Identification and Characterization of BmVta1, a *Bombyx mori* (Lepidoptera: Bombycidae) Homologue for Vta1 That is Up-Regulated in Development

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Abstract

Vps20-associated 1 (Vta1) positively regulates Vacuolar protein sorting 4 (Vps4) to disassemble endosomal sorting complex required for transport III (ESCRT-III) for repeated uses in multivesicular body (MVB) pathway, virus budding and other processes. Currently, these proteins have mainly been studied in yeast and mammalian cells, while identities of them in insects remain largely unknown. We previously identified BmVps4, a Vps4 homologue from *Bombyx mori*. Here, we report the identification of a homologue for Vta1, designated as BmVta1. The BmVta1 cDNA contains an open reading frame of 933 bp and encodes a protein of 311 amino acid residues. We cloned BmVta1, expressed it in *Escherichia coli*, and prepared mouse polyclonal antibodies. Like BmVps4, BmVta1 is well conserved as shown by sequence analysis. Both proteins are localized in cytoplasm as revealed by subcellular location analysis. Interestingly, as revealed by semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR), transcriptions of BmVta1 and BmVps4 are highly up-regulated during silkworm metamorphosis and embryogenesis but down-regulated during larva stages, and are of higher levels in head, silk gland and testis than in Malpighian tube, fat body and ganglion, indicating important and similar roles of them in silkworm development and in silkworm tissues and organs. However, compared to BmVps4, the transcription of BmVta1 changes less drastically during development and is of much higher levels in midgut, ovary and hemolymph, suggesting the existence of distinct requirements of them in silkworm development and in certain tissues and organs.

Key words: expression, silkworm, subcellular location, transcription, Vta1

Multivesicular body (MVB) is formed by inward budding of limiting membrane of late endosome. It was observed many decades ago, but the molecular mechanism underlying its formation remained unknown until the identification of four endosomal sorting complex required for transport (ESCRT-0,II, III) assembled on endosomal membrane by class E Vacuolar protein sorting (Vps) proteins (Hanson and Cashikar 2012, Schuh and Audhya 2014, Odorizzi 2015, Christ et al. 2016). ESCRT-0 (Vps27, Hse1 as in yeast) determines the site for MVB formation via binding to membrane phosphatidylinositol-3-phosphate (PI3P), and recognizes ubiquitin, a signal for entry into MVB pathway, on cargo proteins via its ubiquitin-binding domains. ESCRT-I (Vps23, Vps28, Vps37, Mvb12) and ESCRT-II (Vps22, Vps25, Vps36) deform the limiting membrane to bud inwards and sort cargoes into the forming intraluminal vesicles (ILVs). The assembled ESCRT-III (Vps20, Snf7/ Vps32, Vps24 and Vps2 as core subunits, Vps60 and Vps46/DiD2 as similar components) exists in filament-like structures to cleave the bud neck to release mature ILVs. These ESCRTs form an interaction network via interactions of Vps27-Vps23, Vps28-Vps36 and Vps25-Vps20, and act sequentially to form MVB and sort both endocytic and newly synthesized cargo proteins to target them for degradation or localization in vacuole (mammalian lysosome). Then, the ATPase Vps4 is recruited to disassemble the tightly bound ESCRT-III to enable repeated uses, which is an essential step as dysfunction of Vps4 could completely block MVB sorting pathway. Recently, these proteins have also been shown to be involved in signal transduction regulation, autophagy, apoptosis, cytokinesis, pH stress response, plasma membrane damage repair, virus budding, axon pruning, nuclear pore quality control, nuclear envelope reformation or repair, and are implicated in cancer and neuro-degeneration diseases (Hurley 2015, Alonso et al. 2016, Christ et al. 2016). Notably, ESCRT-III and Vps4 participate in all of these cellular processes and
are considered as the core ESCRT machinery for membrane remodeling.

