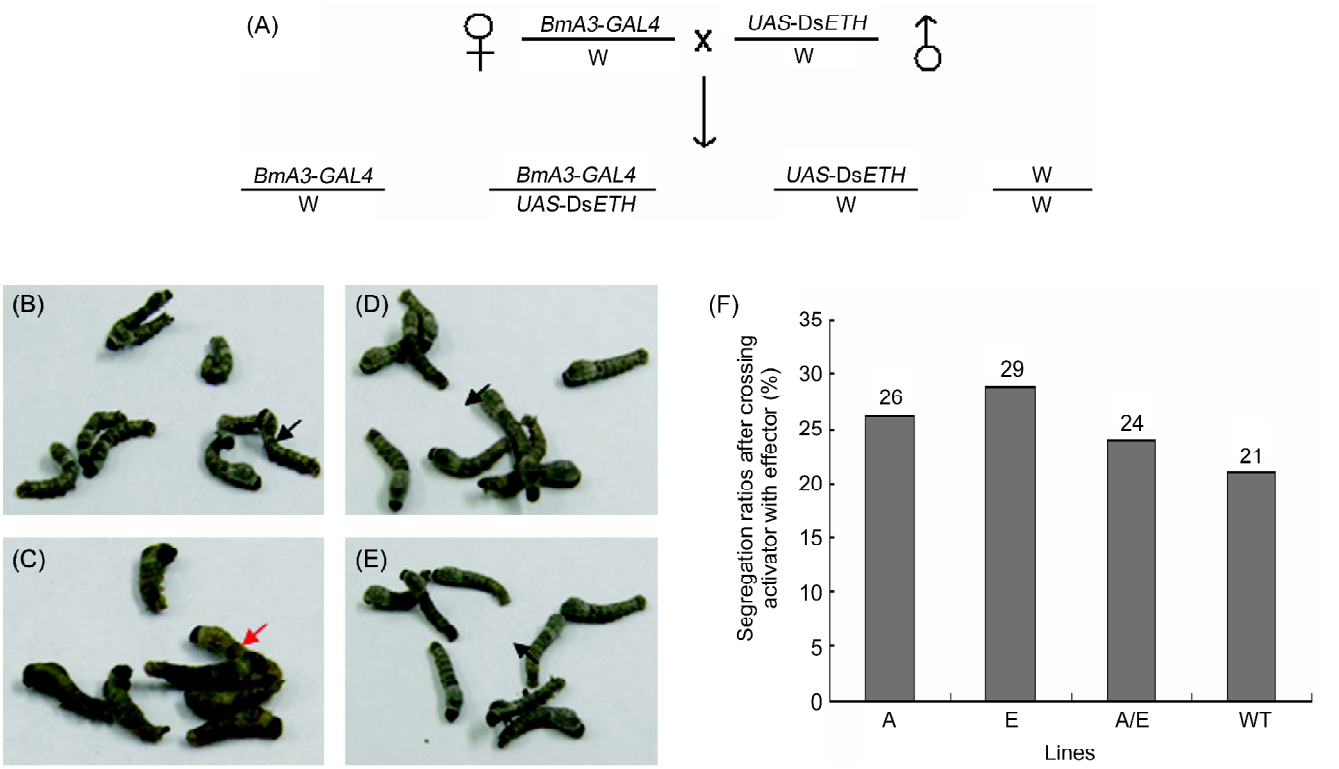

Fig. 2 RNA interference inhibition of ecdysis-triggering hormone expression leads to a lethal ecdysis deficiency at the stage of pharate second instar larvae (A) The genotypes of the offspring from the crossing between the activator and effector silkworms. (C) The lethal ecdysis deficiency was observed at the stage of pharate second instar larvae in the double-positive transgenic silkworm, but not in (B) the activator transgenic silkworm, (D) the effector transgenic silkworm and (E) the wild-type silkworm. (F) The distribution of four genotypes in 100 offspring larvae from the crossing between activator and effector silkworm. Ds*ETH*, double stranded *ETH*; W, wild type; A, activator; *BmA3*, *Bombyx mori* cytoplasmic actin3; E, effector; A/E, activator/effector; WT, wild type.

sequences were derived from the *B. mori* genome.

