Mol Biol Rep (2013) 40:4115–4122
DOI 10.1007/s11033-013-2514-6

Expression and functions of dopa decarboxylase in the silkworm, *Bombyx mori* was regulated by molting hormone

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Received: 18 December 2012 / Accepted: 27 April 2013 / Published online: 3 May 2013

Abstract Insect molting is an important developmental process of metamorphosis, which is initiated by molting hormone. Molting includes the activation of dermal cells, epidermal cells separation, molting fluid secretion, the formation of new epidermis and old epidermis shed and other series of continuous processes. Polyphenol oxidases, dopa decarboxylase and acetyltransferase are necessary enzymes for this process. Traditionally, the dopa decarboxylase (*Bm*Ddc) was considered as an enzyme for epidermal layer's tanning and melanization. This work suggested that dopa decarboxylase is one set of the key enzymes in molting, which closely related with the regulation of ecdysone at the time of biological molting processes. The data showed that the expression peak of dopa decarboxylase in silkworm is higher during molting stage, and decreases after molting. The significant increase in the ecdysone levels of haemolymph was also observed in the artificially fed silkworm larvae with ecdysone hormone. Consistently, the dopa decarboxylase expression was significantly elevated compared to the control. *Bm*Ddc RNAi induced dopa decarboxylase expression obviously declined in the silkworm larvae, and caused the pupae appeared no pupation or incomplete pupation. *Bm*Ddc was mainly expressed and stored in the peripheral plasma area near the nucleus in *Bm*N cells. In larval, *Bm*Ddc was mainly located in the brain and epidermis, which is consisted with its function in sclerotization and melanization. Overall, the results described that the dopa decarboxylase expression is regulated by the molting hormone, and is a necessary enzyme for the silkworm molting.

Keywords Dopa decarboxylase (*Bm*Ddc) · Silkworm, *Bombyx mori* · Molting hormone · RNA interference · Metamorphosis

Introduction

Molting hormone (MH) was initially isolated from silkworm prothoracic gland, which not only controls molting, but also involves in the initialization of genetic program for metamorphosis. In *Bombyx mori*, the metamorphosis type is determined by the juvenile hormone (JH) and MH titer in the haemolymph. Interestingly, the pupation molt will be induced when the secretion of JH is stopped, and ecdysone plays a dominant role when the larval grown into the 4th molt of the last larval stage [1, 2]. MH also has close relationship with cell differentiation [3, 4]. Studies have shown that the 20-hydroxy-ecdysterone can induce cell renewal and cell apoptosis in the pupal wings of lepidoptera insects [5].

Molting includes activation of dermal cells, epidermal cells separation and secretion of molting fluids. Besides, the enzymes proteases and chitinases manufactured by epidermal cells during molting period are deposited in the molting fluid in or between the old cuticle and the epidermis [6–9]. *Bm*Ddc catalyzed dopa to dopamine, involved in the synthesis of melanin, and played a key role in the process of the insect exoskeleton hardening [10]. The increased level of dopamine in the haemolymph also causes delayed pupation [11].

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Though chitin is not considered the main component of ossification and hardening of the epidermis, chitin is a scaffold material associated with cuticle proteins to determine its mechanical properties. They are bound exo and endocuticles or unsclerotized procuticle [12]. Metabolism of chitin is crucial for insect development. During larval molt and pupation, chitin degrading enzymes like proteases and chitinases manufactured by epidermal cells are deposited in the molting fluid in between the old cuticle and the epidermis. Cuticles proteinaceous constituents' degradation by chitinolytic enzymes requires proteases from molting fluid [13].

Meanwhile, dopa decarboxylase (*Bm*Ddc) is one set of the key enzymes that catalyze dopa into dopamine [10]. The aim of the present study is to understand whether *Bm*Ddc is one of the key enzymes in molting and whether MH has any role in the regulation at the time of biological molting processes.