Like assembly of ESCRTs, Vps4 also needs to be assembled into homo-oligomers to function efficiently. It exists as a monomer or dimer in cytosol with low ATPase activity, and can achieve optimal activity when being assembled into hexamer or dodecamer and bound to ATP, ESCRT-III and Vps20-associated 1 (Vta1) (Scott et al. 2005, Azmi et al. 2008, Gonciarz et al. 2008, Xiao et al. 2008, Landsberg et al. 2009, Hill and Babst 2012, Monroe et al. 2014). Vta1 interacts directly with Vps4 to promote or stabilize the oligomerization of Vps4 and stimulate its ATPase activity, acting as an assembly factor and positive regulator for Vps4. Moreover, this regulator can be further regulated by ESCRT-III or ESCRT-III like proteins, which are even more complicated in mammalian cells than in yeast (Shim et al. 2008, Shestakova et al. 2010, Skalicky et al. 2012, Yang et al. 2012). Yeast Vta1 can interact with Vps60 and Vps46/Did2 to increase its stimulatory effect on Vps4. By contrast, besides charged multivesicular body protein 5 (CHMP5)/Vps60 and CHMP1B/Vps46/Did2, lysosomal trafficking regulator-interacting protein 5 (LIP5), the mammalian homologue of Vta1, can also bind to CHMP2A/Vps2-1 and CHMP3/Vps24, and surprisingly CHMP5 can inhibit the stimulation ability of LIP5 on Vps4 (Vild et al. 2015). In addition, LIP5 and Vps4 seem to bind simultaneously to CHMP1B and CHMP2A, and the formation of stable Vps4 complexes require LIP5 binding to ESCRT-III proteins and CHMP5.

Consistently, LIP5 displays more complicated functions than yeast Vta1. Yeast Vta1 apparently plays a general role in MVB pathway, but LIP5 can directly interact with cargo proteins, such as aquaporin 2 (AQP2), to facilitate their entry into MVB pathway (van Balkom et al. 2009). LIP5 is also critically required for virus budding at plasma membrane, such as human immunodeficiency virus type 1 (HIV-1) (Ward et al. 2005), and for intracellular budding and envelopment, such as hepatitis C virus (HCV) (Barouch-Bentov et al. 2016). Depletion of LIP5 can greatly decrease HIV-1 budding by 70%, while depletion of CHMP5 can increase the release of HIV-1 particles (Ward et al. 2005), consistent with the negative regulation of LIP5 by CHMP5. Amazingly, as one of host defense responses, expression of interferon-stimulated gene 15 (ISG15) can disrupt interactions of Vps4, LIP5 and ESCRT-III, blocking their recruitment to virus budding site (Piniceti et al. 2010, Kuang et al. 2011). Recently, LIP5 has also been shown to function in plant defense responses by acting as an important target for pathogen-responsive mitogen-activated protein kinases (MAPKs). The phosphorylation of LIP5 can promote its interaction with Vps4, increase formation of MVBs and exosome-like vesicles, leading to relocation of defense-related molecules to resist pathogen infections, such as Pseudomonas siringae (Wang et al. 2014), and can also increase plant tolerance to heat and salt stresses, which is also dependent on the interaction of LIP5 with Vps4 (Wang et al. 2015). Therefore, although great efforts have been made to illustrate divergent regulations and important roles of Vps4-Vta1, the fifth member of ESCRT machinery due to paralleled significance with other ESCRTs, it is still necessary to further study these regulations in higher species other than yeast to gain better understandings about the underlying mechanisms and physiological or pathological roles for them.

Currently, the MVB pathway has not been much explored in insects. Silkworm (Bombyx mori) is an economic insect and a research model for Lepidoptera insects, and has also been used as a bioreactor to produce valuable bio-molecules. We previously identified a silkworm homologue gene for VTA1, expressed and characterized the protein designated as BmVta1. We found that the transcriptions of BmVps4 and BmVta1 are up-regulated in silkworm metamorphosis and embryogenesis, suggesting their important roles in silkworm development. They are of higher levels in head, silk gland and testis than in Malpighian tube, fat body and ganglion, indicating a similar organ and tissue specificity. Compared to BmVps4, the transcription of BmVta1 changes less drastically during development and is of much higher levels in mid-gut, ovary and hemolymph, indicating distinct regulations and roles for BmVta1.

