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Programmable targeted epigenetic editing using CRISPR system in *Bombyx mori*

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ABSTRACT

DNA methylation has been proven to play roles in regulating gene expression, cell fate, disease determination, and chromatin architecture organization in mammals and plants, and is a significant component of epigenetic modification. Compared to mammals or plants, the status and function of DNA methylation are poorly understood in insects, which is partially due to the lack of efficient manipulation tools. In this study, we show that fusion protein of catalytically inactive Cas9 (dCas9) with TET1 can efficiently demethylate genomic DNA of silkworm *Bombyx mori*, in a programmable target region specific manner. We first developed an all-in-one vector to maximize the targeting efficiency of dCas9-TET1. Then we selected 3 endogenous genes that were previously found to harbor methylated DNA, and designed gRNAs within the methylated region. Co-transfection of dCas9-TET1 and gRNA successfully erased methylation marks near the targeting region, with efficiencies from about 17.50% to 40.00%. Furthermore, targeted demethylation on gene body resulted in increased mRNA transcription level. Unlike the previously widely used decitabine, a methylation inhibitor, dCas9-TET1 is more effective and specific, and has no unwanted impact on whole-genome methylation. DCas9-TET1 provides a powerful tool for investigating the functional significance of DNA methylation in a locus-specific manner, and for exploring the unknown links between methylation and development in insects.

1. Introduction

DNA methylation, which usually refers to the addition of a methyl group to the 5th carbon of cytosine deoxyribotide (5 mC) in CpG dinucleotide, is a significant component of epigenetic regulation of gene expression networks (Holliday and Pugh, 1975). Recently, with the rapid development of high-throughput sequencing, increasing numbers of methylomes have been depicted, including those of humans (Lister et al., 2009), mice (Guo et al., 2013) and Arabidopsis (Lister et al., 2008). In recent decades, numerous studies have highlighted the function of DNA methylation in regulation of gene expression, cell fate, disease determination and chromatin architecture organization in both animals (Li et al., 1992; Reik, 2007) and plants (Kawashima and Berger, 2014; Kim and Zilberman, 2014). In humans, DNA methylation has been frequently reported to be associated with diseases, including cancer (Argentieri et al., 2017; Laird and Jaenisch, 1996). Although DNA methylation plays a similar role in gene silencing, the level across species, patterns of methylation, as well as its role in regulating enzymes are evolutionarily divergent among different organisms (Law and Jacobsen, 2010). We previously reported the single base-resolution methylome of *B. mori* which is one of world's most important economic insects and a powerful model system for Lepidoptera insects researches, and found that only 0.11% of genomic cytosines are 5mCs (Xiang et al., 2010). Hui et al. also revealed a trend of increasing DNA methylation during domestication, through comprehensive comparison of silk gland methylomes of domesticated and wild silkworms (Hui et al., 2013). However, the function of DNA methylation at specific loci remains unclear.

To elucidate the function of methylation, some inhibitors acting on DNA methyltransferases have been synthesized in the last few decades. 5-Aza-2′-deoxycytidine (5-aza-dC, decitabine), a nucleoside analog, is widely used to inhibit DNA methylation by inducing proteasomal degradation of free DNMT1(Palii et al., 2008). However, these inhibitors affect the whole genome, not specific, targeted sites. Owing to the lack of suitable and convenient molecular tools to regulate DNA methylation in specific genes or loci, determining the function of differentially

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methylated regions remains challenging. CRISPR/Cas9, a simple and rapid genome-editing tool, has taken the fields of biological and medical science by storm (Doudna and Charpentier, 2014; Jiang et al., 2013; Sander and Joung, 2014). Cas9 has found application not only for gene knock-out or knock-in by its nuclease activity, but has also been remodeled as dCas9 with 2 mutations (D10A, H840A) to allow for modulation of gene expression and the rewriting of epigenetic marks on chromosomes through its capacity for DNA recombination (Dominguez et al., 2016; Konermann et al., 2015). Recently, it has been demonstrated that DNMT3A and TET1 fused with dCas9 can deepen and erase methylation imprinting in existing gRNAs in human cells (Liu et al., 2016; Vojta et al., 2016).

Our previous studies have demonstrated that the CRISPR system enables efficient genomic editing in *B. mori* (Liu et al., 2014; Ma et al., 2017). Here, we aimed to explore the utility of the CRISPR/dCas9-TET1 system in *B. mori*. We first developed an all-in-one vector to maximize the targeting efficiency of dCas9-TET1, which is an obvious improvement over the traditional binary vector system. Next, we selected 3 endogenous genes that were previously found to harbor methylated DNA, and designed 2 gRNAs per gene within the methylated region. Co-transfection of dCas9-TET1 and gRNA successfully erased methylation imprinting near the gRNA targeting region, with efficiencies in the range of 17.50%–40.00%. We also found that the editing efficiency does not appear to be correlated with the endogenous methylation status and gRNAs position. Furthermore, targeted demethylation on gene body resulted in increased mRNA transcription. DCas9-TET1 provides a powerful tool for investigating the functional significance of DNA methylation in a locus-specific manner, and for uncovering the unknown links between DNA methylation status and specific phenotypes in *B. mori*, and potentially other insects.

