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Effects of $BmKIT_3^R$ Gene Transfer on Pupal Development of *Bombyx mori* Linnaeus Using a *Gal4/UAS* Binary Transgenic System

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ABSTRACT: The pupal stage of the silkworm *Bombyx mori* Linnaeus lasts for approximately two weeks. However, prolongation of pupal duration would reduce the labor required to process and dry fresh cocoons. This study investigated the effects of $BmKIT_3^R$ gene (from the Chinese scorpion *Buthus martensii* Karsch) transfer on the pupal development of *B. mori* using a *Gal4/UAS* binary transgenic system. *Gal4* driven by a pupa-specific promoter *BmWCP4* (from a *B. mori* wing-cuticle protein gene) or *PDP* (from a *B. mori* cocoonase gene), and $BmKIT_3^R$ driven by a *UAS cis*-acting element were used to construct novel *piggyBac*-derived plasmids containing a neomycin-resistance gene (*neo*) controlled by the *Bombyx mori* nucleopolyhedrovirus (BmNPV) *ie-1* (immediate-early gene) promoter and a green fluorescent protein gene (*gfp*) under the control of the *B. mori* actin 3 (A3) promoter. The vector was transferred into silkworm eggs by sperm-mediated gene transfer. Transgenic silkworms were produced after screening for *neo* and *gfp* genes, and gene transfer was verified by polymerase chain reaction and dot-blot hybridization. The larval development of the hybrid progeny of *Gal4*- and *UAS*-transgenic silkworms was similar to that of normal silkworms, but some pupae failed to metamorphose into moths, and the development of surviving pupae was arrested as a result of $BmKIT_3^R$ expression. Moreover, *Gal4* driven by the *BmWCP4* promoter delayed pupal development more effectively than that driven by the *PDP* promoter in the *Gal4/UAS* binary transgenic system. Pupal durations of hybrid transgenic silkworm progeny with *BmWCP4* and *PDP* promoters were approximately 5, 2, and 4 days longer, respectively, compared to corresponding normal silkworms, *BmWCP4/Gal4*, and $UAS/BmKIT_3^R$ transgenic silkworms, respectively. These results suggest new avenues of research for prolonging the pupal duration of silkworms.

KEYWORDS: *Bombyx mori Linnaeus, piggyBac, Gal4/UAS binary transgenic system,* $BmKIT_3^R$*, pupal stage*

INTRODUCTION

The silkworm *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) has been domesticated and is reared on a large scale. Artificial adjustment of silkworm metamorphosis and development is important for adapting industrial processes and improving overall production efficiency. The pupal stage of silkworms is very short and lasts for only about 10−14 days. Pierced cocoons are not suitable for reeling, so pupae are killed by heating before eclosion, and silk is reeled from dried cocoons. Currently, drying 1 ton of fresh cocoon costs about 0.3 ton coal, and this expenditure could be cut down by blocking the pupal development. It would be desirable to extend the pupal duration to reduce the labor required for fresh cocoon processing and drying and even to block pupal development to allow reeling of fresh cocoons in order to improve the grade of raw silk and save on the energy used for drying. A possible effective strategy might be to introduce a toxin gene into the silkworms, which can be expressed during the pupal stage, thus inhibiting pupal development.

$BmKIT_3^R$ is a depressant insect toxin from the Chinese scorpion *Buthus martensii* Karsch. $BmKIT_3^R$ comprises 65 amino acid residues and acts selectively on insects, causing neural paralysis.[1] Our previous studies demonstrated that oviposition decreased by 18.9% and the hatching and final survival rates were approximately 63% and 47.5%, respectively, after transfer of the $BmKIT_3^R$ gene driven by the *B. mori* heat shock protein (HSP) *Bmhsp*20.4 promoter. Some 18.9% of surviving pupae died before developing into moths in the G3 generation, suggesting that development of pupae into moths could be blocked by specific expression of the $BmKIT_3^R$ gene in the pupal stage.[2] Thus, $BmKIT_3^R$ has potential as a candidate reagent for insect pest control.

The spatiotemporal expression of heterologous genes in the silkworm can be controlled using a *Gal4/UAS* binary transgenic system[3] or a tetracycline-inducible gene expression system.[4] A binary gene expression system that allows for the strict control of tissue-specific expression of transgenes has been developed for silkworms, using the yeast transcription factor *Gal4* and upstream activating sequence (*UAS*).[3] Recombinant proteins can be expressed in the middle silk gland in silkworms using this *Gal4/UAS* binary transgenic system.[5] Binary expression systems are of key interest to functional gene analysis by over- or misexpression, and the application of these systems in diverse organisms would allow the study of many biological problems that cannot be addressed in model organisms.[6] This binary system has several advantages for the production of recombinant proteins. First, it facilitates the production of silkworms that can produce recombinant proteins in different tissues. Second, it can be used to generate transgenic lines

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carrying lethal genes or genes encoding toxic proteins,[7,8] because the gene under *UAS* control is not expressed in the absence of *Gal4*.

