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ORIGINAL ARTICLE

Farnesoic acid methyltransferase 6 (BmFAMeT6) interrelates with moltinism of dominant trimolter in silkworm, *Bombyx mori*

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Abstract

Silkworm moltinism is closely related to many important economic characters such as cocoon yield and cocoon filament size. Farnesoic acid methyltransferase (FAMeT) is the rate-limiting enzyme for the conversion of farnesoic acid to methyl farnesoate which acts as juvenile hormone in crustaceans. Silkworm has seven *FAMeT* genes in its genome but the precise role of *BmFAMeT* is still unclear. Our results showed that *BmFAMeT6* expression had a clear difference between two different moltinism strains, the M_3/M_3 (dominant trimolter) and the $+_M/+_M$ (tetramolter). Declining *BmFAMeT6* expression in the M_3/M_3 could increase the number of larval molts/instars and convert trimolter to tetramolter, and injecting ds*BmFAMeT6* could significantly improve the tetramolter rate in several other dominant trimolter strains. These data demonstrated that *BmFAMeT6* is relevant to the moltinism of the dominant trimolter. Interestingly, *BmFAMeT6* expression was high at the early larval stage and low at the final instar. *BmFAMeT6* protein signal was stronger on the first day than on the second day within each instar and was very weak at the last larval instar. *BmFAMeT6*-specific fluorescence was localized on the corpus allatum. It suggests a correlation between *BmFAMeT6* expression and juvenile hormone titers. We speculated that decreasing *BmFAMeT6* could affect the moltinism in the dominant trimolter, which may be interrelated with the close relationship between *BmFAMeT6* and juvenile hormone titers in silkworm.

Keywords *BmFAMeT6* · Moltinism · The number of larval molts/instars · Dominant trimolter · Silkworm

Introduction

Farnesoic acid O-methyltransferase (FAMeT) catalyzes the methylation of farnesoic acid (FA) to methyl farnesoate (MF) with the cofactor S-adenosyl-methionine, which is the final rate-limiting step for the biosynthesis of methyl farnesoate (as juvenile hormone) in crustaceans (Laufer et al. 1987; Holford et al. 2004; Nagaraju 2007). The *FAMeT* gene was first identified in the shrimp *Metapenaeus ensis* De Haan, 1844 (Decapoda) (Gunawardene et al. 2001), subsequently has been isolated from several other crustaceans (Holford et al. 2004; Hui et al. 2008; Duan et al. 2014; Buaklin et al. 2015; Qian and Liu 2019).

In the fruit fly *Drosophila melanogaster* Meigen, 1830 (Diptera) a protein with high homology to crustaceans farnesoic acid O-methyltransferase is encoded by *CG10527* but has no activity on MF biosynthesis and is not essential for survival and fertility (Burtenshaw et al. 2008; Zhang et al. 2010; Wen et al. 2015;). Knockdown experiments have not revealed involvement of *FAMeT* in juvenile hormone (JH) biosynthesis and in the change of oocyte lengths in the desert locust *Schistocerca gregaria* Forskål, 1775 (Orthoptera), which is in accord with the research findings on *Drosophila CG10527* (Marchal et al. 2011). However, the FAMeT enzyme reveals a high preference for FA in the Asian citrus psyllid *Diaphorina citri* Kuwayama, 1908 (Hemiptera) and its expression is consistent with JHs titers in the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann 1824) (Diptera) (Vannini et al. 2010; Marchal et al. 2011; Van Ekert et al. 2015). Further, *FAMeT* transcripts have a high level in the abdomen and a positive change during the vitellogenesis in female adults, but only have a low level in the ovary in the brown planthopper *Nilaparvata lugens* Stal, 1854 (Homoptera) (Liu et al. 2010). In the stingless bee, two

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FAMeT isoforms resulted from alternative splicing exhibit marked differences between workers and queens in representative stages of *Melipona scutellaris* Linnaeus, 1758 (Hymenoptera) (Vieira et al. 2008). These reports indicate that the function of *FAMeT* is different among insect species.

Bombyx mori Linnaeus, 1758 (silkworm), a good model of Lepidoptera, has seven genes (*BmFAMeT1* to *BmFAMeT7*) encoding FAMeT homologues in its genome, but only three (*BmFAMeT5*, *BmFAMeT6* and *BmFAMeT7*) of them have been analyzed by RT-PCR. And the result displayed that the three genes were shown to continuously express from late embryo to adult (Meng et al. 2013). In this study, we produced the near-isogenic lines and proposed that *BmFAMeT6* is most likely related to the moltinism of dominant trimolters by several analysis including RNAi, Western blotting, and immunofluorescence.

Materials and methods

Silkworm strains

Silkworm ZhongXian (the $+_M/+_M$, tetramolter), the M_3/M_3 (dominant trimolter), S4 (dominant trimolter), S6 (dominant trimolter), S11 (dominant trimolter) and HeiShan (dominant trimolter) strains were maintained at the Southwest University in China and were reared under standard conditions 24–26 °C and 70–85% RH with a photoperiod of 12:12 LD.

RNA extraction, cDNA synthesis and cloning

Total RNA was purified using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Then ezDNase was added to the RNA sample incubating for 2 min at 37 °C for the fast removal of genomic DNA from RNA preparations. cDNA was synthesized using oligo (dT) primers and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). The target fragment on the full-length mRNA sequence of *BmFAMeT6* was obtained using specific primers (Online resource 1: Table S1) and then was cloned into pMD19-T vector and verified by sequencing.