2. Material and methods

2.1. Plasmid construction

Silkworm codon-optimized Cas9 was commercially synthesized by company according to silkworm codon preference and kept in our laboratory (Liu et al., 2014). And it was subjected to D10A/H840A substitution by point mutation to construct dCas9, driven by the IE2 promoter, as a basic vector (Dominguez et al., 2016). The IE2 promoter originated from Orgyia pseudotsugata single capsid nuclopolyhedrovirus (Lee et al., 2008). *TET1* core domain sequences were commercially synthesized (Tsingke Co, Ltd., China) with silkworm codon-optimization and inserted into the C-terminal of dCas9 via digestion with *Sph*I and *Hind*III. The expression cassettes of IE1-DsRed and U6-gRNA were cloned using vectors kept in our laboratory and digested with *Xho*I and *Not*I(Liu et al., 2014). All gRNA sequences were commercially synthesized (Tsingke Co, Ltd., China) as oligomers and inserted into *Aar*I sites of vectors. All synthesized sequences and fusion protein used herein are enlisted in supplementary information. All plasmids are available upon request.

2.2. Silkworms, cell culture, transfection and microinjection

Dazao, a widely used strain in *B. mori* experiments, was used in this study. BmE, a *B. mori* embryonic cell line, was maintained in our laboratory and cultured in Grace medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 27 °C.

Before transfection, BmE cells were seeded in 12-well plates. On approaching 70–90% confluence, approximately 2 μg of plasmids were transfected into BmE cells, using X-treme GENE HP DNA Transfection Reagent (Roche, CH). Approximately 72–168 h after transfection, cells were harvested for subsequent detection. Decitabine (Sigma, USA) was dissolved in DMSO at a final concentration of 5 μM.

Embryos microinjection was as described by our previous study (Ma et al., 2017). Firstly, Fresh non-diapause embryos were gathered immediately after oviposition. And then, microinjection was performed within 2 h using a micromanipulator (TransferMan NK2, Eppendorf) and microinjector (Femto Jet 5247, Eppendorf) under microscope (SZX16, Olympus). Approximate 100 embryos were injected in each group. The treated embryos were incubated for 96 h at 25 °C followed by fluorescence screening and DNA extraction.

2.3. Genomic DNA and protein extraction

Transfected cells were harvested at 72–168 h after treatment. Genomic DNA was isolated using E.Z.N.A.® Tissue DNA Kit (Omega, USA) in accordance with the manufacturer's instructions. Total protein was extracted using NP40 lysis buffer containing protease inhibitor phenylmethanesulfonyl fluoride (Beyotime, China). Protein expression was analyzed via western blotting using Flag-tag antibodies (Beyotime, China).

2.4. DNA dot-blot analysis

One microgram of genomic DNA was denatured at 95 °C for 5 min and spotted onto a positively charged nylon membrane (Roche, CH). The spots were air-dried for 5 min and UV-crosslinked (4 min, 1200 J/cm^2). The membrane was blocked with 5% dry milk powder in PBST for 2 h at 25 °C. Thereafter, the membrane was incubated with anti-5-methylcytosine antibody (Abcam, UK) for 2 h at 25 °C or overnight in 4 °C. The membrane was washed thrice with TBST, every 5 min. The membrane was then probed with the secondary antibody for 30 min, followed by another TBST wash.

2.5. Bisulfite sequencing

Approximately 1–2 μg genomic DNA was treated with bisulfite, using the EZ DNA Methylation-Gold Kit (Zymo Research, CA) in accordance with the manufacturer's instructions and amplified using ZymoTaq DNA Polymerase (Zymo Research, CA) with primers designed using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Thereafter, PCR products were cloned into the pEASY-T5 Zero vector (TransGen Biotech, China), followed by Sanger sequencing.

2.6. Quantitative real-time PCR

Total RNA was isolated using the total RNA kit II (Omega, USA) in accordance with the manufacturer's instructions at 72–168 h after treatment. One microgram of RNA was used to synthesize complementary DNA with PrimerScript RT Reagent kit with gDNA Eraser (Takara, Japan). Thereafter, quantitative real-time PCR assay was performed using SYBR® Premix Ex Taq™ II (Takara, Japan) to assess target mRNA abundance relative to that of *Sw22934*, translation initiation factor 4A (internal control). All qPCR analyses were performed as 3 independent experiments.