A method for generating transgenic silkworms has been established using a *piggyBac* transposon as a vector.[9] In the current study, we attempted to develop a *Gal4/UAS* binary transgenic system for the efficient expression of the $BmKIT_3^R$ gene in transgenic silkworm pupae in order to arrest the metamorphosis of pupae into moths. Based on the *piggyBac* transposon, a pigA3GFP-derived[9] effector vector piggyA3GFP-ie-neo-UAS-IT_3^R-PolyA with $BmKIT_3^R$ under the control of a *UAS cis*-acting element was developed. Meanwhile, the activator vectors piggyA3GFP-ie-neo-PDP-Gal4-polyA with *Gal4* driven by the *PDP* promoter of the silkworm cocoonase gene and piggyA3GFP-ie-neo-BmWCP4-Gal4-polyA containing *Gal4* driven by the *BmWCP4* promoter of the silkworm wing-cuticle protein gene were constructed to drive *Gal4* gene expression specifically during the pupal stage of silkworm development. These vectors also contain a neomycin-resistance gene (*neo*) controlled by the *B. mori* nucleopolyhedrovirus *ie-1* promoter. The vector was transferred into silkworm eggs by sperm-mediated gene transfer. Transgenic silkworms were obtained after screening with GFP and G418 antibiotics and verification by polymerase chain reaction (PCR), dot hybridization, and western blotting. Pupal development of the hybrid progeny of the *Gal4*- and *UAS*-transgenic silkworms was significantly inhibited, suggesting that metamorphosis from pupae to moths was arrested by $BmKIT_3^R$ expression.

MATERIALS AND METHODS

Construction of Transgenic Vectors. *piggyA3GFP-ie-neo-UAS-$BmKIT_3^R$-PolyA.* All the primers used in this study were designed according to the sequences deposited in GenBank (Table 1). The PCR product (0.35 kb) amplified from the plasmid pUAST[10] using the primers UAS-1/UAS-2 was digested with *Eco*RI/*Eco*RV and ligated into plasmid pBluescript II SK (+) (Invitrogen, Frederick, MD) to construct the plasmid pSK-UAS. The $BmKIT_3^R$ fragment (0.19 kb) amplified from the plasmid pSK-hsp20.4-IT_3^R-polyA[11] using the primers IT3R-1/IT3R-2 was digested with *Xho*I/*Apa*I and cloned into pBluescript II SK (+) to generate the plasmid pSK-IT_3^R. To construct the plasmid pSK-IT_3^R-polyA, the polyA signal sequence (*fib*L-PA) of the *B. mori* fibroin light chain gene amplified from the plasmid pSK-hsp20.4-IT_3^R-polyA[11] using the primers YCFib-PA-1/YCFib-PA-2 was digested with *Eco*RV/*Kpn*I and ligated into the *Apa*I/*Kpn*I sites of the plasmid pSK-IT_3^R. The fragment of $BmKIT_3^R$-polyA excised from the pSK-IT_3^R-polyA with *Xho*I/*Kpn*I was cloned into the plasmid pSK-UAS to generate the plasmid pSK-UAS-IT_3^R-polyA. The fragment of *UAS-*$BmKIT_3^R$-polyA that was excised from the plasmid pSK-UAS-IT_3^R-polyA with *Eco*RI/*Kpn*I and the *neo* gene controlled by the BmNPV *ie-1* promoter excised from the plasmid pIE-Neo[12] with *Eco*RI were ligated into the plasmid pigA3GFP to generate the transgenic vector piggyA3GFP-ie-neo-UAS-IT_3^R-polyA (Figure 1)

piggyA3GFP-ie-neo-PDP-Gal4-polyA. The *fib*L-PA fragment amplified from the plasmid pSK-hsp20.4-IT_3^R-polyA[11] using the primers TPFib-L-3/TPFib-L-4 was digested with *Xho*I/*Kpn*I and ligated into pBluescript II SK (+) to generate the plasmid pSK-FibL-PA. The *PDP* promoter sequence (1.2 kb) of the silkworm cocoonase gene amplified from the genomic DNA of silkworms (strain Dazao) with the primers PDP-1/PDP-2 was digested with *Bam*HI/*Eco*RV and ligated into the plasmid pSK-FibL-PA to generate the plasmid pSK-PDP-fibL-PA. The fragment of PDP-fibL-PA amplified from pSK-PDP-fibL-PA-1 with the primers PDP-1/FibL-BK was digested with *Bam*HI/*Kpn*I and ligated into pBluescript II SK (+) to construct the plasmid pSK-PDP-fibL-PA-2. The *neo* gene controlled by the BmNPV *ie*6 promoter excised from the plasmid pIE-Neo with *Eco*RI was ligated into the plasmid pSK-PDP-fibL-PA-2 to construct pSK-ie-neo-PDP-fibL-PA. The *Gal4* gene (2.6 kb) amplified from the plasmid pC3G4[10] using the primers GAL4-1/GAL4-2 was digested with *Eco*RV/*Hin*dIII and cloned into the plasmid pSK-ie-neo-PDP-fibL-PA to generate pSK-ie-neo-PDP-Gal4-fibL-PA. The fragment of ie-neo-PDP-Gal4-fibL-PA excised from the plasmid pSK-ie-neo-PDP-Gal4-fibL-PA with *Bam*HI/*Bgl*II was ligated into the plasmid pigA3GFP to generate the transgenic plasmid piggyA3GFP-ie-neo-PDP-Gal4-polyA (Figure 1).

