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# Identification of a midgut-specific promoter in the silkworm *Bombyx mori*

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ABSTRACT

The midgut is an important organ for digestion and absorption of nutrients and immune defense in the silkworm *Bombyx mori*. In an attempt to create a tool for midgut research, we cloned the 1080 bp P2 promoter sequence (P2P) of a highly expressed midgut-specific gene in the silkworm. The transgenic line (P2) was generated via embryo microinjection, in which the expression of EGFP was driven by P2P. There was strong green fluorescence only in the midgut of P2. RT-PCR and Western blot showed that P2P was a midgut-specific promoter with activity throughout the larval stage. A transgenic truncation experiment suggested that regions −305 to −214 and +107 to +181 were very important for P2P activity. The results of this study revealed that we have identified a midgut-specific promoter with a high level of activity in the silkworm that will aid future research and application of silkworm genes.



## 1. Introduction

The silkworm *Bombyx mori* is an economically important lepidopteran model for silk production. Following completion of the draft sequence [1,2], detailed sequencing [3] and resequencing [4] of the silkworm genome, research in the post-genomic era includes discovery, research and application of *B. mori* genes. There are some genes specific to each tissue in the silkworm [5] and we need to create tissue-specific promoters in order to study these genes in detail.

The midgut is an important organ for digestion and absorption in the silkworm. Most silkworm genes specific to the midgut encode hydrolases, binding proteins, transporters and transferases [5], which participate primarily in digestion of the proteins and carbohydrates present in mulberry leaves, which are the natural food source for the silkworm, as well as assimilation of the small molecular products of digestion for growth, development and silk production. The life cycle of the silkworm consists of four developmental stages: egg, larva, pupa and adult moth. Only the larva is involved in food intake; so, most midgut-specific genes are highly expressed in the larval stage.

The midgut is an immune organ that acts in the silkworm as the first line of defense against pathogens. Many pathogens, including *B. mori* nucleopolyhedrovirus (BmNPV), *B. mori* cytoplasmic polyhedrosis virus (BmCPV) and *B. mori* densovirus (BmDNV), invade silkworm larvae mainly via oral infection, and the midgut is the first tissue to be infected. It has been reported that some proteins, including Bmlipase-1 [6], BmSP-2 [7], and BmNOX [8], are expressed specifically in the midgut and have strong antiviral activity against BmNPV in the gut juice. Earlier, we reported that overexpression of *Bmlipase-1* using a systemic promoter can enhance the antiviral capacity of the transgenic silkworm [9], which would be improved by the use of a midgut-specific promoter.

In an attempt to create a tool for the functional research and application of silkworm midgut-specific genes, we analyzed microarray data and selected BGIBMGA014298 [10], a highly expressed midgut-specific gene [5], as a target and the upstream ATG sequence of the P2 promoter (P2P) was cloned. The transgenic silkworm (P2) was constructed, in which EGFP was driven by P2P. The fluorescence of P2 was observed and the expression pattern of EGFP in P2 was analyzed, suggesting P2P is a silkworm midgut-specific promoter with a high level of activity.

## 2. Materials and methods

### 2.1. Analysis of midgut-specific genes

Silkworm midgut-specific genes were analyzed using microarray data [5]. We selected highly expressed BGIBMGA014298 as a target after bioinformatics analysis. BGIBMGA014298 expression was detected in different larvae developmental stages and different tissues of day 3 fifth instar larvae.

### 2.2. Cloning the promoter sequence and construction of the transgenic silkworm

The translation start site (ATG) of BGIBMGA014298 was located in the 1081 bp of scaffold 1148 in our Silkworm Genome Database (SilkDB) [10]. The 1080 bp upstream sequence with a typical

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promoter pattern was cloned (P2P) using the *Dazao* genome. P2P, Enhanced Green Fluorescent Protein (EGFP), and the SV40 termination signal were added to *piggyBac* [3× p3 DsRed af], which is a basic transgenic vector with the 3× P3-DsRed-sv40 gene as a report marker, to generate the transgenic vector *piggyBac* [P2-EGFP-SV40-3× p3 DsRed af] (pb-P2) (Fig. 2A). Microinjection with non-diapause *Dazao* embryos and screening transgenic silkworms were done as described [9,11,12] and we obtained a total of 5 transgenic lines (Supplementary Table 1).

### 2.3. Analysis of the expression pattern of P2P

We selected two transgenic lines at random for fluorescence observation, which revealed green fluorescence only in the midgut of transgenic lines. One transgenic line (P2) was selected at random for molecular detection. RNA and protein were extracted from samples of the hemocyte, fat body, silk gland, anterior midgut and posterior midgut of P2 dissected from day 3 fifth instar larvae. RNA was extracted from samples of P2 2, 4, 6 and 8 days old eggs, hatched silkworm, first instar molt, second instar, second instar molt, third instar, third instar molt, fourth instar, fourth instar molt, fifth instar larvae, wandering silkworm, 4 days old pupa and adult moth. These RNA samples were treated with RNase-free DNase I and reverse-transcribed into cDNA [9,11,12], which was used for analysis of BGIBMGA014298, EGFP, and internal control sw222934 [9,11,12] by RT-PCR. The primers are given in Supplementary Table 2. The protein samples were detected with EGFP and reference GAPDH antibodies.