3. Results

3.1. Construction of an all-in-one vector for dCas9-TET1 fusion protein

To select an appropriate cell line, we measured the genomic DNA methylation levels during different developmental stages, using a methylation-specific dot-blot assay, using an antibody specifically binding with DNA oligonucleotides harboring 5 mC (Fig. S1a). The signal intensity was high in the *B. mori* embryo, and decreased during subsequent development. DNA methylation in the posterior silk gland in $IV4^{th}D$, $V3^{th}D$ or $V7^{th}D$ was significantly lower than that in the whole embryo. Therefore, BmE, a *B. mori* embryonic cell line, was used in the following experiment. We constructed a dCas9-TET1 fusion protein via inclusion of the core catalytic domain of TET1 to the C-terminal of

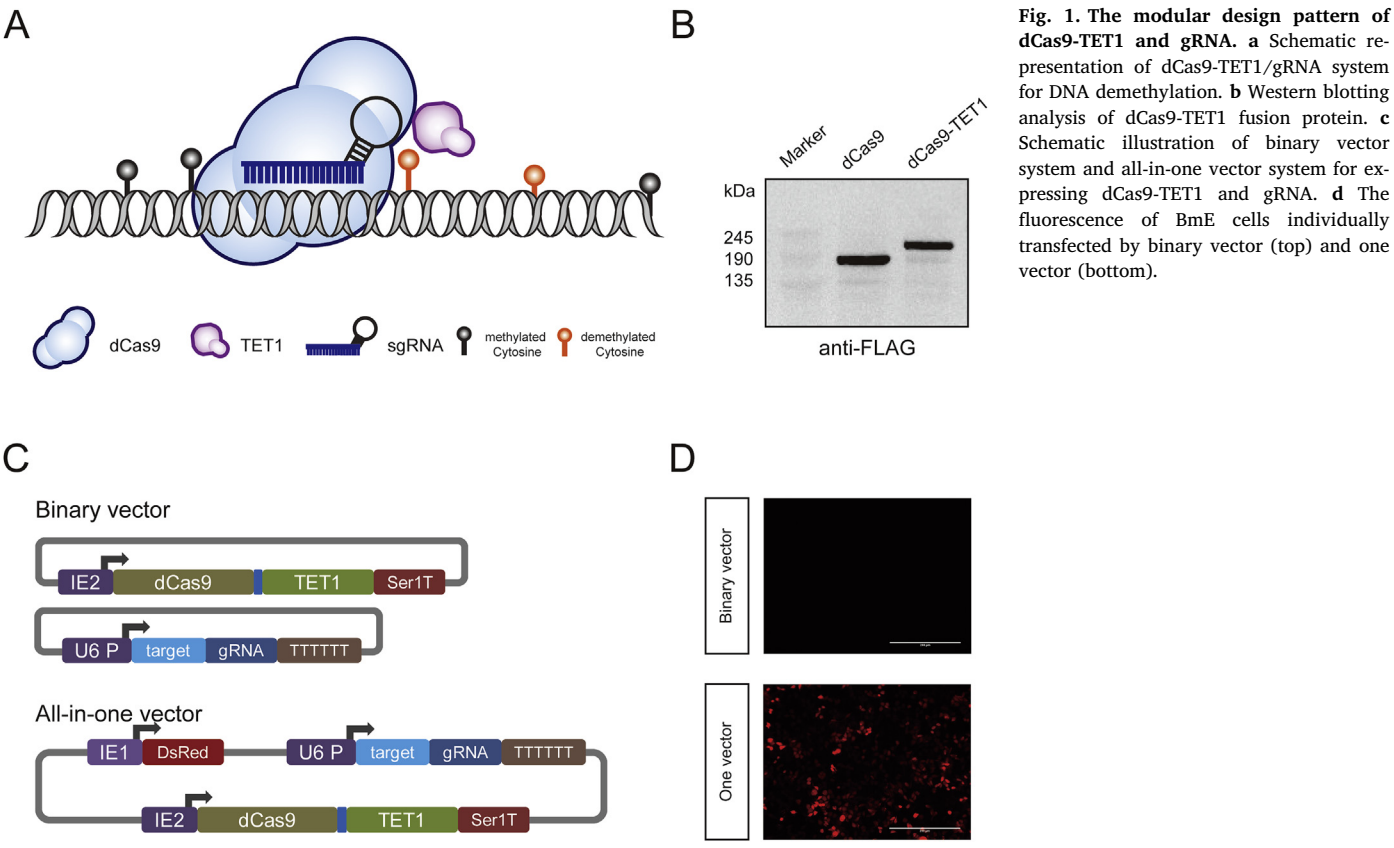

Fig. 1. The modular design pattern of dCas9-TET1 and gRNA. a Schematic representation of dCas9-TET1/gRNA system for DNA demethylation. **b** Western blotting analysis of dCas9-TET1 fusion protein. **c** Schematic illustration of binary vector system and all-in-one vector system for expressing dCas9-TET1 and gRNA. **d** The fluorescence of BmE cells individually transfected by binary vector (top) and one vector (bottom).

dCas9 with a polypeptide linker (Fig. 1a), under the control of the IE2 promoter (Fig. 1c), which has strong activity in BmE cells. The dCas9-TET1 expression vector was transfected into BmE cells. Western blotting analysis with Flag-tag antibody revealed successful expression of the fusion protein. The protein size was as expected, and its expression level was comparable to that of dCas9 (Fig. 1b).