RNAi inhibition of *ETH* leads to the lethal ecdysis deficiency in pharate second instar larvae

Insects undergo multiple developmental stages during their life cycle, and each transition requires molting and ecdysis to produce a new epidermis and shed the old cuticle. *ETH* activates the ecdysis behavior by direct actions on the central neurons system [14]. Positive transgenic silkworm was screened by *EGFP* fluorescence signal, and moths of the effector line and the activator line were intercrossed [**Fig. 2(A)**]. The larvae from this crossing protocol contained four different genotypes that were identified by PCR analysis of the larvae genomic DNA: (1) wild type, [A(-)E(-)]; (2) activator lines, [A(+)E(-)]; (3) effector lines, [A(-)E(+)]; and (4) double-positive (activator/effector) lines, [A(+)E(+)]. The segregation ratio of the progeny of the cross was nearly 1:1:1:1, as expected, indicating that the transgenes were stably inherited in a Mendelian fashion [**Fig. 2(F)**]. At the stage of pharate second instar larvae, severe ecdysis deficiency proved lethal to the double-positive silkworm, as identified by PCR [**Fig. 2(C)**, red arrow]. This was not observed in the control larvae [**Fig. 2(B,D,E)**, black arrow].

ETH expression was knocked down in the lethal pharate second instar larvae

Real-time quantitative PCR analysis showed that *ETH* expression was markedly knocked down at mRNA level in the double-positive pharate second instar larvae, but no changes in *ETH* expression were found in the control larvae [**Fig. 3(A)**]. The *GAL4* gene was highly expressed in the double-positive pharate second instar larvae and the activator transgenic larvae, but it was not expressed in the effector transgenic larvae and the wild-type larvae [**Fig. 3(B)**].

ETH and Ds*ETH* expression patterns were analyzed at mRNA level in transgenic and wild-type silkworm

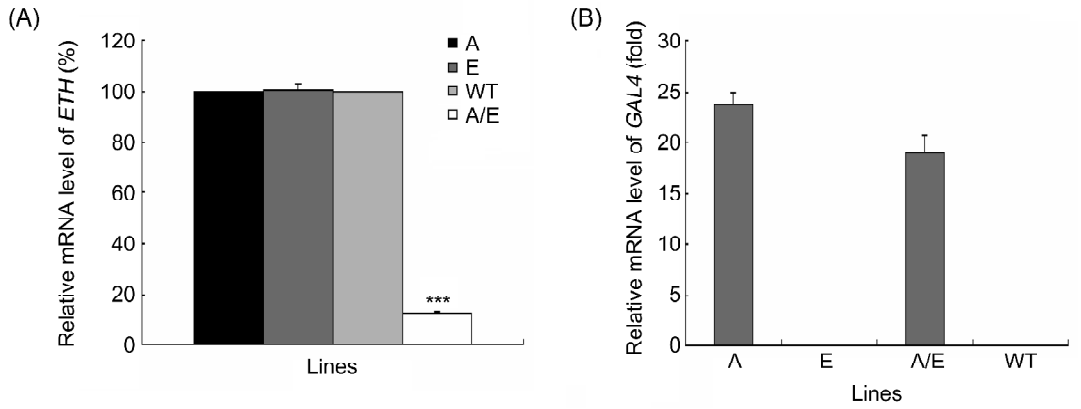

Fig. 3 Real-time quantitative PCR analysis of ecdysis-triggering hormone (*ETH*) and *GAL4* expression in silkworm with different genotypes (A,B) RNA samples from 20 pharate second instar larvae of the activator, effector, double-positive and wild-type silkworm, respectively, were prepared and subjected to real-time quantitative PCR. Data are shown as the mean±SD. Three independent experiments were performed. A, activator; E, effector; A/E, activator/effector; WT, wild type. ***P<0.005.

ETH gene is expressed specifically in epitracheal gland of insects [18]. The *ETH* expression level increases before pre-ecdysis and then declines after ecdysis stage in insects [19]. We assayed *ETH* expression at different developmental stages in silkworm. Total RNA was prepared from 7 d embryos, first instar larvae, and pharate second instar larvae from double-positive silkworm and wild-type silkworm. As quantified by real-time quantitative RT-PCR, the *ETH* mRNA expressions in the wild-type silkworm [**Fig. 4(A)**] as well as the single transgenic lines (data not shown) were high at pharate second instar larval stage and relatively low at first instar stage. In contrast, only in the double-positive transgenic lines, Ds*ETH* RNA molecules, which serve as RNAi against *ETH*, were produced at high levels at all developmental stages, including the pharate second instar larval stage [**Fig. 4(B)**].