piggyA3GFP-ie-neo-BmWCP4-Gal4-polyA. The *BmWCP4* promoter of the silkworm wing-cuticle protein gene was amplified from genomic DNA using primers WCP4-1/WCP4-2 and the recovered PCR products (1.12 kb) were digested with *Eco*RI/*Sma*I and ligated into pSK-ie-neo-PDP-Gal4-fibL-PA to generate the plasmid pSK-BmWCP4-Gal4-polyA. The *neo* gene controlled by the BmNPV *ie-1* promoter excised from the plasmid pIE-Neo with *Eco*RI was cloned into pSK-BmWCP4-Gal4-polyA to generate pSK-ie-neo-BmWCP4-Gal4-polyA. The fragment of ie-neo-BmWCP4-Gal4-polyA (5.8 kb) excised from the pSK-ie-neo-BmWCP4-Gal4-polyA with *Bam*HI/*Bgl*II was ligated into the plasmid pigA3GFP to generate the transgenic plasmid piggyA3GFP-ie-neo-BmWCP4-Gal4-polyA (Figure 1).

Introduction of the Foreign Gene into Silkworm Eggs. Sperm-mediated gene transfer was performed according to Zhao et al.[13] The transgenic vector at a concentration of 1 $\mu g/\mu L$ was mixed

Table 1. Primers Used for Construction of Plasmids and PCR[a]

primer name	sequence	restriction enzyme	reference sequences GenBank no.
UAS-1	ctg aat tcg cat gcc tgc agg tcg	*Eco*RI	Brand and Perrimon, 1993[10]
UAS-2	ttg ata tcc aat tcc cta ttc aga g	*Eco*RV	
IT3R-1	cac tcg aga tgg acg gct ata ttc gc	*Xho*I	Wang et al., 2011[2]
IT3R-2	cag ggc cct caa ccg cat gta ttg c	*Apa*I	
YCFib-PA-1	ggg ata tca aat tgt gtt tgc gtt agg	*Eco*RV	M76430
YCFib-PA-2	gcg gat ccg gta ccc act gtc caa tcc acc gtc	*Bam*HI/*Kpn*I	
DEGFP1	tgg aat tca tgg tga gca agg gcg agg	*Eco*RI	AB593377
DEGFP2	ttg gat cct tac ttg tac agc tcg tcc atg	*Bam*HI	
NEO-1	ctg ata tca tga ttg aac aag atg g	*Eco*RV	EF550208
NEO-3	agc tcg agc tag agg tcg acg g	*Xho*I	
GAL4-1	agg ata tca tga agc tac tgt ctt cta tcg	*Eco*RV	Brand and Perrimon, 1993[10]
GAL4-2	gca agc ttg cac agt tga agt gaa ctt gcg g	*Hin*dIII	
PDP-1	ttg gat ccg aat tct aca tcc ata acc ctg g	*Bam*HI/*Eco*RI	EU420085
PDP-2	ttg ata tct tca gtt tcg atc cgg cg	*Eco*RV	
WCP4-1	gtg aat tca cca cgc gcc aag aga gc	*Eco*RI	AB262389
WCP4-2	tac ccg ggt ttg ctt tgg aca caa aag cc	*Sma*I	
TPFib-L-3	ggc tcg agc aaa ttg tgt ttg cgt tag g	*Xho*I	M76430
TPFib-L-4	gcg gta ccc act gtc caa tcc acc gtc	*Kpn*I	
FibL-BK	gcg gta cca gat ctc act gtc aat cca ccg tc	*Kpn*I/*Bgl*II	M76430

[a]Underlining indicates the recognition site of the restriction enzyme used for cloning.