Thereafter, we constructed 2 gRNA vectors targeting *BGIBMGA000958*, a gene with a hypermethylated first intron (Hui et al., 2013). The gRNAs were designed to be maximally proximal to the selected region of methylation (Fig. 2a). The vectors were co-transfected with dCas9-TET1 expression vector into BmE cells, and cells were harvested 72 h post transfection. Bisulfite sequencing analysis revealed that the methylation level of all 10 5 mC sites within the gRNA targeting region were lower than that in the untransfected cells, indicating that dCas9-TET1 directly mediated targeted demethylation in BmE cells (Fig. 2b and c), with an albeit much lower efficiency than that with mammalian cells (Liu et al., 2016; Vojta et al., 2016). We speculated that the reduction in transfection efficiency may have resulted from our use of a binary vector that delivers dCas9-TET1 and gRNA separately. Thus, we constructed an all-in-one vector, wherein dCas9-TET1 and gRNA expression cassettes were combined in a single plasmid (Fig. 1c). To eliminate the influence of transfection efficiency, we also included a fluorescent reporter cassette containing DsRed under the control of the IE1 promoter, in the vector (Fig. 1c and d).

The same gRNAs were incorporated in the all-in-one vector and transfected into BmE cells. Transfected cells were selected via fluorescence-activated cell sorting (FACS) analysis for subsequent experiments. Bisulfite sequencing was performed to detect methylation status. As shown in Fig 2b and 12 TA-clones of every group were sequenced, and samples from the all-in-one vector revealed an obvious reduction in methylation levels in comparison with the binary vector. In the all-in-one vector group, methylation levels decreased by approximately 17.50%, which was approximately 2.1-fold higher than that in the binary vector (Fig. 2c). Methylation was even completely obliterated on some loci, indicating the erasure of DNA methylation by dCas9-TET1 with gRNA in BmE cells.

3.2. DCas9-TET1 mediated efficient targeted demethylation at various genomic loci

We examined the methylation editing efficiency and effective range of the all-in-one system at various genomic loci. *BGIBMGA004109*, *BGIBMGA002379*, and *BGIBMGA001471* are having appropriately methylation fragments in introns or exons, the first containing 8 5mCs; middle, 5 5mCs; last, 9 5mCs (Table S1). Every gene was assigned 2 gRNAs proximal to the region of methylation. Positive cells were harvested via FACS 72 h after transfection. Bisulfite sequencing revealed that almost all detected methylation loci were apparently demethylated (Fig. 3a), and some loci were completely demethylated. Comparing all 4 genes, the editing efficiency varied considerably, from approximately 17.50%–40.00% (Fig. 3b). This phenomenon is consistent with our previous studies reporting that the CRISPR system has different editing efficiencies at different loci (Liu et al., 2014). We suspected that this is influenced by base composition of target sites or complex chromosome structure. Furthermore, in the present study, 5mC-1 in *BGIBMGA004109*, the farthest 5 mC site, approximate 191 bp upstream of the PAM sequence, is prominently demethylated. The editing range approached 200 bp, similar to the range in the mammalian genome (Liu et al., 2016; Vojta et al., 2016). However, confoundingly, the editing efficiency displayed no apparent correlation with endogenous methylation status or gRNA position in these detected genes (Fig. 3c).

To further verify whether the CRISPR-dCas9 system could function in *B. mori*, we processed embryonic transient assay by microinjecting the all-in-one vectors into early embryo. *BGIBMGA004109* and *BGIBMGA001471*, both with 9mCs and high methylation level, were choosed. Positive embryos were harvested via fluorescence microscope 96 h after microinjecting (Fig. S1b). Bisulfite sequencing revealed that the target sites were successfully demethylated. The demethylation