Most importantly, the study of molting mechanism has important implication to understand insect life cycle, their growth and development, and particularly, in the control and management of pests. This study also focuses on revealing the process of molting ecdysone and their relationship with the dopa decarboxylase. The regulation mechanism of MH during molting period revealed the impact of major enzymes on physiological and biochemical processes and provided a theoretical basis for the production practices.

Materials and methods

Bombyx mori larvae

Hybrid strain silkworm (Jing Song × Hao Yue) provided by the Silkworm and Mulberry Station of Zhejiang University, Hangzhou, China were used for this experiment. The silkworms were reared in an incubator chamber. The temperature and humidity were maintained at 25 ± 1 °C and 70–85 % with a photoperiod of 12:12 LD. Silkworms were reared on mulberry leaves (Collected from Zijingang campus, Zhejiang University, Hangzhou, China mulberry garden).

Molting hormone administration

Phytogenous ecdysteroid (MH-III) was extracted from Radix achyranthes which was a cholest-7-ene-6-one carbon skeleton (C27) (Tian Can Bao Pharmaceutical limited company, Huzhou, China). Double-distilled water was added to make diluted solution. The silkworms were fed on mulberry leaves evenly sprayed with MH-III solution at the rate of 200 μg/10 ml of water/100 g leaf/100 worms.

RNA extraction and cDNA synthesis

The total RNA was extracted from silkworm tissues using RNAiso Plus, a commercial RNA extraction kit according to the manufacturer's instructions (Takara Biological Engineering Limited Company, Dalian, China). The isolated RNA was measured in NanoDrop 2000 Spectrophotometer. First strand cDNA was synthesized using the Toyobo (Shanghai) Biotechnology Limited Company's ReverTra Ace qPCR RT Kit, according to the manufacturer's instructions.

Quantitative real-time PCR and data analysis

*Bm*Ddc-specific primers were set as QPCR-Ddc-F: 5′-CTTGGACTGCGGTGATGG-3′ (fwd); QPCR-Ddc-R: 5′-TAGCCGTGCCCTGGATTA-3′ (rev). The *B. mori* actinA3 specific primers used as internal for this experiment were *Bm*RT-PCR-actinA3-F: 5′-GCGCGGCTACTCGTTCACTACC-3′ (fwd) and *Bm*RT-PCR-actinA3-R: 5′-GGATGTCCACGTCGCACTTCA-3′ (rev).

Total RNA was isolated using RNeasy Mini Kit (QIAGEN) from the whole larvae tissues. A sample (2 μg) of total RNA was used as template to synthesize cDNA using superscript II reverse transcriptase (invitrogen).

Real-time PCR was performed on the resulting cDNAs using pairs of primers mentioned above with ABI7300 (Ambion, Foster City, CA, USA) and using the fluorescence dye SYBR®Premix Ex Taq™ (TAKARA). The two-step amplification protocol consisted of a 30 s at 95 °C followed by target amplification via 40 cycles at 94 °C for 5 s, 60 °C for 31 s. After PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the products. The transcript levels of the targeted fragments were normalized with actinA3 transcript levels in the same samples. All reactions were performed in triplicate.

dsRNA-mediated transcript depletion

*Bm*Ddc dsRNA was synthesized and purified according to the instructions provided by the Promege's T7 RiboMAX™ Express RNAi System with the primers as following: T7-*Bm*Ddc: 5′-GGATCCTAATACGACTCACTATAGGCTTGGACTGCGGTGATGG-3′ (fwd), and T7-*Bm*Dbc: 5′-GGATCCTAATACGACTCACTATAGGTAGCCGTGCCCTGGATTA-3′ (rev). All samples were stored at −20 °C temperature. For RNAi experiments, 10 μl of *Bm*Ddc dsRNA was injected into the larva of fifth instar. The same quantity of physiological water (0.9 % sodium chloride) was injected as control.