Recombinant protein expression, purification and production of the antibody

The ORF sequence of *BmFAMeT6* has cloned into pET32 a (+) vector by *EcoR* I and *Xho*I sites (Online resource 1: Table S1), and then the *BmFAMeT6*-pET32 a (+) plasmid was transformed into *E. coli* strain BL21 cells. The recombinant protein containing the His-tag was induced to express by IPTG and subsequently purified by affinity chromatography using a Ni^{2+} column. The polyclonal anti-*BmFAMeT6* from the male adult rabbit was produced by JinSiRui Biotechnology Company (China) (Online resource 1: Fig. S1).

RNAi

Primers for the dsRNA synthesis were listed in Online resource 1: Table S2 and the dsRNA was synthesized by the T7 RiboMAX™ Express RNAi System (Promega). The integrity of dsRNA was confirmed by non-denaturing agarose gel electrophoresis (Online resource 1: Fig. S2). The dsRNA was diluted to the concentration at 6 μg/μL using RNase-free water for injection.

Experimental larvae were chosen to base on similar weight within the strain in the same instar. After surface-sterilization with 70% alcohol, larvae were cold anesthetized and injected liquid using a homemade capillary syringe through the third or fourth spiracle into hemocoel to avoid bleeding as much as possible. Each larva was injected twice, and for the first injection 2 μL solutions were used on the left side at 0 h, and for the second injection 3 μL solutions were used on the right side at 12 h after entering the second molting process. The control larvae were injected with RNase-free water (ddH_2O) or dsRNA of *EGFP* (ds*EGFP*) under the same conditions, and the injected individuals were kept at 25 °C.

Semi-quantitative RT-PCR

The primers listed in Online resource 1: Table S3, according to their CDS sequence, were designed for the expression analysis of *BmFAMeT6* (KC888750), *BmACAT* (AB274988, encoding acetoacetyl CoA thiolase), *BmHMGR* (AB274990, encoding 3-hydroxy-3-methylglutaryl-CoA reductase), *BmHMGS* (AB274989, encoding 3-hydroxy-3-methylglutaryl-CoA synthase), *BmMevK* (AB274991, encoding mevalonate kinase), *BmMevPK* (AB274992, encoding Phosphomevalonate kinase), *BmMevPPD* (AB274993, encoding diphosphomevalonate decarboxylase), *BmIPPI* (DQ311366, encoding isopentenyl-diphosphate delta isomerase), *BmFPPS* (AB274995, encoding farnesyl diphosphate synthase), *BmCYP15C1* (AK289313, encoding cytochrome P450 monooxygenase), *BmJHAMT* (AB113578, encoding juvenile hormone acid methyltransferase), *BmKr-h1* (AB360766, encoding Kruppel homolog 1), *BmMet* (AB359911, encoding methoprene-tolerant receptor), *BmECR* (D43943, encoding ecdysone receptor), *BmUSP* (AB182582, encoding RXR type hormone receptor), *BmJHE* (AY489292, encoding juvenile hormone esterase), *BmJHEH* (AY377854, encoding juvenile hormone epoxide hydrolase), *BmJHDK* (AY363308, encoding juvenile hormone diol kinase) and *BmJHBP* (AY286477, encoding juvenile hormone binding protein). Templates were cDNAs from the larval head or body without midgut, and PCR reactions were carried out in 25 cycles. *BmActin3* was used as an

internal control. The amplified products were detected by 1% agarose gel electrophoresis.

Quantitative RT-PCR

Quantitative RT-PCR was performed to measure the expression levels of *BmFAMeT6* and *BmJHAMT* using the ABI Prism 7000 sequence detection system (Applied Biosystems, USA) with a SYBR Premix Ex-Taq kit (Takara). The primers designed for qRT-PCR are listed in Online resource 1: Table S4. The *B. mori* gene of translation initiation factor 4A (sw22934) was used as an internal control.

Western blotting

Concentrations of total proteins from the larval head of the third or fourth instar were estimated using the bicinchoninic acid (BCA). The sample with 30 μg protein was separated using 12% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences). The *BmFAMeT6* protein and tubulin protein on the membrane were detected with anti-BmFAMeT6 (1:1000 dilution) and anti-tubulin (1:5000 dilution), respectively, as primary antibody, and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2000 dilution, Sigma-Aldrich) as the secondary antibody.

Immunofluorescence analysis

The brain-corpora cardiaca-corpora allata complexes were taken out in pH 7.4 PBS and washed with wash buffer (0.1% Tween-20 in PBS) after removing the fat body and other sundries, then the complexes were fixed with 4% paraformaldehyde at 4 °C for 12 h. The fixed complexes were dialyzed with dialysate I (1 M pH 6.8 Tris, 10% Triton-X 100, 2% BSA, 0.05 M NaCl) at 4 °C for 2 days and dialysate II (4 volume anhydrous ethanol and 1 volume DMSO) at −20 °C for 4 h. Then the complexes washed with wash buffer to incubate it in polyclonal anti-BmFAMeT6 (1: 500) at 4 °C for 3 days. After rigorous washing, the tissues were incubated with Alexa Flour 488 (1:1000, Beyotime) at 4 °C for 3 days. Next, the tissues were counterstained the nuclei with DAPI (Sigma) for 3 h, and then washed with wash buffer before placing it in 50%, 60%, 70%, 80%, 90%, 100% ethanol gradients in turn for 5 min respectively. Finally, the stained tissues were transferred in 70% glycerol for microscopic observation under fluorescence after adding antibody quencher (Chen et al. 2019).