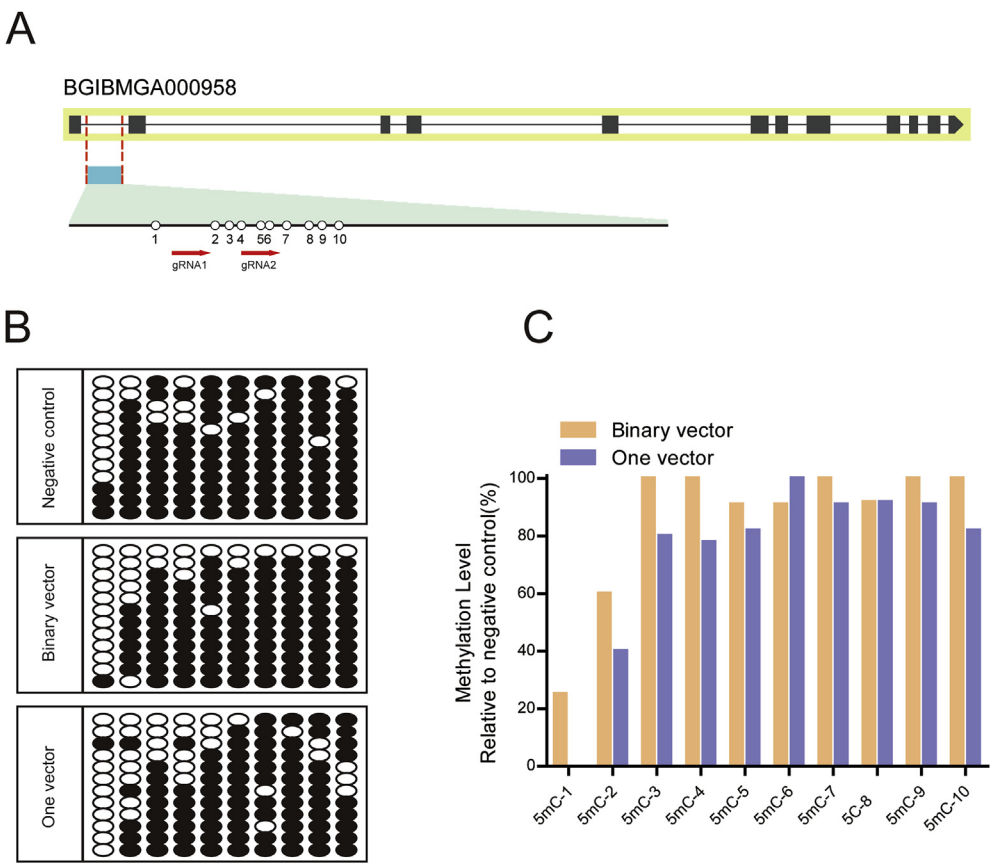

Fig. 2. DCas9-TET1 regulating DNA demethylation on *BGIBMGA000958*. **a** Schematic of *BGIBMGA000958* targeted by dCas9-TET1. The 5 mC locus was annotated on the first intron by circle. Red arrows mean gRNA1 and gRNA2. **b** Bisulfite sequencing of 3 samples. Solid circles mean methylated cytosine and hollow circles mean unmethylated cytosine. **c** The editing efficiency by binary vector and one vector on target region, compared to negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

efficiencies *BGIBMGA004109* and *BGIBMGA001471* were 18.63% and 11.69%, respectively (Fig. S1c). The editing efficiency was lower than BmE cells, we suspected that the promoter activity in embryo might be lower than BmE cells, resulting in not enough expression of dCas9-TET1 protein. This speculation was also supported by our previous observations that Cas9 editing efficiency was higher in cell than embryo (Ma et al., 2017). Taken together, we showed that dCas9-TET1 could induce target demethylation in *B.mori* cell line and vivo, providing an efficient editing tool to explore DNA methylation function in silkworm or even insects.

3.3. Targeted demethylation in the gene body caused gene upregulation

Numerous studies have focus on epigenetic phenomena, especially DNA methylation, because of their significant roles in cancer (Arai et al., 2010; Dawson and Kouzarides, 2012; Muraki et al., 2009). Tumor-suppressor genes are frequently aberrantly methylated during tumorigenesis and cancer progression. Promoter hypermethylation has been frequently reported in suppressing/silencing gene expression, whereas hypomethylation activates gene expression (Madrigano et al., 2012; Tao and Freudenheim, 2010). However, few studies have analyzed promoter or gene body methylation in insects, despite some methylomes having been reported, and theories or viewpoints regarding the function of insects methylation are scare (Jjingo et al., 2012; Teixeira and Colot, 2014). Hence, we determined the potential effects of dCas9-TET1 mediated demethylation at the gene body on transcriptional activity, via qPCR analysis, using dCas9 protein without a TET1 domain as the negative control. As illustrated in Fig. 4a, after treatment with dCas9-TET1 and gRNAs, mRNAs were upregulated relative to the negative control by approximately 1.2- to 1.7-fold. These results demonstrate that gene body demethylation promotes transcriptional activity in *B. mori*, consistent with traditional theories (Métivier et al., 2008; Weber et al., 2007). Furthermore, we hypothesized that reductions in methyl groups would alleviate the inhibition of ribosome binding and transcription on the template strand. Interestingly, the extent of demethylation was not apparently reflected in the degree of gene upregulation (Fig. 4b).