**Materials and Methods**

**Materials**
The silkworm P50 is maintained in our lab and the larvae are reared on fresh mulberry at 26 °C. The first day larvae of fifth instar were dissected to obtain tissues and organs including fat body, silk gland, Malpighian tube, hemolymph, midgut, ovary, testis, head and ganglion, and then frozen in liquid nitrogen and stored at −80 °C. The silkworm samples of fertilized eggs (right after oviposition), larva (from first to fifth instar, right after molt), pupa (right after prepupa) and moth (right after pupa) were also collected. RevertAid First Strand cDNA Synthesis Kit was from Thermo Scientific. The T4 DNA ligase, restriction enzymes, PCR reagents were obtained from TakaRa Company (Dalian, China), pET30a vector was from Novagen. The insect expression vector pBl2/V5-His was from Invitrogen. The pEGFP-C1 was a gift from Dr Qiang Wang in the Institute of Life Science (Jiangsu University, China). Primers were synthesized by Sangon Biotech (Shanghai, China). The monoclonal antibody against α-tubulin was from Protein tech Group (IL).

**Bioinformatic analysis**
To identify silkworm homologues for Vta1, the sequence of yeast Vta1 was used as a query to BLAST silkworm genome database (http://silkworm.genomics.org.cn/). Multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/) and GeneDoc 3.2. The protein sequences of Vta1 from different species were aligned, including B. mori (NP_001040410), Homo sapiens (NP_057569), Saccharomyces cerevisiae (NP_013282), Caenorhabditis elegans (NP_492139), Drosophila melanogaster (NP_647640), Mus musculus (Q9CR26), Arabidopsis thaliana (NP_194405), and Candida albicans (EEQ42051.1). The phylogenetic tree was constructed by MEGA 3.1 using neighbor-joining method and the homology-modeling was carried out by SWISS-MODEL (http://swissmodel.expasy.org/).

**Gene cloning**
The total RNA was extracted from silkworm samples using Trizol reagents. The RevertAid First Strand cDNA Synthesis Kit was used for reverse transcription. To amplify the full length open reading frame (ORF) of BmVta1, the following PCR primers were used: the forward primer 5'-GTCGAATTCATGGCAAACATTCCTG-3' (EcoR I) and the reverse one 5'-GGACTCGAGTGCAGCTGGATCAC-3' (Xho I), and the underlined bases indicate sites for restriction endonucleases in parenthesis. The primers were designed according to a silkworm genomic ORF (NP_001040410), the predicted protein sequence of which is of the highest similarity to yeast Vta1. The PCR reaction included (50 μl) 1 μl diluted cDNA, 1.5 mM MgCl2, 50 pmol primers, 0.5 mM dNTP mix and 1 unit Taq DNA polymerase (TaKaRa, China). The PCR was performed

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on a thermal cycler (Model 2720, Applied Biosystems) using the following condition: 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s, and 15 min for final extension step. The agarose gel (1%) electrophoresis was conducted to separate the PCR products and was visualized by a nucleic acid dye GRred staining (Generay Biotech Co., Shanghai, China).

To express the BmVta1 in Escherichia coli, the PCR fragment was digested with EcoR I and Xho I, purified and subcloned into pET30a vector to obtain the construct pET30a-BmVta1. To investigate the subcellular localization of BmVta1, the fragment was subcloned into a modified insect expression vector pIB/5-V-EGFP to express an EGFP-BmVta1 fusion protein in silkworm BmN cells. The vector contains a promoter of the immediately early 2 (ie-2) gene from baculovirus and is commonly used to express proteins in insect cells. The EGFP ORF was obtained by PCR from the parent construct pEGFP-C1 with forward primer 5'-GGCGGTACCCTGTTGAGCGCAAGGG-3' (Kpn I) and reverse primer 5'-CTCCCTCTCAATGCCTGTCAG-3' (EcoR I). The resultant construct was named as pIB-EGFP-BmVta1, and all constructs were verified by DNA sequencing (Invitrogen, Shanghai, China). Similarly, the pIB-BmVps4-EGFP was constructed to examine the subcellular location of BmVps4. The primers 5'-GTTGGATCCATGGAGCAGAGGCCGC-3' (BanH I) and 5'-CCGGCTCGAGATTACTTGTGGACCGTTCG-3' (Xho I) were used for subcloning of EGFP ORF. The 5'-GGCGGTACCCTGTTGAGCGCAAGGG-3' (Kpn I) and 5'-GTTGGATCCATGGAGCAGAGGCCGC-3' (BanH I) were used for subcloning of BmVta1 protein in PBS buffer or a dialysis buffer (25 mM Tris–HCl, pH 7.5, 100 mM NaCl), dialyzed against PBS or a dialysis buffer (25 mM Tris–HCl, pH 7.5, 100 mM NaCl), transferred into centrifuge tubes and stored at −70 °C. Protein concentration was determined by a Bio-Rad protein assay.