Discussion

In this paper, we described a new approach for efficient and specific inhibition of an endogenous gene in transgenic silkworm by RNAi based on the binary *GAL4/UAS* system.

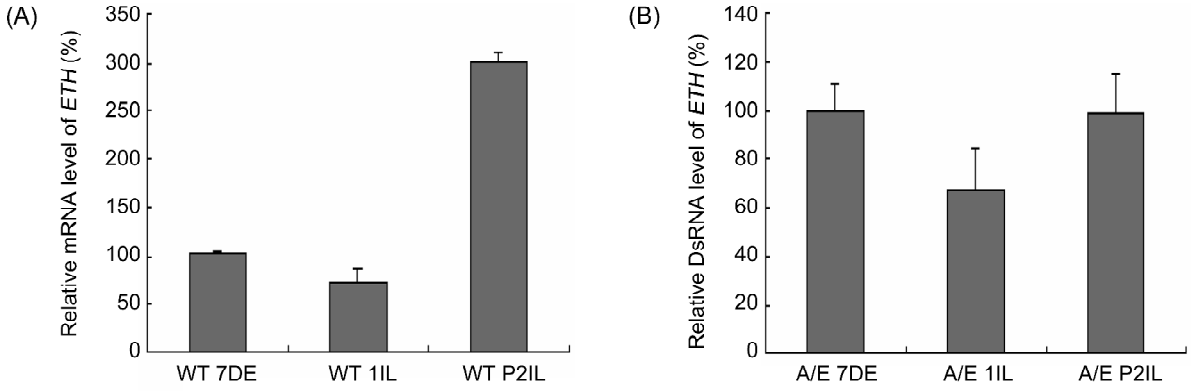

Fig. 4 Real-time quantitative PCR analysis of the expression pattern of ecdysis-triggering hormone (*ETH*) and double-stranded *ETH* (A) RNA samples from 50 7 d embryos, 50 first instar larvae and 20 pharate second instar larvae of wild-type silkworm were prepared and subjected to real-time quantitative PCR analysis. (B) RNA samples from 50 7 d embryos, 50 first instar larvae and 20 lethal larvae of the double-positive transgenic silkworm were prepared and subjected to real-time quantitative PCR analysis. Data are shown as the mean±SD. Three independent experiments were done. 1IL, first instar larvae; P2IL, pharate second instar larve; 7DE, 7 d embryos; DsRNA, double-strand RNA; A, activator; E, effector; A/E, activator/effector; WT, wild-type.

Tissue-specific knockdown of a gene could be achieved by selecting an appropriate promoter to drive the expression of the activator *GAL4*. We believe this system will become more powerful as different types of *GAL4* transgenic silkworm are generated.

The system was tested with *ETH* as the target gene in the current study. Two lines of transgenic silkworm, the *UAS*-driven *ETH* RNAi effector line and the *BmA3*-driven *GAL4* activator line, were established using the piggyBac-mediated transgenesis technique. After crossing the two strains, severe ecdysis deficiency accompanied by a dramatic decrease in *ETH* mRNA level proved lethal to nearly all the double-positive transgenic silkworm at the stage of the pharate second instar larval. This phenomenon was consistent with the result from a recent study of *Drosophila* [19] in which mutations in the *ETH* gene lead to a lethal ecdysis deficiency.

ETH is an integration factor for regulating the ecdysis behavior of insects [14–16,19,20]. Our study of *ETH* expression pattern sin silkworm showed higher *ETH* RNA levels at the stage of the pharate second instar larval than that at the first instar larval. This suggests *ETH* may play more important roles in the ecdysis process at the second instar larval stage, which may explain the lethal phenotype at the second instar larval stage observed in the study.

Although the *UAS/GAL4*-mediated RNAi system has been successfully used in many other species, this is the first report, to our knowledge, of its application in silkworm. The approach developed here allowed us to study the function of silkworm genes *in vivo* in specific cell types and at different development stages.