3.4. DCas9-TET1 is more effective and specific than decitabine

Some inhibitors acting on DNA methyltransferases have been synthesized in the last few decades to study the function of methylation and for cancer therapy. Decitabine, a remarkable inhibitor with a long treatment history and broad applications, has been used to treat myeloid leukemia (Yang et al., 2006). As a pyrimidine analog, it can covalently trap DNMT1 upon its incorporation into DNA in place of cytosine, thereby irreversibly inhibiting enzyme activity (Patel et al., 2010). We assessed decitabine-mediated demethylation effects in *B. mori*. Upon treating BmE cells with decitabine for 160 h, bisulfite sequencing and qPCR analyses were performed (Figs. S2 and S3). Decitabine prominently decreased methylation status by approximately 7.07%–34.17% and increased mRNA levels of the 4 targeted genes by approximately 1.2- to 2.3-fold. Levels of demethylation and mRNA upregulation were comparable between dCas9-TET1 and decitabine treatments. Nevertheless, because of its non-specificity with significant effects on whole-genome methylation levels, decitabine has unpredictable side effects. However, dCas9-TET1 fusion protein prevented unwanted influence on whole-genomic methylation. To compare the differences, we treated BmE cells with decitabine for 72, 120 and 168 h. Dot-blot analysis revealed that decitabine prominently influenced demethylating (Fig. 5a). However, treatment with dCas9-TET1 at the same intervals did not yield a discernible change in genome-wide methylation (Fig. 5a). Previous studies have reported that *B. mori* has 2 DNA methyltransferases, DNMT1 and DNMT2 (Mitsudome et al., 2015). The former was the target of and degraded by decitabine; the latter's homolog catalyzed tRNA methylation (Goll et al., 2006). After

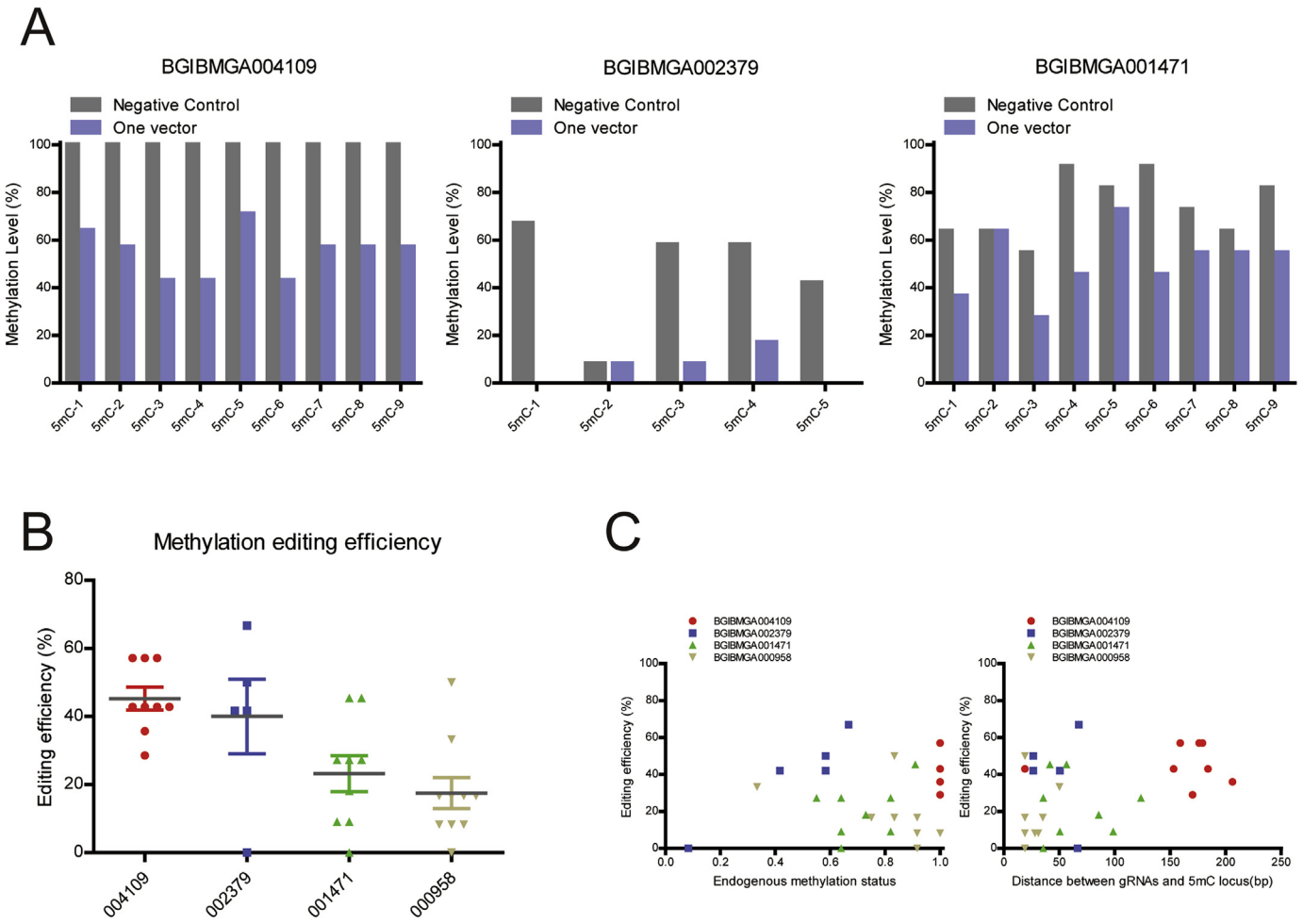

Fig. 3. Dcas9-TET1 regulating DNA methylation in different gene body. a The editing efficiency of *BGIBMGA004109*, *BGIBMGA002379* and *BGIBMGA*001471 by one vector. **b** The methylation editing efficiency of the 4 genes. **c** The correlation among endogenous methylation status, distance of gRNA and 5 mC locus and editing efficiency.

treatment with decitabine, DNMT1 and DNMT2 were gradually upregulated with time. The dCas9-TET1 treated sample did not exert a prominent effect on methyltransferase expression (Fig. 5b and c). The present results show that dCas9-TET1 is more effective and specific than decitabine, an excellent substitute for demethylation in insects.