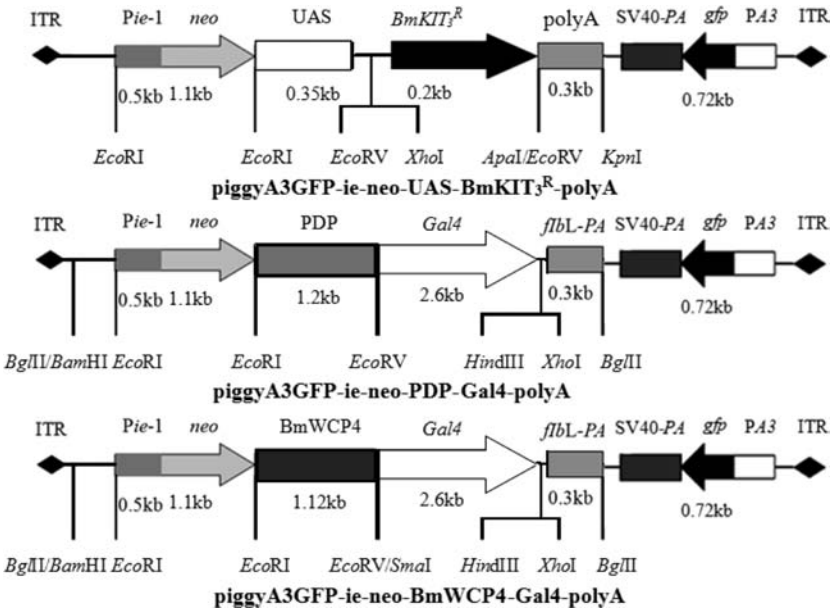

Figure 1. Maps of the transgenic vectors. Physical maps of the transgenic vectors piggyA3GFP-ie-neo-PDP-Gal4-polyA, piggyA3GFP-ie-neo-BmWCP4-Gal4-polyA, and piggyA3GFP-ie-neo-UAS-BmKIT$_3^R$-polyA. ITR, inverted terminal repeats of *piggyBac* transposon; P_{A3}, *B. mori* A3 cytoplasmic actin gene promoter; *gfp*, green fluorescent protein gene; SV40-PA, polyA signal sequence of SV40; $P_{ie\text{-}1}$, immediate early stage gene promoter of *B. mori* nucleopolyhedrovirus; *neo*, neomycin resistance gene; PDP, promoter of silkworm cocoonase gene; BmWCP4, promoter of silkworm wing cuticle protein gene; *Gal4*, yeast transactivator Gal4 protein; UAS, Gal4 protein corresponding upstream activation sequence (UAS); BmKIT$_3^R$, depressant insect toxin gene of *Buthus martensii* Karsch; *fib*L-PA, polyA signal sequence of *B. mori* fibroin light chain gene.

with helper plasmid pigA3,[14] which carries an actin A3-promoter-transposase expression cassette, at a ratio of 1:1, and injected into the copulatory pouch of copulated female moths (strains Dazao, 3-166 and 3-247). After spawning, embryos were incubated at 25 °C under a relative humidity of 85−90% for 10 days until hatching. The larvae were reared on mulberry leaves at room temperature.

Screening and Identification of Transgenic Silkworms. Newly hatched larvae were reared on mulberry leaves coated with 10 μg/mL G418 until approximately 10% of the larvae remained. Surviving silkworms were observed under a stereomicroscope (Olympus SZX12, Tokyo, Japan), and fluorescent individuals were fed with normal mulberry leaves.

Genomic DNA extracted from larvae or moths with green fluorescence were identified by PCR using primers including *gfp*-specific (DEGFP1/DEGFP2) and *neo*-specific (NEO-1/NEO-3) (Table 1). Genomic DNA extracted from a fluorescent G1-generation moth was denatured by boiling and dotted onto a nylon membrane. DNA hybridization with a DIG-labeled *gfp* probe, membrane washing, and signal detection were carried out using a DIG DNA labeling and detection kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Genomic DNA from a normal silkworm and plasmid pigA3GFP were used as negative and positive controls, respectively.

Detection of *BmKIT$_3^R$* Expression in Hybrid Offspring of *Gal4*- and *UAS*-Transgenic Silkworms. Homogenates of transgenic silkworms were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (Figure 2K). The concentrations of the stacking and separating gels were 5% and 15%, respectively. The primary and secondary antibodies were mouse anti-*BmKIT$_3^{R2}$* and horseradish peroxidase (HRP)-conjugated goat antimouse immunoglobulin G (IgG) (Beijing Biosynthesis Biotechnology, Beijing, China), respectively.

Successive Rearing of Transgenic Silkworms. G0-generation fluorescent moths were mated with each other to generate a G1 generation. The G2 generation was generated by mutual crossing between fluorescent moths from generation G1. G3−G7 generations were obtained in the same way as the G2 generation and were reared on mulberry leaves.

Hybrid Descendants of *Gal4*- and *UAS*-Transgenic Silkworms. The G7 generation of *Gal4*-lines were crossed with the G7 *UAS/BmKIT$_3^R$*-transgenic line, and the hybrid offspring were reared on mulberry leaves. Data on larval and pupal durations and survival rates were recorded.