Enzyme activities assay

Dopa decarboxylase activities were determined according to Noguchi et al. [10] and Nagatsu et al. [14] methods with slight modification. Frozen silkworm tissues (head, haemolymph and epidermis) were grinded in liquid nitrogen and homogenized in ice-cold phosphate buffer solution (pH 7.0) containing 0.2 M sucrose and 0.05 % phenylthiourea. After centrifugation at 10,000×*g* for 1 min, the supernatant was used for enzyme assay. The assay was performed in the solution (total volume: 50 µl) containing 0.1 M phosphate buffer (pH 7.2), 0.3 mM EDTA, 0.17 mM ascorbic acid, 0.01 mM pyridoxal phosphate, and 0.5 mM dopa and 0.1 mM pargyline hydrochloride. The mixture was equilibrated at 25 °C for 10 min, and then the reaction was done for 30 min by adding 5 µl enzyme preparation. After the reaction was terminated by adding 10 µl 3 M trichloroacetic acid, the reaction mixture was centrifuged at 20,000×*g* for 10 min at 4 °C and a 5 µl aliquot of the supernatant was directly analyzed by the HPLC-ECD system to measure produced dopamine levels. *Bm*Ddc activity was calculated from the amount of dopamine formed enzymatically.

Hormone titer assay in vivo

HPLC was used to determine the hormone titer under the set values: column length: 15 cm, diameter: 4.6 mm, stainless steel column; stationary phase: ODS (C18 column), granularity 5 m; mobile phase: acetonitrile, methanol, water volume ratio of 1:2:4; flow rate: 0.8 ml/min; column temperature: 30 °C; the injection volume: 5 µl; UV detector, detection wavelength 246 nm. Standard molting hormone was purchased from Sangon (Shanghai) Limited Company for this experiment.

Construction of recombinant plasmid pET-30a-c(+)/*Bm*Ddc

The *Bm*Ddc mature gene sequence was PCR amplified from pMD18-T/*Bm*Ddc using the forward primer (5′-GAATTCATGGAGGCCCCTGACTTC-3′) and reversed primer (5′-CTCGAGCTTCTTTTGGCTTTTAAG-3′) with restriction enzymes *Eco*Rl and *Xho*l, respectively. The process of PCR was under the following conditions: 94 °C, 2 min; 35 cycles of 94 °C, 1 min; 55 °C 1 min; and 72 °C, 90 s; followed 72 °C, 8 min. The products with the expected size (1,437 bp) were purified using a DNA Gel Extraction Kit (Axygen). The *Bm*Ddc gene was cloned into the corresponding sites of pET-30a-c(+), yielding the recombinant plasmid named pET-30a-c(+)/*Bm*Ddc, and followed by sequencing to ensure the accuracy of its sequence and open reading frame (ORF), stored at −20 °C for use.

Expression of *Bm*Ddc in *Escherichia coli* and antibody preparation

The verified *Bm*Ddc sequence was cloned into pET-30a-c(+) (Novagen, Darmstadt, Germany) vector, and transformed into *E. coli* BL21 competent cells. The culture was then induced with IPTG (final concentration at 1 mM) and further cultured for another 10 h. *E. coli* cells were collected at 500 g for 5 min, and re-suspended with PBS (NaCl 137 mmol/l, KCl 2.7 mmol/l, Na_2HPO_4 4.3 mmol/l, KH2PO4 1.4 mmol/l). SDS-PAGE sample buffer (200 mM Tris–HCl, pH 8.3, 4 % SDS, 400 mM DTT, 20 % glycerol, 2 mM EDTA, 0.05 % bromophenol blue) was added, heated in a boiling water bath for 5 min, centrifuged at 25,000×*g* for 10 min at 4 °C, and the supernatants were collected. The protein was resolved on 10 % SDS–polyacrylamide gel and then stained with Coomassie Brilliant Blue R250 to visualize the protein bands.

The fusion protein was expressed and purified (Ni–NTA Spin Columns, QIAGEN, Germany) and subsequently used as antigens to immunize rabbit for antibody production. After four times of immunization, serum was collected (HuaAn Biotechnology Co., Ltd. Hangzhou, China), then purified antibody according to manual (Montage® Antibody Purification Kit and Spin Columns with PROSEP®-A Media, Milipore, Boston, MA, USA).