To prepare mouse polyclonal antibodies, 0.2 ml (1 mg/ml) BmVta1 protein in PBS buffer was thoroughly mixed with 0.2 ml Freund’s complete adjuvant for the first immunization to a Balb/c mouse and with Freund’s incomplete adjuvant for additional immunizations. A blood sample of mouse ear was taken to test the antibody titer by Western blot. One week after the first immunization, the mouse was sacrificed and the whole blood sample was collected to obtain polyclonal antiserum. To verify the effectiveness of antiserum, it was applied in Western blot to detect the recombinant protein BmVta1 expressed in E. coli and the endogenous BmVta1 protein in silkworm larva and pupa.

**Subcellular localization analysis**

The BmN cells were cultured in TC100 medium supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin at 27 °C. The recombinant plasmids pIB-EGFP, pIB-EGFP-BmVta1, and pIB-BmVps4-EGFP were, respectively, transfected into BmN cells by liposome method as reported previously (Guo et al. 2015, Lu et al. 2017). Briefly, 8 μl cellfectin II reagent was diluted into 100 μl TC100 medium, mixed thoroughly and incubated for 30 min at room temperature, then was mixed with 100 μl TC100 medium containing 3 μg of plasmid and incubated for another 30 min to form liposomes. The cells were plated into 6-well culture plates with 8 × 10⁵ cells per well for 15 min at room temperature, then the medium was removed and the well was refilled with 2 ml fresh TC100 medium containing 1.5% FCS and no antibiotics. The liposomal mixture was slowly added into the well and incubated at 27 °C for 5 h, then the medium was replaced by TC100 medium containing 10% FCS and two antibiotics. The cells were incubated at 27 °C for 72 h, then examined by a fluorescence microscope (Nikon ECLIPSE Ti-E, Japan).

**Results**

Molecular cloning and bioinformatic analysis

The cDNA sequence of BmVta1 contains an ORF of 933 bp (Supp Fig. 1 [online only]). It encodes a peptide of 311 amino acid residues with a predicted molecular weight of 34.3 kDa and an isoelectric point of 5.1. **Figure 1** shows the sequence alignment and **Fig. 2A** for phylogenetic tree analysis. BmVta1 is similar to Vta1 proteins from various species: *A. thalana* Vta1 (identities = 38%, positives = 56%), *S. cerevisiae* Vta1 (identities = 22%, positives = 40%), *C. albicans* Vta1 (identities = 43%, positives = 57%), *C. elegans* Vta1 (identities = 37%, positives = 53%), *H. sapiens* Vta1
identities = 48%, positives = 63%), M. musculus Vta1 (identities = 47%, positives = 62%), D. melanogaster Vta1 (identities = 57%, positives = 70%). BmVta1 is more similar to Vta1 proteins from human, mouse and fruit fly than those from fungi and plants, and is of highest sequence-similarity to fruit fly Vta1, consistent with their relationship in evolution. Moreover, BmVta1 also contains the N terminal microtubule Interacting and trafficking (MIT) domains, the middle linker region, the C terminal VSL (Vta1/SBP1/LIP5) domain, all of which are well conserved and are also indicated by the homology-modeling (Fig. 2B). Using the cloned BmVta1 sequence to BLAST the silkworm genome database (http://silkworm.genomics.org.cn/), we could not find other genes of significant similarity to BmVta1, indicating it is the only homologue in silkworm for Vta1. The cloned cDNA sequence of BmVta1 was submitted to Genbank (EU583493).