Results

Production of the near-isogenic lines of dominant trimolter

The larval molt times before wandering varies with strains, which is named "moltinism" in the silkworm. Larvae of standard *B. mori* strains undergo molting four times and thus have five larval instars, and these larvae are conventionally termed "tetramolter". Similarly, larvae undergo molting three times and thus have four larval instars, and are termed "trimolter" (Zhou et al. 2017). HeiShan strain is a dominant trimolter whose offspring by mating with tetramolter show trimolter phenotype, and was used as the donor parent. And ZhongXian (the $+_M/+_M$) strain with tetramolter trait was used as the receptor parent in this work. To generate the near-isogenic lines of dominant trimolter, the moltinism trait in HeiShan strain was transferred into the receptor parent to displace its tetramolter traits. The specific process of transferring was as follows: HeiShan mated with the receptor parent to achieve the first transfer-crossing of dominant trimolter trait. The hybrid progeny with trimolter phenotype mated also with the receptor parent to continue the back-cross breeding. After five times of the back-cross breeding, the offspring with trimolter phenotype inbred to produce progenies. Then by inter-batch selection to above posterity, the group where all individuals had trimolter trait was chosen and then stabilized using self-crossing to establish new dominant trimolter strain (Fig. 1). Since the genetic composition of offspring is half from the father and the other half from the mother, the new dominant trimolter strain shared theoretically 96.875% similarity with the receptor parent on hereditary substance. So the new dominant trimolter strain (the M_3/M_3) and the receptor parent strain (the $+_M/+_M$) constituted the near-isogenic lines of dominant trimolter.

Screening genes by differential expression between the M_3/M_3 and the $+_M/+_M$

JH modulates molting and growth of insects via stage-specific changes in titers and plays a leading role in maintaining larval morphology and characteristics (Riddiford 1994; Truman and Riddiford 1999; Riddiford et al. 2003; Muramatsu et al. 2008; Daimon et al. 2015; Zeng et al. 2017). And *CYP15C1* (a key gene for JH biosynthesis) mutation (*mod*) showed that JH is not necessary for the first or second instar larvae of *B.mori* (Daimon et al. 2012). In order to find the genes causing moltinism difference between the near-isogenic lines of the dominant trimer (the M_3/M_3 and the $+_M/+_M$), the expressions of nineteen genes involved in JH biosynthesis (11 genes including *BmACAT*, *BmHMGS*, *BmHMGR*, *BmMevK*, *BmMevPK*, *BmMevppD*, *BmIPPI*, *BmFPPS*, *BmJHAMT*, *BmCYP15C1* and *BmFAMeT6*), degradation (3 genes

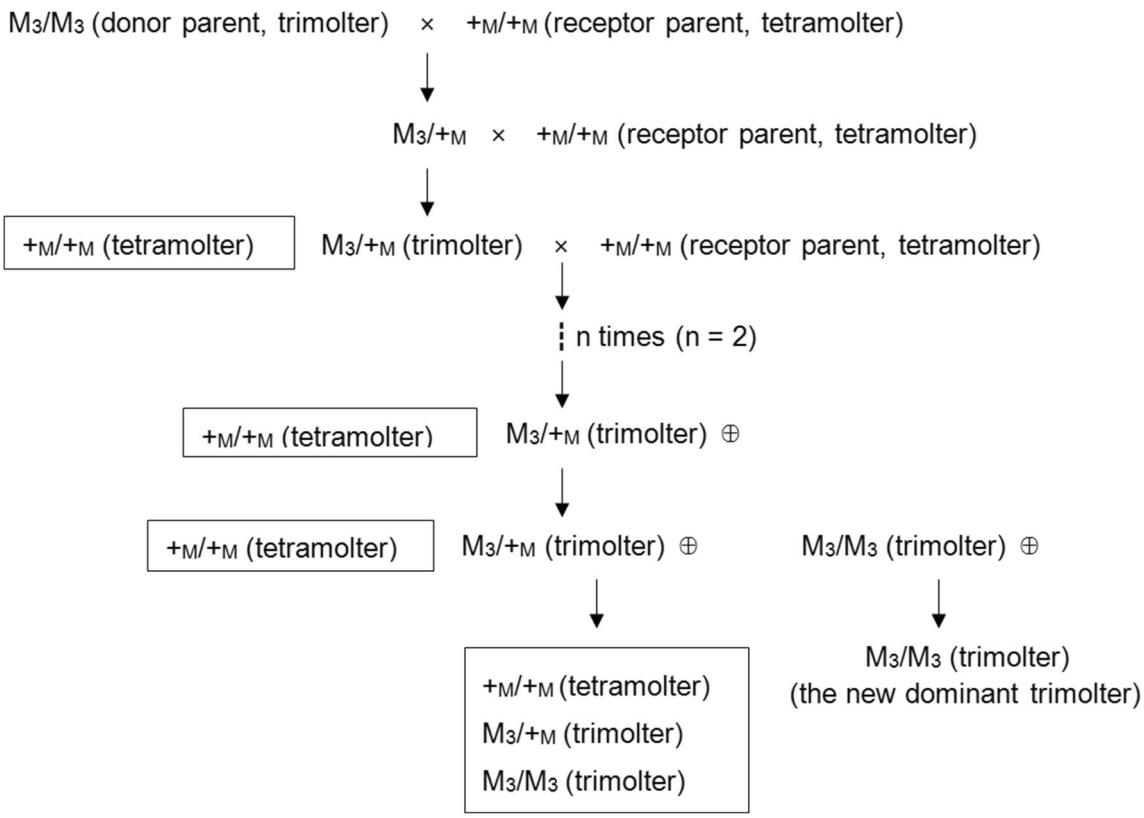

Fig. 1 A flowsheet of generation of new dominant trimolter strain of *B.mori* by importing moltinism character from dominant trimolter strain (M_3/M_3, donor parent) into tetramolter strain ($+_M/+_M$, receptor parent). Black box indicates eliminated offspring; × indicates hybridization crossing; ⊕indicates self- crossing