Immunohistochemistry

*Bm*N cells were mixed in 4 % formaldehyde/PBS for 10 min. After washing the fixed cells with PBS, they were permeabilized in 0.5 % Triton X-100/PBS, followed by washing with PBS. The fixed cells were blocked in blocking solution (10 % FBS, 0.3 % Triton X-100/PBS) for 1 h at 37 °C. Primary antibody were added with a dilution factor of 1:1,000 and incubated at 37 °C for 2 h, then washing with washing buffer (2 % FBS, 0.3 % Triton X-100/PBS), the cells were then incubated with FITC-conjugated goat anti-rabbit secondary antibody (HuaAn, Hangzhou, China) in 37 °C for an hour. The fixed cells were washed extensively. DAPI was added before observation under a Zeiss laser confocal microscope [15].

The silkworm tissues were fixed in 4 % paraformaldehyde overnight at 4 °C. The tissues were dehydrated through graded alcohols and embedded in paraffin. Four micrometer sections were cut on a Leica microtome and placed on slides. The slides were washed in PBST simply, then blocked in 5 % goat serum diluted in PBST (0.2 % Triton X-100) for around 30 min to reduce the non-specific binding of antibodies, incubated with the mixture of two primary antibody diluted in blocking solution for overnight at room temperature. The slides were then washed in PBST (0.2 % Triton X-100) for 3 × 5 min. After wash, the

mixture of two secondary antibody conjugated with FITC (1:400 diluted in blocking buffer) were added to the slides and incubated at room temperature for about 105 min (keep dark). The slides were washed in PBST (0.2 % Triton X-100) for 5 min for three time (keep dark), then mounted with DAPI (18 μl), then cover slip. Pictures were taken using Zeiss laser confocal microscope [16–19].

Statistical analysis

Microsoft Excel and ACDsee v5.0 were used to plot graphs and figures. All the experiments were performed in three independent biological replication and the reactions of each sample were carried out in triplicate.

Results

Expression levels of *Bm*Ddc in different tissues

All the tissues were obtained from the larvae of the fifth instar. *Bm*Ddc were mainly expressed in the epidermis, head and reproduction organs whereas the expression level was very low in the haemolymph. Even though the expression level of this enzyme is low in haemolymph at this stage, we came to the conclusion that this enzyme is expressed successfully in haemolymph based on the evidence of molting related enzymes' biosynthesis pathways. The low transcript level may results from their instant decomposition because high speed of translation for enzyme protein (Fig. 1).

Comparative expression of *Bm*Ddc in different stage

The expression of the *Bm*Ddc was high at the late younger stages of silkworm. Both have high expression level in the molting stage larvae from the second instar (Fig. 2).

Changes in the *Bm*Ddc expression were mainly observed in the head and reproduction organs and epidermis tissues of the later instars of fifth stage. However, the expression level was sharply increased in the epidermis tissues of the spinning stage larvae (last days of fifth instars larvae), then the expression lever decreased sharply in all these tissues except for haemolymph (Fig. 3).

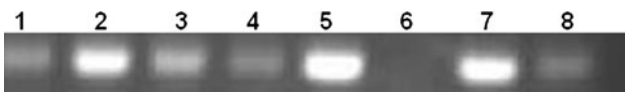

Fig. 1 Distribution of *Bm*Ddc in different tissues. *Lane 1* midgut, *lane 2* epidermis, *lane 3* fat body, *lane 4* silk gland, *lane 5* head, *lane 6* haemolymph, *lane 7* ovaries or testes, *lane 8* malpighian tubule