Protein expression and preparation of polyclonal antibodies

The recombinant BmVta1 was expressed in E. coli with N terminal His and S fusion tags and a predicted molecular weight of ~40 kDa. As shown in Fig. 3A (Lane 6), the appearance of a protein band with a size of ~40 kDa indicated the successful expression of BmVta1, which was confirmed by Western blots using an anti-His monoclonal antibody (B, Lane 6) and home-made mouse polyclonal antiserum (C, Lane 3) and verified by Mass Spectrometry (data not shown). The successful detection of N terminal fusion tags expressed alone also proved the effectiveness of anti-His monoclonal antibody (B, Lane 4).

As shown in Fig. 3C, the effectiveness and specificity of home-made antiserum were examined using Western blot. The expressed BmVta1 in E. coli could only be detected when the culture was induced by IPTG (C, Lane 3). Besides the major protein band, a minor band down below could also be observed. This may be caused by partial degradation of recombinant BmVta1 as no proteins from E. coli could be detected when BmVta1 was not expressed (C, Lane 1, 2).
Notably, the endogenous protein of BmVta1 in silkworm larva and pupa could also be detected and appeared only as a single band (C, Lane 4, 5), indicating the usefulness of this antiserum in profiling the expression of BmVta1.

Transcription profiles of BmVta1 and BmVps4
As shown in Fig. 4A, the transcription of BmVta1 was at the lowest level in the third instar of silkworm larva, increased continuously in the fourth and fifth instar, peaked in pupa, moth and oosperm, and decreased in the first and second instar, suggesting a close relationship with the silkworm development. The transcription of BmVps4 was similarly regulated but in a more drastic manner as it was barely detectable in the third instar and sharply increased in pupa, moth and oosperm.

As shown in Fig. 4B, the transcription of BmVta1 in silkworm tissues and organs was also analyzed. The samples of fat body, Malpighian tube, midgut, ovary and hemolymph were firstly analyzed (left panel), then more samples of head, silk gland, ganglion and testis were also tested (right panel). For better comparison, the fat body sample was included in both experimental batches. The transcriptions of both BmVta1 and BmVps4 were of higher levels in head, silk gland and testis than in Malpighian tube, fat body and ganglion. However, the transcription of BmVta1 was of much higher levels in midgut, ovary and hemolymph, while that of BmVps4 is much lower.

Together, these results indicate a globally similar transcription profiles for BmVta1 and BmVps4 during silkworm development and in some tissues and organs, and also suggest delicate but distinctive regulations and possibly different roles for them in certain developmental stages or tissues and organs.

Subcellular localizations of BmVta1 and BmVps4
To investigate the subcellular location of BmVta1, the plasmids pBEGFP, pBEGFP-BmVta1 and pB-BmVps4-EGFP were separately transfected into BmN cells. The GFP signal was observed under a fluorescence microscope, and the DAPI (4',6-diamidino-2-phenylindole) dye was used to reveal the nucleus. As shown in Fig. 5, the fusion proteins EGFP-BmVta1 and BmVps4-EGFP were located mainly in cytosol, consistent with known properties of Vta1 and Vps4 proteins. As a control, the EGFP expressed alone was broadly distributed in cells. We also constructed another plasmid pB-BmVta1-EGFP, but no GFP signals could be observed after transfection. One of reasons may be that the EGFP fusion tag caused disruption of protein folding or stability as Vta1 exists as a dimer with C terminus forming the essential dimerization domain.
of MVB sorting pathway in yeast cells (Shiflett et al. 2004). and ESCRTs, deletion or knockout of Vta1 causes only mild defects may be that unlike the severe defects caused by dysfunction of Vps4 and ESCRTs, deletion or knockout of Vta1 causes only mild defects of MVB sorting pathway in yeast cells (Shiflett et al. 2004).