including *BmJHE*, *BmJHEH* and *BmJHDK*) and signal transmission (5 genes including *BmJHBP*, *BmMet*, *BmECR*, *BmUSP*, *BmKr-h1*) in the larval body without midgut were analyzed by RT-PCR in day 1 of the third instar (3L1D) and day 2 of the third instar (3L2D). *BmFAMeT6* and *BmJHAMT*, but not the other seventeen genes, were obviously different in the band brightness of PCR products between the M_3/M_3 and the $+_M/+_M$ (Fig. 2a). To verify the reliability of RT-PCR detection, the expression levels of *BmFAMeT6* and *BmJHAMT* were analyzed by qRT-PCR. The results showed that the expression of *BmFAMeT6* in M_3/M_3 was significantly increased in 3L1D and 3L2D compared with $+_M/+_M$, while *BmJHAMT* was only highly expressed in 3L2D instead of 3L1D (Fig. 2b, c). These data indicate that the expression of *BmFAMeT6* is different between the near-isogenic lines of the dominant trimolter.

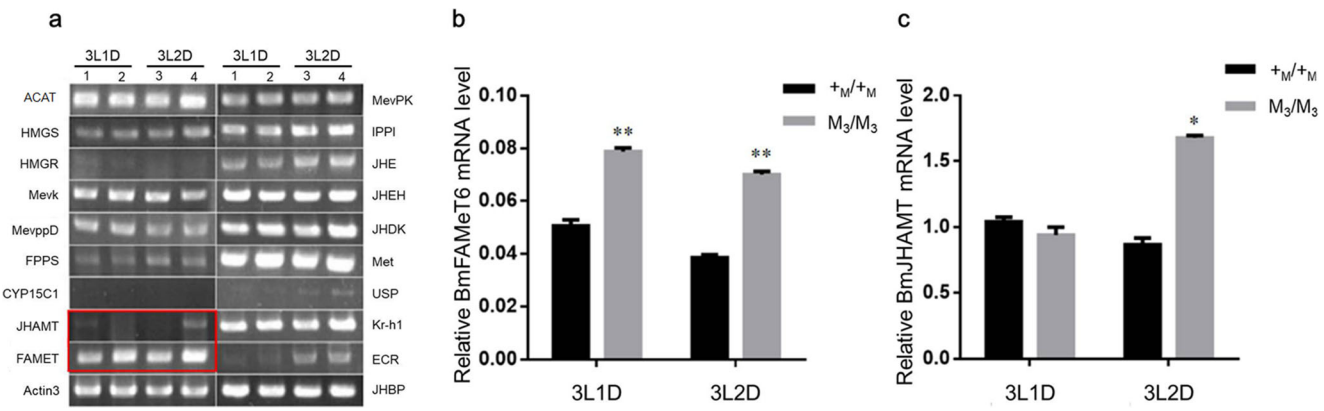

Fig. 2 Expression of genes related to JH in both the M_3/M_3 and the $+_M/+_M$. **a** RT-PCR analysis for JH pathway-related genes. 1, 3 indicate the M_3/M_3; 2, 4 indicate the $+_M/+_M$. The red box indicates the difference-expression of *BmFAMeT6* and *BmJHAMT* between strains. **b** qRT-PCR analysis for *BmFAMeT6*. **c** qRT-PCR analysis for *BmJHAMT*. Data represent the mean of three values, and error bars indicate S.E.M. * and ** indicate significance at $p < 0.05$ and $p < 0.01$, respectively. 3LD1 indicates day 1 of the third instar; 3LD2 indicates day 2 of the third instar

Cloning *BmFAMeT6* and purifying expressed protein *in vitro*

In order to study the function of *BmFAMeT6*, we referenced KC888750 in GenBank and cloned the 706 bp cDNAs sequence containing the ORF reading frame of *BmFAMeT6* from the larval head of the third instar of the M_3/M_3 (Fig. 3a). Furthermore, the complete coding sequence of *BmFAMeT6* was cloned to the prokaryotic expression vector (Fig. 3a), and mass protein was induced to express by 0.2 mM IPTG (Fig. 3b). The molecular weight of the recombinant protein was about 49 kDa as estimated by electrophoresis (Fig. 3b, c), which is consistent with 49.2 kDa of the predicted weight of *BmFAMeT6*. The recombinant protein of *BmFAMeT6* was purified in high yield and purity (Fig. 3d).