RESULTS

Identification of Transgenic Vectors. To express *BmKIT$_3^R$* specifically in the pupal stage, two activator vectors piggyA3GFP-ie-neo-PDP-Gal4-polyA, piggyA3GFP-ie-neo-BmWCP4-Gal4-polyA, and an effector piggyA3GFP-ie-neo-UAS-IT$_3^R$-polyA (Figure 1) were constructed, respectively. These vectors were verified by digestion with restriction enzymes and specific DNA bands corresponding to the expected sizes were detected (data not shown). The vectors were also identified by sequencing, and the results confirmed that the transgenic vectors had been constructed successfully.

Screening and Identification of Transgenic Silkworms. The transgenic vector was mixed with the helper plasmid and injected into copulatory pouches of copulated female moths that were then allowed to oviposit. The eggs were incubated at 25 °C. Fluorescence in the eggs was observed under a fluorescence microscope. Green fluorescence in some eggs (Figure 2A) indicated that the vector had been introduced successfully into these eggs. Transgenic silkworms should exhibit neomycin resistance if the *neo* gene was successfully introduced into the genome and was expressed correctly. Larvae were reared on mulberry leaves coated with 10 mg/mL G418 from the second instar stage. Obvious developmental differences were seen among silkworms in response to G418, and successive deaths occurred. Green fluorescence could be observed in some surviving larvae (Figure 2B) and pupae (Figure 2C) of *UAS*-transgenic silkworms (strain 3-247). Simultaneously, some worms with green fluorescence could be also observed among the *PDP/Gal4*-transgenic (strains 3-

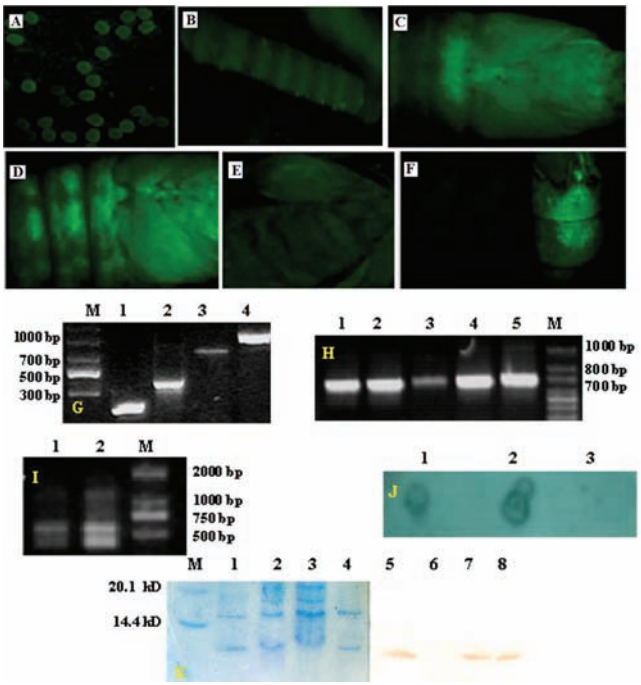

Figure 2. Screening and identification of transgenic silkworms. (A) Silkworm eggs laid by female moths following sperm-mediated gene transfer. (B, C) G0-generation fluorescent silkworm larva and pupa from the *UAS*-transgenic line (strain 3-247), respectively. (D, E) G0-generation fluorescent silkworm pupa (strain 3-247) and moth (strain 3-166) from the *PDP/Gal4*-transgenic line. (F) Normal pupa and G0-generation transgenic pupa from *BmWCP4/Gal4*-transgenic silkworm under fluorescent field (left, normal pupa; right, transgenic pupa). (G) PCR identification of G0-generation *UAS/BmKIT3*^{R}-transgenic silkworm; M, DNA marker, lanes 1−4, PCR products representing *BmKIT3*^{R} (0.2 kb), *UAS* (0.35 kb), *gfp* (0.72kb), and *neo* (1.1 kb), respectively. (H) PCR identification of G0-generation *PDP-Gal4* transgenic line using the primer pair DEGFP-1/DEGFP-2; lanes 1−5, five fluorescent G0-generation silkworms. (I) PCR identification of G0-generation *BmWCP4-Gal4*-transgenic line. M, DNA marker; lanes 1 and 2, two fluorescent G0-generation silkworms. (J) Identification of G1-generation fluorescent silkworms from the *UAS*-transgenic line by dot hybridization with a DIG-labeled *gfp* probe. 1, a transgenic silkworm; 2, vector pigA3GFP; 3, normal silkworm. (K) SDS-PAGE and western blots of *BmKIT*$_3^R$ expressed in poisoned transgenic hybrid silkworms. M: protein marker; lane 3: normal pupa; lanes 1, 2, and 4: poisoned transgenic hybrid pupae: *UAS/BmKIT*$_3^R$ (strain 3-247) × *BmWCP4/Gal4* (strain Dazao), *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-247), and *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-166), respectively. Lanes 5−8 illustrate western blots corresponding to lanes 1−4. The primary antibody was mouse anti-*BmKIT3*^{R2}, and the secondary antibody was HRP-conjugated goat antimouse IgG.