4. Discussion

In this study, we assessed targeted demethylation editing using dCas9-TET1, the reconstructed form of CRISPR system in *B. mori*. DCas9 was fused with the catalytic domain of TET1 on the C-terminal to predictably erase methylation at target sequences. Furthermore, to maximize targeting efficiency, we fused dCas9-TET1 protein and the gRNA expression cassette on a single plasmid additionally containing a DsRed reporter used to select positively transfected cells. Compared to the traditional binary vector, the all-in-one vector system improved the demethylation efficiency by approximately 2.1-fold. Three other genes containing methylated regions were selected to verify the utility of CRISPR/dCas9-TET1 system in silkworm, and the demethylation ratio was approximately 17.50%–40.00%. The editing range was within approximately 200 bp, and the editing ratio and range were similar to mammalian dCas9-TET1. What's more, embryonic transient assay verified that dCas9-TET1 could function in vivo. As a traditional demethylation agent, decitabine displayed comparable demethylation effects in *B. mori*; however, it exerted unwarranted effects on DNA methyltransferases and whole-genome methylation. DCas9-TET1 proved to be an optimal substitute to obliterate DNA methylation in *B. mori*.

Moreover, irrespective of dCas9-TET1 or decitabine treatment, qPCR analysis of these target genes revealed that gene body demethylation (exon or intron) would increase mRNA transcription, concurrent with promoter demethylation in vertebrates and plants (Ehrlich and Lacey, 2013). However, a few studies on gene body methylation suggested that it may regulate alternative splicing in several animal genomes (Assaf et al., 2010; Flores, 2012). Furthermore, gene body methylation may help regulate gene family expansion and functional diversification of gene families, leading to phenotypic variation (Asselman et al., 2016). However, these conclusions were drawn from genome-wide sequencing and pattern analysis, not specific gene or locus. The present study is the first, to our knowledge, to highlight the direct regulation of transcription by gene body methylation status in insects. Certainly, gene body methylation may not be simply attributed to the regulation of transcription. Furthermore, no obvious association has been reported among demethylation ratios, endogenous methylation status, distance between gRNAs and 5 mC locus, and the levels of gene upregulation. We speculate that some undetected 5 mC regions exist around these target genes, probably with unknown function.

Since the silkworm methylome has been reported, insect DNA methylation is receiving increasing attention. Hui et al. investigated DNA methylation during domestication through comprehensive comparison of silk gland methylomes of domesticated and wild silkworms (Hui et al., 2013). Numerous studies have been conducted on the functions