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References

1 Quan GX, Kanda T, Tamura T. Induction of the white egg 3 mutant phenotype by injection of the double-stranded RNA of the silkworm white gene. Insect Mol Biol 2002, 11: 217–222
2 Tabunoki H, Higurashi S, Ninagi O, Fujii H, Banno Y, Nozaki M, Kitajima M *et al*. A carotenoid-binding protein (CBP) plays a crucial role in cocoon pigmentation of silkworm (*Bombyx mori*) larvae. FEBS Lett 2004, 567: 175–178
3 Ohnishi A, Hull JJ, Matsumoto S. Targeted disruption of genes in the *Bombyx mori* sex pheromone biosynthetic pathway. Proc Natl Acad Sci USA 2006, 103: 4398–4403
4 Gui ZZ, Lee KS, Kim BY, Choi YS, Wei YD, Choo YM, Kang PD *et al*. Functional role of aspartic proteinase cathepsin D in insect metamorphosis. BMC Dev Biol 2006, 25: 6–49
5 Uhlirova M, Foy BD, Beaty BJ, Olson KE, Riddiford LM, Jindra M. Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of Broad-Complex in insect metamorphosis. Proc Natl Acad Sci USA 2003, 100: 15607–15612
6 Isobe R, Kojima K, Matsuyama T, Quan GX, Kanda T, Tamura T, Sahara K *et al*. Use of RNAi technology to confer enhanced resistance to BmNPV on transgenic silkworms. Arch Virol 2004, 149: 1931–1940
7 Dai HJ, Jiang RJ, Wang J, Xu GJ, Cao MX, Wang ZG, Fei J. Development of a heat shock inducible RNAi system in silkworm. Biomol Eng 2007, 24: 625–630
8 Grabher C, Wittbrodt J. Efficient activation of gene expression using a heat-shock inducible Gal4/Vp16-UAS system in medaka. BMC Biotechnol 2004, 4: 1–6
9 Liqun L, Lee T, Nardine T, Null B, Reuter J. Using the MARCM system to positively mark mosaic clones in *Drosophila*. Dros Inf Serv 1999, 82: 102–105
10 Hartley KO, Nutt SL, Amaya E. Targeted gene expression in transgenic *Xenopus* using the binary Gal4-UAS system. Proc Natl Acad Sci USA 2002, 99: 1377–1382
11 Imamura M, Nakai J, Inoue S, Quan GX, Kanda T, Tamura T. Targeted gene expression using the GAL4/UAS system in the silkworm *Bombyx mori*. Genetics 2003, 165: 1329–1340
12 Negeri D, Eggert H, Gienapp R, Saumweber H. Inducible RNA interference uncovers the *Drosophila* protein Bx42 as an essential nuclear cofactor involved in Notch signal transduction. Mech Dev 2002, 117: 151–162
13 Mollereau B, Wernet MF, Beaufils P, Killan D, Pichaud F, Kuhnlein R, Desplan C. A green fluorescent protein enhancer trap screen in *Drosophila* photoreceptor cells. Mech Dev 2000, 93: 151–160
14 Kim YJ, Zitnan D, Galizia CG, Cho KH, Adams ME. A command chemical triggers an innate behavior by sequential activation of multiple peptidergic ensembles. Curr Biol 2006, 16: 1395–1407
15 Zitnan D. Kingan TG, Hermesman JL, Adams ME. Identification of ecdysis-triggering hormone from an epitracheal endocrine system. Science 1996, 271: 88–91
16 Park Y, Kim YJ, Dupriez V, Adams ME. Two subtypes of ecdysis-triggering hormone receptor in *Drosophila* melanogaster. J Biol Chem 2003, 278: 17710–17715
17 Dai HJ, Xu GJ, Thomas JL, Wang ZG, Jiang RJ, Fei J. Efficient and stable generation of transgenic silkworm, *Bombyx mori* with the lepidopteran derived transposon piggyBac. Chinese Science Bulletin 2005, 15: 1617–1621
18 Zitnan D, Hollar L, Spalovska I, Takac P, Zitnanova I, Gill SS, Adams ME. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*. J Exp Biol 2002, 205: 3459–3473
19 Park Y, Filippov V, Gill SS, Adams ME. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. Development, 2002, 129: 493–503
20 Zitnan D, Zitnanova I, Spalovska I, Takac P, Park Y, Adams ME. Conservation of ecdysis-triggering hormone signaling in insects. J Exp Biol 2003, 206: 1275–1289