247, 3-166) (Figure2D,E) and *BmWCP4/Gal4*-transgenic silkworms (strain Dazao) (Figure 2F).

Genomic DNA extracted from mated G1-generation moths was used as a template for PCR detection. By using the primer pairs IT3R-1/IT3R-2, UAS-1/UAS-2, DEGFP-1/DEGFP-2, and NEO-1/NEO-3 (Table 1), respectively, specific bands representing the *BmKIT3*^{R} (0.2 kb), *UAS* (0.35 kb), *gfp* (0.72 kb), and *neo* (1.1 kb) genes could be detected in *UAS/BmKIT*$_3^R$-transgenic silkworms (strains 3-247 and 3-166) (Figure 2G), suggesting that the silkworms were transgenic. *gfp*-specific PCR products could be amplified from the genomic DNA of *PDP/Gal4*-transgenic (strain 3-247) (Figure 2H) and *BmWCP4/Gal4*-transgenic silkworms (strain Dazao) (Figure 2I), respectively. The fluorescent silkworms were allowed to develop into moths, which copulated with other fluorescent moths and laid eggs.

In addition to PCR identification, a G1-generation fluorescent silkworm with the *UAS-BmKIT*$_3^R$ expression cassette (strain 3-247) was identified by dot hybridization with a DIG-labeled *gfp* probe (Figure 2J). As expected, the *gfp* probe also hybridized specifically with the genome of the G1-generation fluorescent moth, indicating that *gfp* had been integrated into the genome.

To detect *BmKIT*$_3^R$ expression in the hybrid offspring of *Gal4*- and *UAS*-transgenic silkworms, homogenates of hybrid offspring pupae were subjected to SDS-PAGE and western blotting, and a specific signal band corresponding to *BmKIT*$_3^R$ could be detected (Figure 2K).

Development and Survival of Hybrid Offspring of *Gal4*- and *UAS*-Transgenic Silkworms. The pupal durations of strains Dazao, 3-247, and 3-166 were 5, 10, and 10 days, respectively, and those of the hybrid offspring 3-247 × Dazao, 3-247 × 3-166, and 3-166 × Dazao were 7, 10, and 7 days, respectively. Development of the *Gal4*- and *UAS*-transgenic lines was similar to that of the corresponding nontransgenic lines (Figure 3). G7-generation *Gal4*-transgenic silkworms were

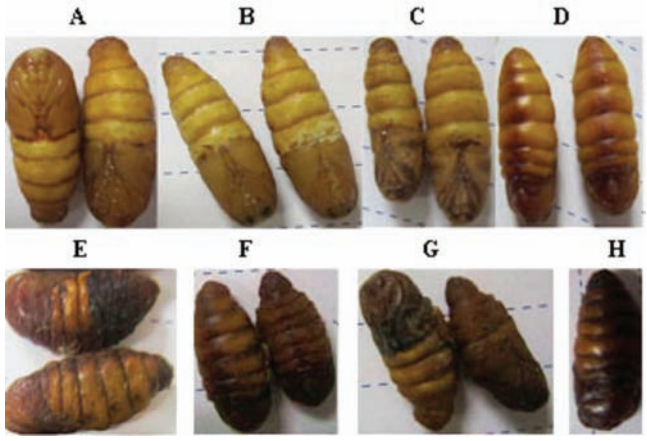

Figure 3. Abnormalities of poisoned pupae. (A−D) Pupae of *PDP/Gal4* transgenic silkworms (strain 3-166), *PDP/Gal4* transgenic silkworms (strain 3-247), *BmWCP4/Gal4* transgenic silkworms (strain Dazao), and *UAS/BmKIT*$_3^R$ transgenic silkworms (strain 3-247), respectively. (E−H) Dead pupae of *UAS/BmKIT*$_3^R$ (strain 3-247) × *BmWCP4/Gal4* (strain Dazao), *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-166), *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-166), and *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-247). The dead pupae were investigated and recorded when they appeared the symbol of abnormalities.

crossed with G7 *UAS/BmKIT*$_3^R$-transgenic silkworms, and the larval durations of all cross combinations were normal; however, some pupae failed to metamorphose into moths (Figure 3). These pupae did not decay within several months at room temperature, and the development of the surviving pupae was arrested (Figure 4). The pupal durations of *UAS/BmKIT*$_3^R$ (strain 3-247) × *BmWCP4/Gal4* (strain Dazao), *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-247), and *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-166) were approximately 5, 2, and 4 days longer, respectively, compared to the corresponding normal silkworms. These results indicate that the *BmKIT*$_3^R$ gene was not expressed in the larval stage of