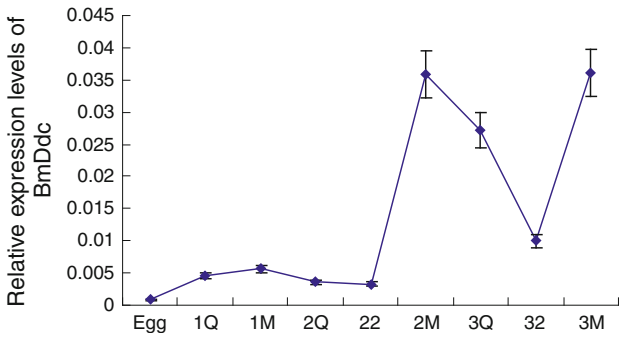

Fig. 2 Expression of *Bm*Ddc at different stages of larvae. *Egg* silkworm egg, *1Q* newly hatched larvae, *1M* molting larvae of first instar, *2Q* newly molted larvae of second instar, *22* second day larvae of second instar, *2M* molting larvae of second instar, *3Q* newly molted larvae of third instar, *32* second day larvae of third instar, *3M* molting larvae of third instar

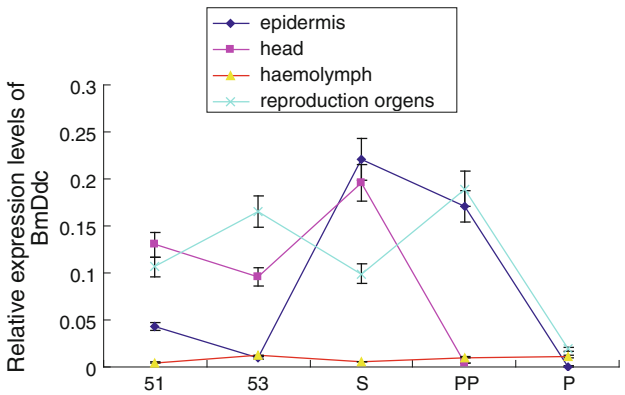

Fig. 3 The changes in the expression levels and the expression in different tissues of *Bm*Ddc from the fifth instar larvae to pupae. *51* First day of fifth instar, *53* third day of fifth instar, *S* matured larva, *PP* pre-pupa, *P* pupa

Changes of enzyme expression after MH administration

The level of *Bm*Ddc expression was dramatically increased at 12 h after the administration of molting hormone, then the expression level was similar to control (Fig. 4).

dsRNA-mediated transcript depletion of *Bm*Ddc

The injection of exogenous dsRNA relatively has large impact on the development of silkworm. The *Bm*Ddc expression level was significantly decreased about 25 % at 24 h, though the effect at 48 h is not as impressive as the 24 h (Fig. 5).

dsRNA-mediated molting patterns

The phenotypes of some incomplete pupation and failure in pupation larvae emerged as moth after *Bm*Ddc dsRNA's

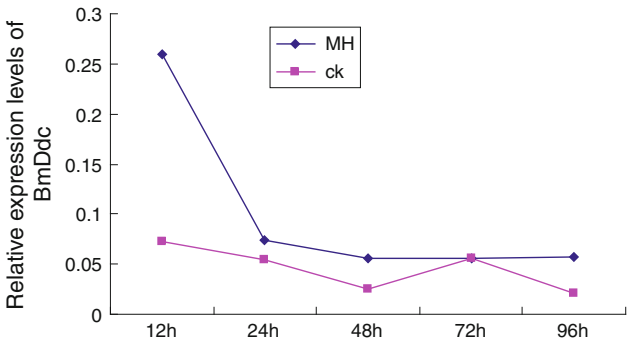

Fig. 4 Changes of *Bm*Ddc expression after MH administration

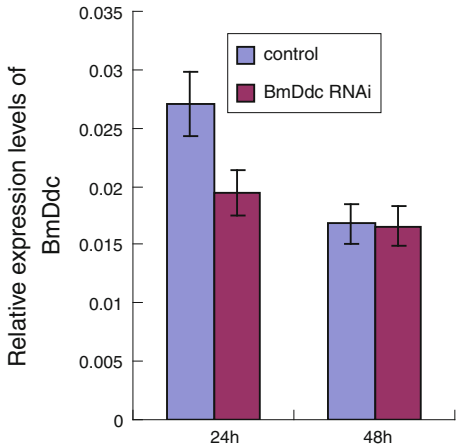

Fig. 5 Expression of *Bm*Ddc gene's after injection of *Bm*Ddc dsRNA. The expression levels of *Bm*Ddc gene in silkworm (injected with dsRNA) after 24 and 48 h at 25 °C

injection. Finally, the abnormal pupa and lava bodies of silkworm slowly decayed, turned black and died. This reveals that *Bm*Ddc RNAi hinders the tanning of the epidermal layers and therefore, the silkworm epidermis failed to form hard shells and become pupae (Fig. 6).