Knockdown of *BmFAMeT6* in larvae of the dominant trimer

The dsRNA of *BmFAMeT6* (ds*BmFAMeT6*) was injected into the larvae of the M_3/M_3 at the second molting process. Each larva was injected with 2 μL solution at 0 h and 3 μL solution at 12 h after entering the molting process. The control larvae were injected with ds*EGFP* or ddH_2O under the same conditions and each group consisted of 30 individuals. There were 24, 25, and 22 living individuals in the ddH_2O, the ds*EGFP* and the ds*BmFAMeT6* group, respectively (Table 1). Interestingly, the survival individuals with ds*BmFAMeT6* entered the third molting process, the midterm of fourth instar and the fourth molting process, while most of the survival larvae with ddH_2O or ds*EGFP* were in the late third instar, the third molting process and the first half of the final instar at 116 h, 141 h, and 229 h after injection, respectively (Fig. 4a-c). The larvae in the ds*BmFAMeT6* group shortened the duration of the third and fourth instars and showed an extra molt to the fifth instar. And qRT-PCR analysis displayed that the expression of *BmFAMeT6* was substantially down-regulated in the larvae injected ds*BmFAMeT6* (Fig. 4d). Further, the two control groups, the ds*EGFP* group and the ddH_2O group, were similar in the results. These suggested that shortening the time span in the third and fourth instars, increasing the number of larval molts, and converting trimolter to tetramolter were induced by the decrease of the *BmFAMeT6* expression, and *BmFAMeT6* was probably related to the moltinism in the M_3/M_3. Also, there were very few larvae having five instars in the ds*EGFP* group or the ddH_2O group, which was similar to the investigation from the group of uninjected larvae and should be caused by environmental factors.

To corroborate whether *BmFAMeT6* influence the moltinism of dominant trimolter, RNAi was applied to three other dominant trimolter strains S4, S6, and S11 to record the molt times before the onset of spinning. There were 30 larvae for ds*FAMeT6* and 50 larvae for ddH_2O per strain. Results showed that the tetramolter rates in living individuals were 70.59% and 23.81%, 71.43% and 25.58%, 72.73% and 18.18% in the ds*FAMeT6* group and the ddH_2O group in S4, S6 and S11 strain, respectively (Table 2). Compared with the control group, the tetramolter rate of the experimental groups was increased about 3 times, 2.8 times, and 4 times in S4, S6, and S11 strains, respectively. These indicated that injection of ds*BmFAMeT6* into several other dominant trimolter strains also could increase the larval molt times and

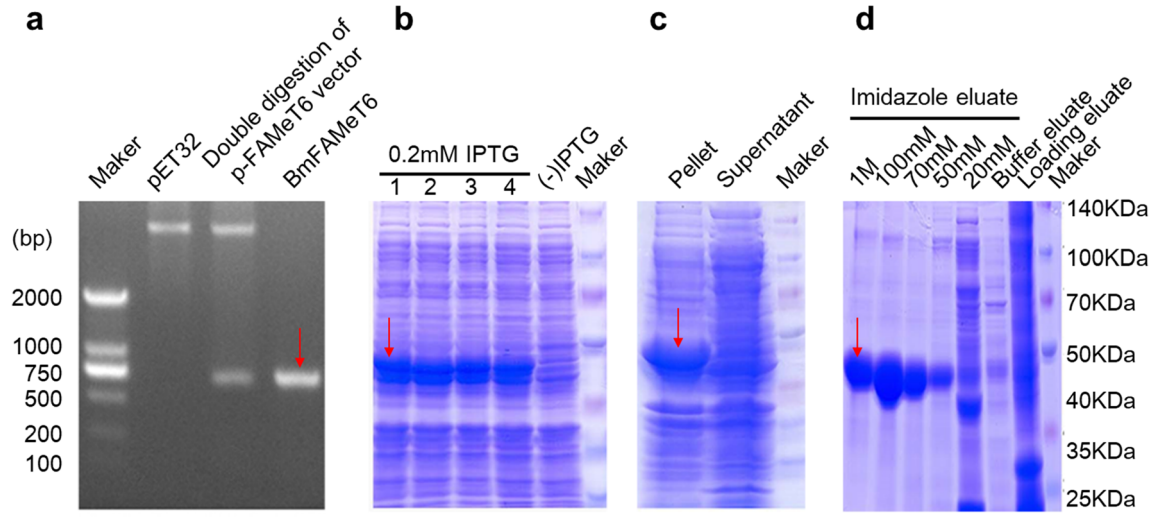

Fig. 3 Production of recombinant *BmFAMeT6*, cloning, and in vitro expression, and purification. **a** clone and expression vector construction of *BmFAMeT6* ORF sequence. **b** inducing expression of *BmFAMeT6* protein by 0.2 mM IPTG. **c** Detection of *BmFAMeT6* protein in supernatant and pellet of cell lysate. **d** Effect of various concentrations of imidazole eluate in the purification of *BmFAMeT6* protein. The red arrow indicates the target band

Table 1 Detailed data for RNAi effect of *BmFAMeT6* on moltinism phenotype in the M_3/M_3 strain of *B. mori*

Treatment	Larvae injected (N)	Survival larvae (N)	Death larvae (N)	Larvae molting four times (N)	Tetramolter rate in survival larvae (%)
ddH_2O	30	24	6	1	4.17%
ds*EGFP*	30	25	5	2	8%
ds*BmFAMeT6*	30	22	5	22	100%
un-injection	30	30	0	2	6.67%

transform trimolter to tetramolter. This is similar to the investigation from the M_3/M_3.