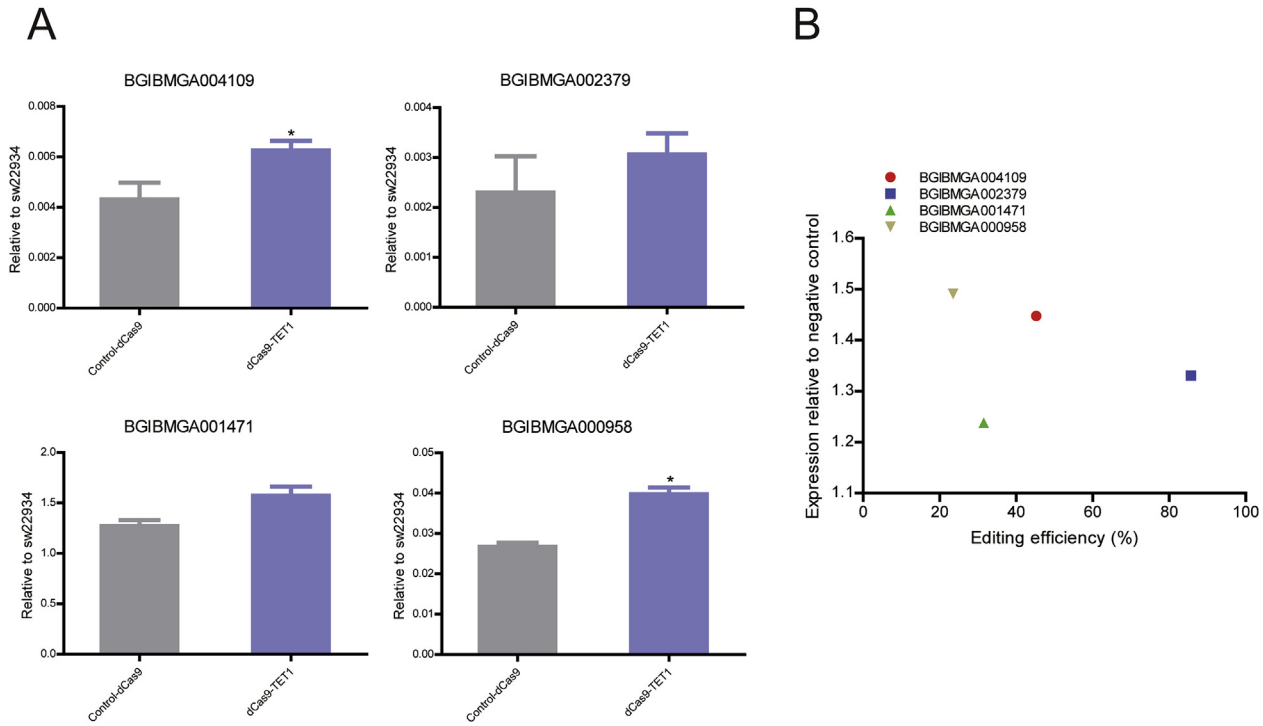

Fig. 4. The effect of up-regulating gene expression by dCas9-TET1. a QPCR shows mRNA transcription is activated by dCas9-TET1 treatment, dCas9 as negative control. *P* value of < 0.05 was considered statistically significant. **b** The correlation between editing efficiency and mRNA activity level.

of DNA methylation in *B. mori*, simultaneously during the compilation of the present data. Zhang et al. reported that Krüppel homolog 1(Kr-h1) can inhibit transcription in steroidogenic enzymes by directly binding its promoter and inducing DNA methylation; it was initially reported that DNA methylation contributed to transcriptional repression of ecdysone via juvenile hormone signaling (Zhang et al., 2018). Furthermore, Xu et al. reported that tissue- and stage-specific gene expression can be regulated through promoter methylation during insect development (Xu et al., 2018). These reports imply that epigenetic factors significantly contribute to transcription, alternative splicing, metamorphosis, and domestication. The dCas9-TET1 constructed herein can be used to further elucidate the role of DNA methylation in specific genes or loci as a powerful tool, in *B. mori*, Lepidopterans, and other insects. Future studies are required to further elucidate the functions of DNA methylation in insects.

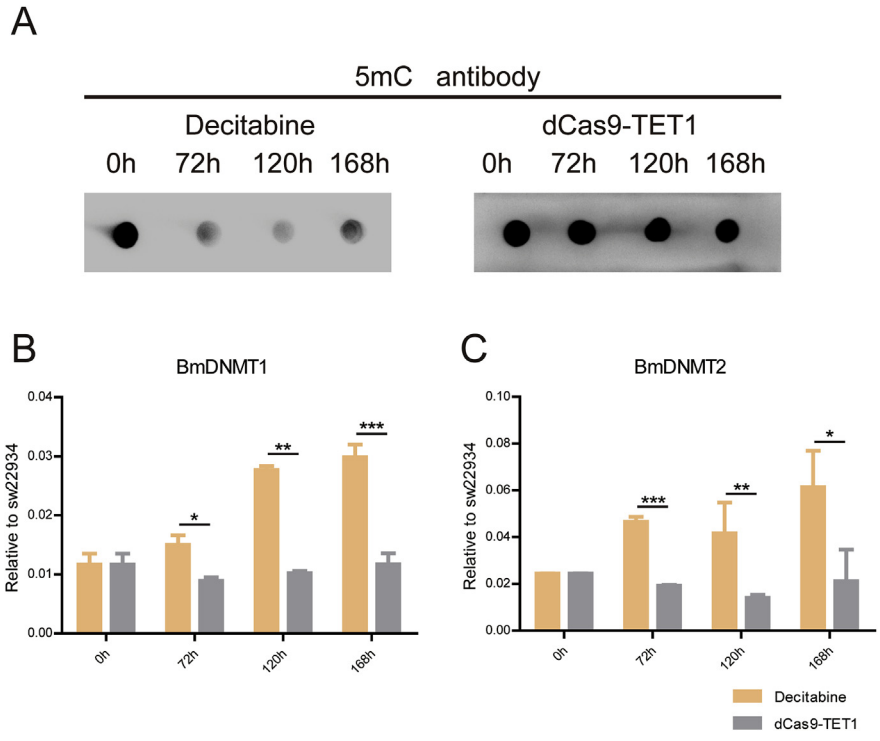

Fig. 5. The effect of decitabine and dCas9-TET1 on whole genome methylation and DNA methyltransferases. a DNA dot blotting of 1 μg genomic DNA treated by decitabine and dcas9-TET1 for about 0 h, 72 h, 120 h and 168 h **b** QPCR of *BmDNMT1* treated with decitabine or dCas9-TET1 fusion for about 0 h, 72 h, 120 h and 168 h **c** QPCR of *BmDNMT2* treated with decitabine or dCas9-TET1 fusion for about 0 h, 72 h, 120 h and 168 h.

Declaration of interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.04.013.

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