Changes of dsRNA-mediated enzyme activities

*Bm*Ddc were mainly functional in the epidermis during molting stages. The investigation of *Bm*Ddc activity after *Bm*Ddc dsRNA-mediated interference showed that the *Bm*Ddc activities were a little lower compare to the control at 24 h post-injection, but the rebound phenomenon appeared at 48 h post-injection (Fig. 7).

*Bm*Ddc expression of in *E. coli*

We expressed *Bm*Ddc in *E. coli,* and obtained a 55 kDa protein, this was consistent with the expected results (Fig. 8).

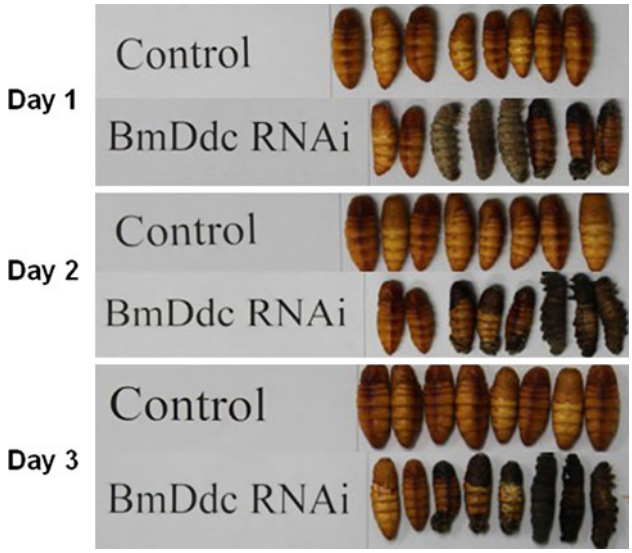

Fig. 6 Patterns of pupa after larva's were injected with dsRNA in day wise manner

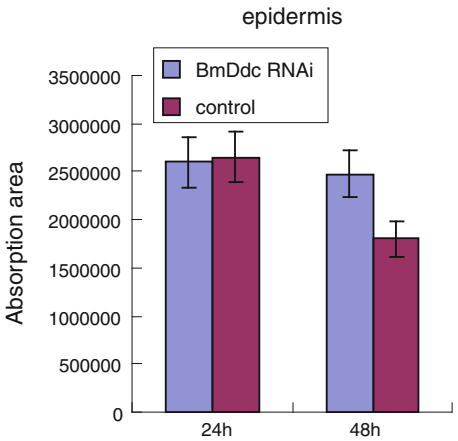

Fig. 7 *Bm*Ddc activity change after RNAi

*Bm*Ddc localization in *Bm*N cells and tissues

In order to give a clear view of the functional location of *Bm*Ddc in *B. mori*, we prepared the first antibody and bought the FTIC-labeled secondary antibody. *Bm*Ddc was mainly expressed and stored in the peripheral plasma area near the nucleus in *Bm*N cells (Fig. 9).

*Bm*Ddc was mainly located in the brain and epidermis, especially in brain surface and nerve layer and in dermal cells, but few existed in the testis or ovary (Fig. 10). The head and epidermis are the main tissues for tanning, sclerotization and melanization. The high expression of *Bm*Ddc in these two tissues is consisted with its function in sclerotization and melanization.