Detection of *BmFAMeT6* mRNA and protein in the larval head

We analyzed the presence of mRNA and the encoded protein of *BmFAMeT6* in the larval head of 3L1D, 3L2D, day 1 of the fourth instar (4L1D) and day 2 of the fourth instar (4L2D) in both the M_3/M_3 and the $+_M/+_M$. RT-PCR analysis showed that the *BmFAMeT6* transcript appeared in all samples although with differences in the levels. The expressions of the *BmFAMeT6* in the M_3/M_3 were high in 3L1D and 3L2D, but low in 4L1D and 4L2D. In the $+_M/+_M$, the expressions of the *BmFAMeT6* were moderate in 3L1D, 3L2D, 4L1D and 4L2D (Fig. 5a). The results of Western blotting indicated that the protein signals of *BmFAMeT6* were strong in 3L1D and 3L2D of the M_3/M_3, 3L1D and 4L1D of the $+_M/+_M$, but weak in 4L1D and 4L2D of the M_3/M_3, 3L2D and 4L2D of the $+_M/+_M$ (Fig. 5b). The third instar was more than the fourth instar on the *BmFAMeT* protein signal as well as the mRNA level in the M_3/M_3. And the protein signals were stronger on the first day than the second day within all observed instars, excepting the fourth instar (the final larval instar) of the M_3/M_3 where signals were equally weak in both periods.

Location BmFAMeT6 in the brain - corpus cardiacum - corpus allatum complex

The corpora allata is a very small ellipsoid tissue connected to the brain through the corpus cardiacum in the larval head. In order to reduce the loss of small organs with low visibility in

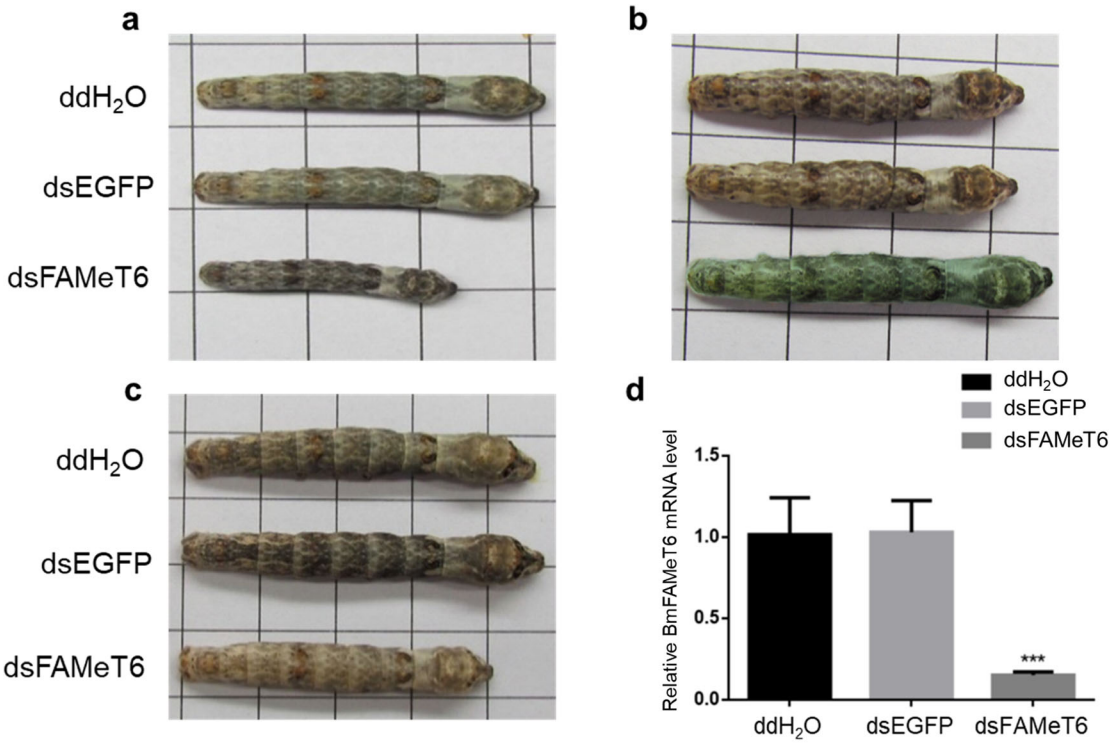

Fig. 4 Effect of injecting ds*BmFAMeT6* into the larvae of the M_3/M_3 at the second molting process. **a** larvae at 116 h after injection. **b** larvae at 141 h after injection. **c** larvae at 229 h after injection. The side length of each small dark square grid under larvae represents 1 cm. **d** Expression levels of *BmFAMeT6* in the injected larvae by qRT-PCR. Data represent the mean of three values, and error bars indicate S.E.M. *** indicate significance at $p < 0.001$

Table 2 Detailed data for RNAi effect of *BmFAMeT6* on moltinism phenotype in S4, S6 and S11 strains of *B. mori*

Strain	Treatment	Larvae injected (N)	Survival larvae (N)	Death larvae (N)	Larvae molting four times (N)	Tetramolter rate in survival larvae (%)
S4	ddH_2O	50	42	8	10	23.81%
	ds*BmFAMeT6*	30	17	13	12	70.59%
S6	ddH_2O	50	43	7	11	25.58%
	ds*BmFAMeT6*	30	21	9	15	71.43%
S11	ddH_2O	50	44	6	8	18.18%
	ds*BmFAMeT6*	30	22	8	16	72.73%

the immunohistochemical experiment, the brain with attached corpora cardiaca-allata were analysed as wholemounts. Clear fluorescence was only observed in the corpora allata but not in the brain or corpus cardiacum at 4L1D and 4L2D in the $+_M/+_M$ (Fig. 6a, b). No fluorescence signals in the corpora allata were found at day 1 of the fifth instar (5L1D) and day 2 of the fifth instar (5L2D) in the $+_M/+_M$ (Fig. 6c, d), and at 4L1D and 4L2D in the M_3/M_3 (Fig. 6e, f). The fourth instar of the M_3/M_3 such as the fifth instar of the $+_M/+_M$ is the last larval instar, where no *BmFAMeT6* fluorescence signals were found.