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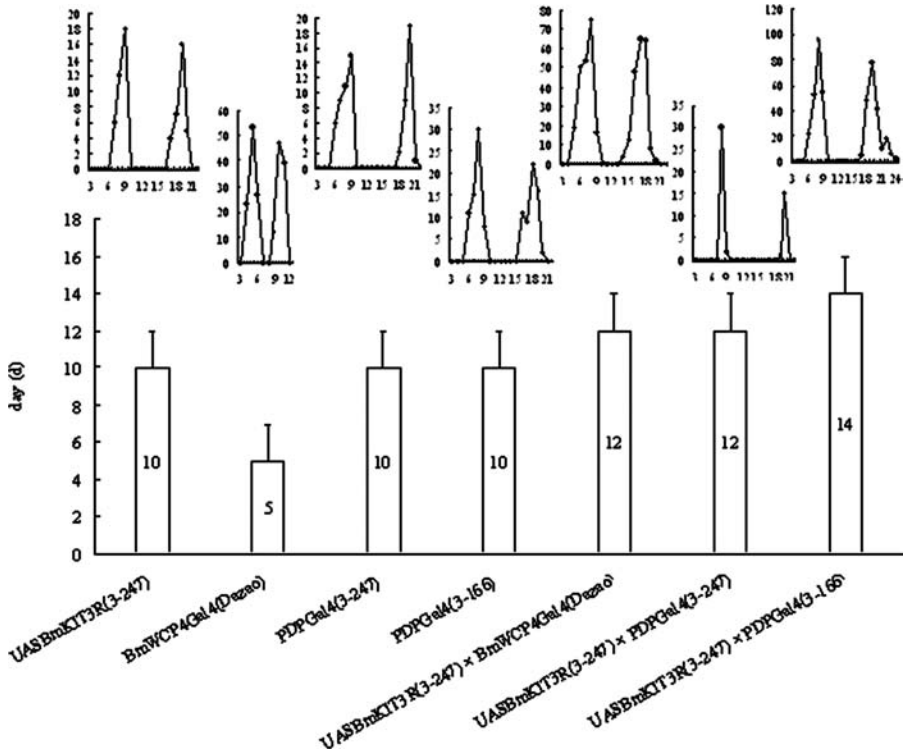

Figure 4. Duration of pupal stages in *UAS/BmKIT*$_3^R$ × *PDP/Gal4* and *UAS/BmKIT*$_3^R$ × *BmWCP4/Gal4* silkworms. Comparison of duration of pupal stages in *UAS/BmKIT*$_3^R$ × *PDP/Gal4* and *UAS/BmKIT*$_3^R$ × *BmWCP4/Gal4* silkworms are presented on the *x* axis. Controls for single system of *Gal4/UAS* as well as wild-type 3-247, 3-247 × Dazao, and 3-247 × 3-166 were included. *B. mori* transgenic and wild-type controls were fed on normal mulberry leaves. Columns represent mean duration of pupal stages based on the difference between the numbers of days corresponding to the last and first peaks in the curves illustrated above each bar (the 3-247 × Dazao and 3-247 × 3-166 groups are not illustrated because the data were collected in former experiments under the same breeding conditions). The *x* axis of each curve represents the days of the pupation process. The *y* axis of each curve represents the number of pupae and moths. The numbers of pupae and moths were recorded three times a day. Bars represent standard deviations. For each group, the duration of each combination was the result of independent measurements based on more than 100 silkworms. In the case of the *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-166) group, there were three peaks in the curve, and poisoned pupae were only observed during the third peak. The duration of the pupal stage in this *UAS/BmKIT*$_3^R$ (strain 3-247) × *PDP/Gal4* (strain 3-166) was therefore calculated as the difference between the third and first peaks.

hybrid offspring of *Gal4*- and *UAS*-transgenic lines and that, while some pupae failed to metamorphose into moths, the development of the surviving pupae was significantly inhibited as a result of *BmKIT*$_3^R$ expression.

DISCUSSION

The silkworm *B. mori* is a holometamorphic silk-spinning insect with a pupal duration of about 10−14 days. Uniform silkworm development is expected during rearing, but intensive cocoon harvesting means that silk-producing manufacturers are unable to complete silk reeling during the short 10−14 day span. Therefore, the conventional strategy, called cocoon drying, involves killing the pupae inside the cocoon before eclosion. However, the purchasing and drying of cocoons are labor-intensive, expensive, and energy-consuming processes. In addition, the quality of silk from fresh cocoons is better than that from dried cocoons. It would therefore be desirable to prolong pupal duration in order to reduce the labor required for fresh cocoon processing and drying and potentially even to block pupal development to allow reeling of fresh cocoons thus improving the grade of the raw silk and saving on the energy used for drying.