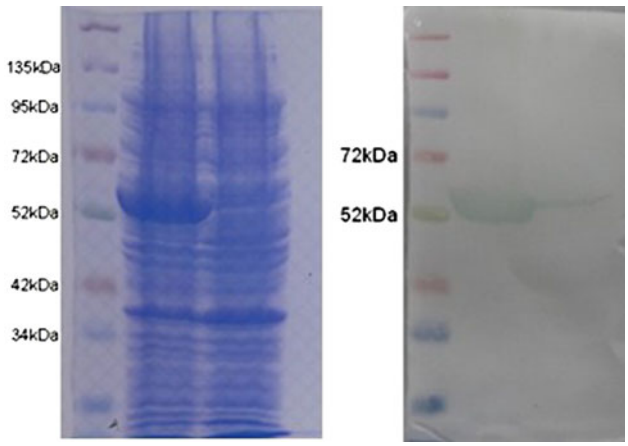

Fig. 8 Western blotting result of *Bm*Ddc expressed in *E. coli*

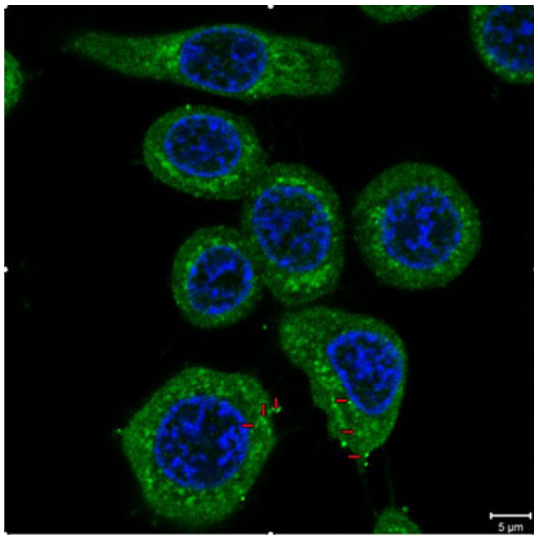

Fig. 9 *Bm*Ddc localization in *Bm*N cells (*Green* FITC, *Blue* DAPI). (Color figure online)

Discussion

The silkworm, *B. mori* is an important economic insect and model organism of Lepidoptera in the fields of genetics, physiology and biochemistry. Molting in silkworms is closely associated with their growth and other physiological processes. Ecdysis is a cascade process of PTTH initiated hormone mediated by gene expression and interaction. Study of molting in insects not only understands the normal growth and development of multiple important processes, but also helps to understand eukaryotic gene regulation of important models. Similarly, the use of synthetic interferometer during molting process helps to unlock chemicals for pest control [20, 21].

Silkworm molting is regulated by photoperiod and temperature. Under these influential factors, the brain synthesizes and release prothoracicotropic hormone (PTTH) to direct the precise timing of the molt by stimulating the prothoracic glands. This stimulation synthesizes and releases MH called ecdysone. Following which, molting and metamorphosis are both regulated by ecdysone and JH secreted from corpora allata. Subsequently, molting and metamorphosis takes place under the influence of PTTH, ecdysone and the JH [22].

Molting hormone or molting sterol was initially isolated from silkworm prothoracic gland. The initial effects of ecdysone on epidermal cells are stimulated by the synthesis of molting hormones. The hormone is bound to a protein receptor, which induces the chromosome to appear bubble and the transcription from mRNA further induces intracellular to synthesis dopa decarboxylase and phenol oxidase to promote epidermis tanning and hardening.

The synthesis of new epidermal protein including dopa decarboxylase and phenol oxidase was initiated when the ecdysone titers decreased [23]. Therefore, ecdysone plays an important role, especially for molting and metamorphosis including insect growth, development and reproduction [24].

This work suggested that dopa decarboxylase is one set of the key enzymes in molting, which closely related with the role of ecdysone in the regulation at the time of biological molting processes. The quantitative expression analysis of dopa decarboxylase in different stages and tissues showed that the expression of dopa decarboxylase in early stage of silkworms are consistent with the molting process where the expression peak of dopa decarboxylase in silkworm is higher during molting stage, and later decreases after molting.

Further, changes in the *Bm*Ddc expression were mainly observed in the head and epidermis tissues of the early instars of fifth stage with low level of expression in haemolymph. However, the expression level was sharply increased in the epidermis tissues of the spinning stage larvae (last days of fifth instars larvae), then the expression lever decreased sharply in all these tissues except for haemolymph.