Discussion

The silkworm is an important economic insect, its moltinism is closely related to many important economic characters such as cocoon yield, cocoon silk quality and cocoon filament size (Chen 2006). Near-isogenic lines, due to the small genetic difference, have great advantages in studying the genetic information of important characters and were widely used in animals and plants (Shen et al. 2001; Miao et al. 2005;). Here, we generated the near-isogenic lines of dominant trimolter using classical transfer hybridization in silkworm breeding. These lines provide an excellent resource for the study of the molecular mechanism of moltinism regulation that underlies plasticity in the number of larval instars.

In the silkworm, the number of larval instars in a strain is generally fixed although can be modified by the environment and genetic signals (Shen et al. 2001; Miao et al. 2005;). So far, several signaling molecules (*FOXO*, *JHE*, *CYP15C1*, *Met*, *JHAMT*, *Kr-h1* and *E93*) have been shown to be involved in the regulation of moltinism (Shinoda and Itoyama 2003; Tan et al. 2005; Konopova and Jindra 2007; Daimon et al. 2012; Zeng et al. 2017). In this study, the *BmFAMeT6* expression had an obvious distinction between the near-isogenic lines of the dominant trimolter (the M_3/M_3 and the $+_M/+_M$), suggesting that *BmFAMeT6* is probably related to the moltinism of dominant trimolter. Especially, knockdown of *BmFAMeT6* in the M_3/M_3 induced extra molting and convert trimolter to tetramolter. And injecting ds*BmFAMeT6* could promote the tetramolter rate to increase significantly in several other dominant trimolter strains. These demonstrate that *BmFAMeT6* is relevant to the moltinism of dominant trimolter. In crustaceans, many studies reveal that *FAMeT* plays an important role in reproduction (Wainwright et al. 1998; Zapata et al. 2003; Duan et al. 2014; Buaklin et al. 2015). However, *FAMeT* mRNA is constitutively expressed throughout the molt cycle in *M. ensis* (Gunawardene et al. 2002), and its level is higher in pre-molt than inter-molt in the mud crabs *Scylla olivacea* (Herbst, 1796) (Decapoda) (Sunarti et al. 2016). The shrimps can't develop to the final stage of molt cycle when *FAMeT* is knocked down in the white shrimp, *Litopenaeus vannamei* Boone, 1931 (Decapoda) (Hui et al. 2008). By

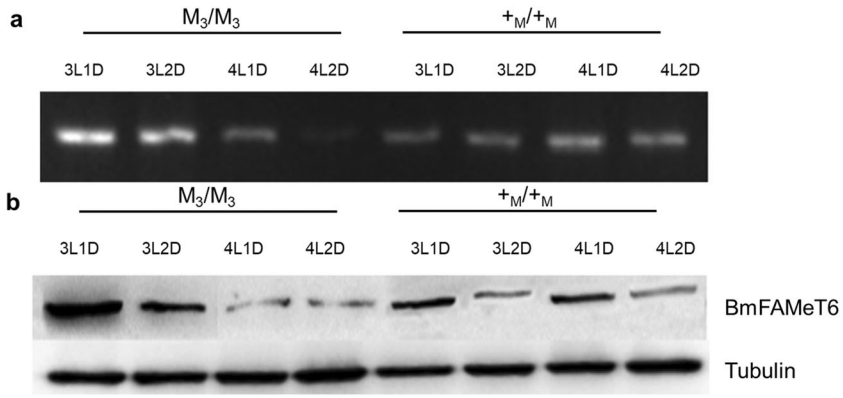

Fig. 5 Expression detection of *BmFAMeT6* in the larval head of the M_3/M_3 and the $+_M/+_M$. **a** RT-PCR analysis. **b** Western blotting analysis. 3LD1 indicates day 1 of the third instar; 3LD2 indicates day 2 of the third instar. 4LD1 indicates day 1 of the fourth instar; 4LD2 indicates day 2 of the fourth instar