Scorpion toxins have largely been classified into two types. Long-chain toxins contain 60−70 amino acid residues cross linked by four disulfide bridges. Short-chain toxins are composed of 30−40 amino acid residues cross linked by three or four disulfide bridges.[15,16] There are three toxin gene types in Chinese scorpion *Buthus martensii* Karsch (BmK), α-neurotoxin, excitatory, and depressant insect toxins.[17] BmKIT$_3^R$ is a depressant insect toxin with selective neural paralysis effects on insects. The introduction into the silkworm genome of *BmKIT*$_3^R$ gene that can be expressed specifically in the pupal stage or the regulated expression of a critical gene controlling metamorphosis represent promising strategies for prolonging pupal duration. In our previous studies, we found that the development of silkworm larvae and pupae could be blocked by introducing the *BmKIT*$_3^R$ gene driven by a heat shock protein (hsp) 20.4 promoter[2] and an *egt* (ecdysteroid glucosyltransferase) gene controlled by a hsp 23.7 promoter.[18] These findings suggest that pupal duration may be prolonged by *BmKIT*$_3^R$ or *egt* gene transfer and expression, specifically at the pupal stage. As expected, some pupae died due to the expression of BmKIT$_3^R$ protein specifically at the pupal stage; furthermore, these poisoned pupae were mummified and not putrescent.

The *Gal4/UAS* system has been used previously in silkworms.[3,5,19] The current study investigated the pupal development of hybrid offspring between transgenic silkworms with the *Gal4* gene driven by the *PDP* or *BmWCP4* promoters and silkworms with the *BmKIT*$_3^R$ gene driven by a *UAS cis*-acting element. The results showed that, although the larval

duration of all cross combinations was normal, some pupae failed to metamorphose into moths and the development of surviving pupae was arrested, suggesting that the $BmKIT_3^R$ gene was expressed specifically in the pupal stage and not in the larval stage; that is, the *PDP* and *BmWCP4* promoters were pupal-stage specific. Moreover, the pupal durations for *UAS*/$BmKIT_3^R$ (strain 3-247) × *BmWCP4*/*Gal4* (strain Dazao), *UAS*/$BmKIT_3^R$ (strain 3-247) × *PDP*/*Gal4* (strain 3-247), and *UAS*/$BmKIT_3^R$ (strain 3-247) × *PDP*/*Gal4* (strain 3-166) were approximately 5, 2, and 4 days longer, respectively, compared to the corresponding normal silkworms, suggesting that *Gal4* driven by the *BmWCP4* promoter was more effective than that driven by the *PDP* promoter in terms of delaying pupal development. It seems likely that this difference was related to the expression level, expression phase, and expression distribution of $BmKIT_3^R$ at the pupal stage. In *B. mori*, the wing-cuticle protein gene *BmWCP4* is expressed specifically in the epidermis at the onset and midstage of pupation and is responsible for the formation of the pupal cuticle during larval−pupal metamorphosis.[20] In contrast, the cocoonase gene is expressed specifically in the midgut, maxillae, and crop during the later pupal stage (pharate adult stage),[21−24] suggesting that $BmKIT_3^R$ expression in the pupae of *UAS*/$BmKIT_3^R$ × *BmWCP4*/*Gal4* silkworms occurred earlier than in the pupae of *UAS*/$BmKIT_3^R$ × *PDP*/*Gal4* silkworms.

Even though some pupae failed to metamorphose into moths and the development of surviving pupae was arrested in hybrid offspring of *BmWCP4*/*PDP*/*Gal4* and *UAS*/$BmKIT_3^R$ transgenic silkworms, the development of the surviving pupae could be not blocked completely. In the future, more pupa-specific promoters and more toxic genes are required to further extend pupal development using the *Gal4*/*UAS* binary transgenic silkworm system.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

A3, *Bombyx mori* actin 3; *Bmhsp*, *Bombyx mori* heat shock protein; $BmKIT_3^R$, a gene from the Chinese scorpion *Buthus martensii* Karsch; BmNPV, *Bombyx mori* nucleopolyhedrovirus; *BmWCP4*, a *B. mori* wing-cuticle protein gene; *egt*, ecdysteroid glucosyltransferase gene; *fib*L, the *Bombyx mori* fibroin light chain gene; *gfp*, green fluorescent protein gene; HRP, horseradish peroxidase; hsp, heat shock protein; *ie-1*, BmNPV immediate-early gene; IgG, immunoglobulin G; *neo*, neomycin-resistance gene; PCR, polymerase chain reaction; *PDP*, a *B. mori* cocoonase gene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; *UAS*, upstream activating sequence

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