The significant increase in the ecdysone levels of haemolymph was observed in the artificially fed silkworm larvae with ecdysone hormone. Consistently, the *Bm*Ddc expression was significantly elevated compared to the control.

*Bm*Ddc was mainly expressed in the peripheral plasma area near the nucleus in *Bm*N cells and was visible mainly in the brain surface and the nerve tissues. It was also distributed ubiquitously in the epidermis cells. This was in accordance with its function which was mainly in the process of molting and metamorphosis.

Overall, the results described that the *Bm*Ddc expression is regulated by the molting hormone, and is a necessary enzyme for the silkworm molting. *Bm*Ddc RNAi induced

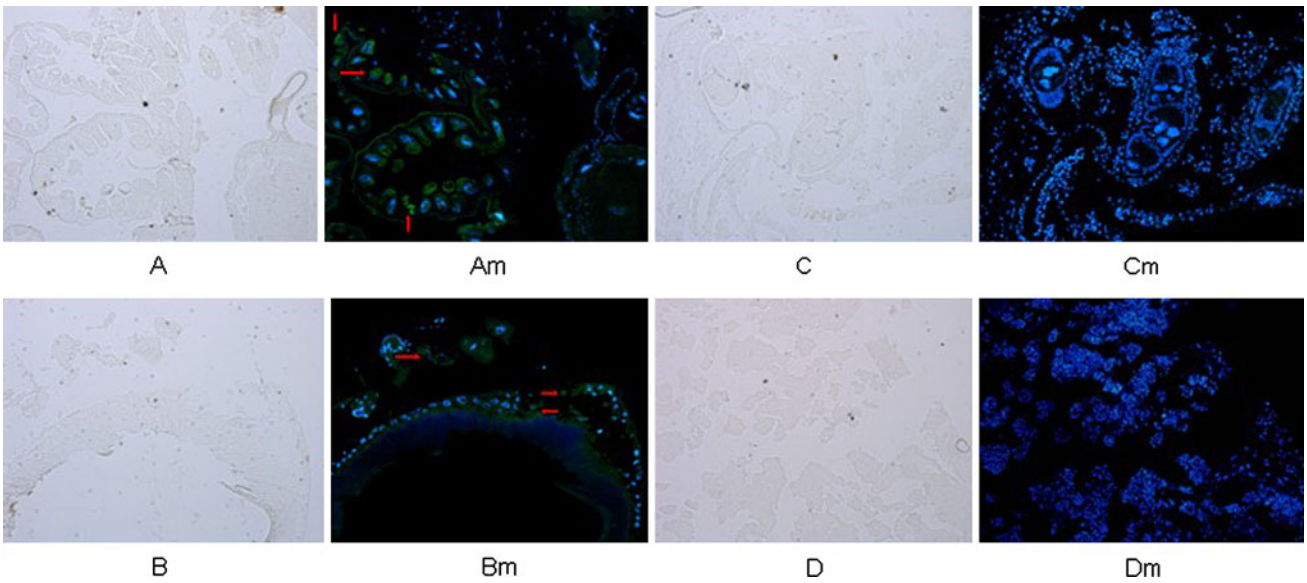

Fig. 10 *Bm*Ddc localization in tissues of silkworm larval. (*Green* FITC, *Blue* DAPI). *A/Am* head, *B/Bm* epidermis, *C/Cm* testis, *D/Dm* ovary. (Color figure online)

*Bm*Ddc expression subtle decline in the silkworm larvae even though some pupa's appeared incomplete pupation phenomenon and failure in pupation.

Acknowledgments The work was supported by the National Basic Research Program of China under Grant No. 2012CB114601 and the National Natural Science Foundation of China (No. 30972141/C120110), the Key project of Zhejiang Government (No. 2011C14006), the Science and Technology Innovation Team of Zhejiang Province (No. 2010R50031) and Chinese Universities Scientific Fund.

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