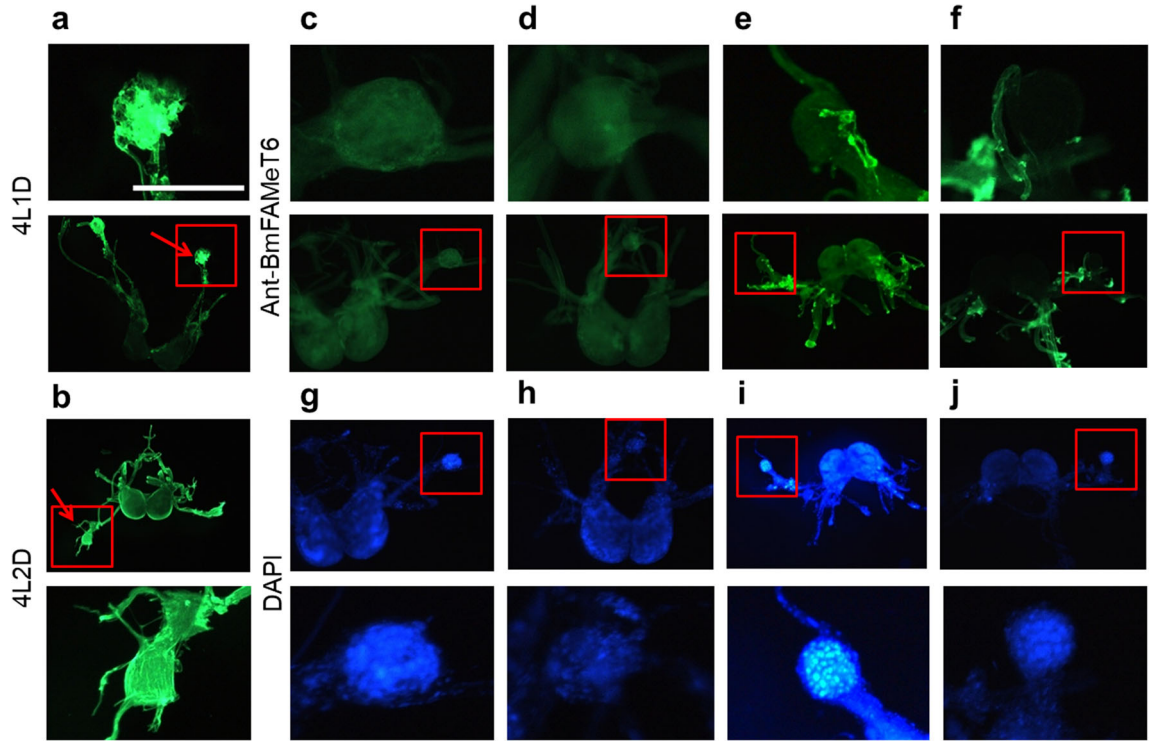

Fig. 6 Immunofluorescence analysis of BmFAMeT6 in the brain - corpus cardiacum – corpus allatum complex. a 4LD1 of the $+_M/+_M$. b 4LD2 of the $+_M/+_M$. c day 1 of the fifth instar (5LD1) of the $+_M/+_M$. d day 2 of the fifth instar (5LD2) of the $+_M/+_M$. e 4LD1 of the M_3/M_3. f 4LD2 of the M_3/M_3. g 5LD1 of the $+_M/+_M$ for DAPI. h 5LD2 of the $+_M/+_M$ for DAPI. i 4LD1 of the M_3/M_3 for DAPI. j 4LD2 of the M_3/M_3 for DAPI. The red arrow indicates the fluorescence signal. The red box indicates the magnified area. Scale bar 200 μm

RNAi silence of *FAMeT* down-regulates the expression of the ecdysone receptor gene (*EcR*) and silence of *EcR* decreases the expression of *FAMeT* as well in the giant freshwater prawn, *Macrobrachium rosenbergii* De Man, 1879 (Decapoda) (Qian and Liu 2019). These indicate that *FAMeT* is also interrelated to the molt of crustacean.

Immunohistochemical analysis has confirmed the presence of *FAMeT* in the corpus allatum of *D. melanogaster* ring gland (Burtenshaw et al. 2008). The *FAMeT* is highly expressed in the corpus allatum of *N. lugens* (Liu et al. 2010) and *S. gregaria* (Marchal et al. 2011). In *D. citri*, the *FAMeT* mRNA is detected in the head-thorax containing the corpora allata (Van Ekert et al. 2015). Our data exhibited that *BmFAMeT6*-specific fluorescence was strictly limited to the corpora allata, and *BmFAMeT6* transcript and protein were discovered in the larval head containing corpus allatum in silkworm. Therefore, the *BmFAMeT6* is expressed in the corpus allatum of the silkworm, what is concordand with other studied insects.

The JH titer is high at the beginning of each instar and then debase to a low level, and gradually decrease from the early larval stage to the early stage of the final instar and then disappear (Furuta et al. 2013; Meng et al. 2015). The concentration of JH titers in the dominant trimolter is high during the second instar (Meng et al. 2015). We tested *BmFAMeT6* mRNA and protein at the early stage of the third and fourth instar in the larval head with corpus allatum. The results show that the expression level of *BmFAMeT6* was higher at the beginning of each instar and decreased gradually from the early larval stage to the final instar, which was consistent with the change of JH titers. And the *BmFAMeT6* fluorescence signal was located on the corpus allatum at the fourth instar (an un-last instar stage) of the $+_M/+_M$. These suggested that there seemed to be a correlation between *BmFAMeT6* expression and JH titers, which was similar to the report from *C. capitata* (Van Ekert et al. 2015).

In the silkworm, precocious larval-pupal transitions could be induced by the loss of or low levels of JH (Fukuda 1944; Tan et al. 2005; Daimon et al. 2012; Wang et al. 2012). The trimolting phenotype in the dominant trimolter is most likely due to the greater concentration of JH titers during the second larval instar (Meng et al. 2015). This indicates that the disorder of JH titers is associated with moltinism mutation. Our immunohistochemical analysis revealed that *BmFAMeT6* is specifically expressed in the corpus allatum, the only organ producing and secreting JHs in the *Bombyx* larva. *BmFAMeT6* expression probably had a close correlation with the JH synthesis and secretion. Particularly, the reduction of *BmFAMeT6* expression induced extra molt to turn trimolter to tetramolter. These indicated that descending *BmFAMeT6* could change the larval molt times in the dominant trimolter, which might be

due to the close relationship between *BmFAMeT6* and JH titers. We will try to elucidate the relationship between *BmFAMeT6* and JH titers in further research.

Conclusion

Our results provide new information for moltinism regulation in dominant trimolter of silkworm, and display the possible biological function of *FAMeT* in Lepidoptera for the first time.

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Declarations

Competing interests The authors declare no competing or financial interests.

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