However, as introduced earlier, the physiological and pathological significance of Vta1/LIP5 is greatly appreciated in mammalian and plant cells, especially in host defense responses against both biotic and abiotic stresses. Moreover, the additional layers of interactions and regulations of LIP5 also indicate the existence of multiple roles for Vta1/LIP5 in higher species than in yeast. In present study, we found that BmVta1 is of higher similarity to mammalian LIP5 than those of plant or fungus, indicating it may have similar functions like mammalian LIP5. Both BmVps4 and BmVta1 are of higher levels of transcription in head, silk gland and testis than in Malpighian tube, fat body and ganglion, suggesting similar roles for them in these tissues and organs. Notably, the transcription of BmVta1 is of much higher levels in midgut, ovary and hemolymph than that of BmVps4, indicating specific roles for BmVta1. The midgut is a major digestive organ for insects, and also the initial place in silkworms for the infection of baculovirus Bombyx mori nuclear polyhedrosis virus (BmNPV) (Han et al. 2014, Hao et al. 2015, Wang et al. 2016). It is thus tempting to speculate a role for BmVta1 in silkworm defense responses as well as in the infection and replication of BmNPV. This is also supported by a recent finding of a requirement of Vps4 for the entry and egress of another baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Li and Blissard 2012). To our knowledge, this is the first time to observe the significantly higher levels of transcription of BmVta1 in ovary than that of BmVps4, while both are at high levels in testis. Although the reasons are unknown, it may suggest a specific requirement to up-regulate BmVta1 or down-regulate BmVps4 in the development or functions of ovary in female moth. Unlike vertebrate blood system, insect hemolymph functions mainly to transport nutrients or metabolites rather than oxygen, and acts as a major immune system for insects. It is thus possible that the higher transcription of BmVta1 in silkworm hemolymph may indicate a role of it in silkworm immune responses. In all, the successful identification and characterization of BmVta1 provides a good start to further investigate its roles in these interesting processes.

Discussion

In this study, we successfully identified and cloned a silkworm homologue for Vta1. Like BmVps4, BmVta1 is also a well conserved protein, and both are localized mainly in cytoplasm, consistent with reported properties of Vps4 and Vta1 proteins. We also found that the transcriptions of BmVta1 and BmVps4 are up-regulated during silkworm metamorphosis and embryogenesis, indicating their important roles in silkworm development. It is known that silkworm embryogenesis and metamorphosis involve active cell division, growth, tissue and organ patterning, and the latter also requires autophagy and apoptosis for degeneration of larva tissues and organs. As these molecular processes in yeast and mammalian cells require the functions of ESCRTs, Vps4 and Vta1 (Michelet et al. 2010, Rusten et al. 2012), it is conceivable that their silkworm homologues are also required for corresponding processes essential for silkworm development.

Most of current studies are performed at the molecular and cellular levels, it is thus necessary to carry out studies at the level of organism to more accurately evaluate the roles of these proteins. As presented in this study, it is clear that the transcriptions of BmVta1 and BmVps4 are tightly and dynamically regulated during silkworm growth and development. They are only up-regulated when being in a great need for the smooth proceeding of metamorphosis and embryogenesis, and become down-regulated when being in the larva stages for growth, indicating they may play more important roles in silkworm development rather than growth. Although recent studies have begun to reveal the involvement of ESCRTs in the development of C. elegans, D. melanogaster, M. musculus and A. thalianahas (Michelet et al. 2010), the roles of Vps4 and Vta1/LIP5 have not been experimentally explored. As far as we know, this is the first time to implicate Vps4 and Vta1/LIP5 in the development of silkworm, a research model for Lepidoptera insects and an economic insect in many countries.

Currently, the physiological roles of Vta1 are much less investigated compared to those of ESCRTs and Vps4. One of the reasons may be that unlike the severe defects caused by dysfunction of Vps4 and ESCRTs, deletion or knockout of Vta1 causes only mild defects of MVB sorting pathway in yeast cells (Shiflett et al. 2004).

It is thus tempting to speculate a role for BmVta1 in silkworm defense responses as well as in the infection and replication of BmNPV. This is also supported by a recent finding of a requirement of Vps4 for the entry and egress of another baculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Li and Blissard 2012). To our knowledge, this is the first time to observe the significantly higher levels of transcription of BmVta1 in ovary than that of BmVps4, while both are at high levels in testis. Although the reasons are unknown, it may suggest a specific requirement to up-regulate BmVta1 or down-regulate BmVps4 in the development or functions of ovary in female moth. Unlike vertebrate blood system, insect hemolymph functions mainly to transport nutrients or metabolites rather than oxygen, and acts as a major immune system for insects. It is thus possible that the higher transcription of BmVta1 in silkworm hemolymph may indicate a role of it in silkworm immune responses. In all, the successful identification and characterization of BmVta1 provides a good start to further investigate its roles in these interesting processes.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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Disclosure

The authors declare no conflict of interest.

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