### 2.4. Identification of the critical region of P2P activity

To identify the critical region affecting P2P activity, we cloned five truncated sequences of P2P; P2-T1–T5 promoters. The five truncated promoters were added to the transgenic basic vector *piggyBac* [3× p3 EGFP afm] [9,11,12] to drive EGFP in order to generate transgenic vectors pb-P2-T1, -T2, -T3, -T4 and -T5 (Supplementary Fig. S1A). We generated one transgenic line of each vector using *Dazao* embryos (Supplementary Table 1); P2-T1–T5, respectively. RNA was extracted from samples of the anterior midgut, posterior midgut, silk gland, hemocyte and fat body of P2 (P2-T1, -T2, -T3, -T4 and -T5, respectively) for analysis of EGFP by qPCR at day 3 of the fifth instar; sw222934 was used as the control [9,11,12].

## 3. Results and discussion

Specific promoters are important research tools. Midgut-specific promoters have been reported in the mosquito [13–15]. To create a midgut-specific promoter of *B. mori*, BGIBMGA014298 with a high level of expression only in the silkworm midgut was selected after analysis of microarray data [5]. The RT-PCR results revealed that this gene was expressed only in the midgut of male and female silkworms and throughout the larval stage (data not shown). Bioinformatics analysis showed there was typical promoter pattern in the 1080 bp sequence upstream of the ATG of BGIBMGA014298, which was cloned and named the P2 promoter (P2P). The TATA box, transcript start site (TSS) and translation start site (ATG) was located at –30, +1 and +182, respectively (Fig. 1). The transgenic vector pb-P2 was constructed in which the expression of EGFP was controlled by P2P (Fig. 2A) and then the transgenic silkworm (P2) was generated by microinjection [9,11,12] via a single insertion in an intergenic region (Supplementary 3).

The normal *Dazao* has no green fluorescence, because there is no EGFP in its genome [1,2,10]; but there was blazing green fluorescence in the midgut of P2 (Fig. 3). The fluorescence was so


**Fig. 1.** Analysis of the P2 promoter sequence. The black boxes represent the TATA box, the transcript start site (TSS) and the translation start site (ATG), respectively. Numbers indicate the position of each boldface nucleotide and TSS was set as +1. The underlined sequences are the predicted transcription factor-binding sites (continuous line) and 5′ UTR (broken line).

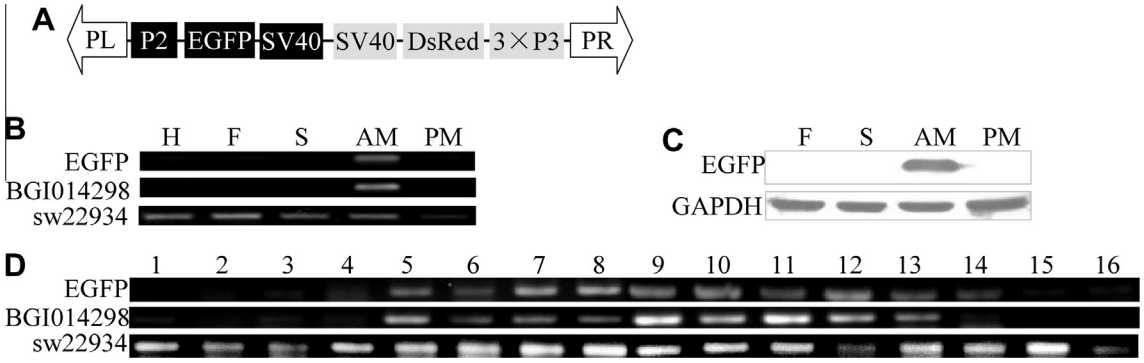


**Fig. 2.** Analysis of P2 promoter activity in the transgenic silkworm. (A) A schema of the pb-P2 vector. *piggyBac* [3× p3 DsRed af] is a basic transgenic vector with the 3× P3-DsRed-sv40 gene as a report marker. PL and PR, left and right terminal inverted repeats, respectively. P2, P2 promoter; EGFP, Enhanced Green Fluorescent Protein; SV40, polyadenylation signal. (B) RT-PCR results for different tissues of transgenic silkworm P2. RNA was extracted from samples of the hemocyte (H), fat body (F), silk gland (S), anterior midgut (AM) and posterior midgut (PM) of P2 and then reverse-transcribed to cDNA. The cDNA templates were used for RT-PCR analysis of EGFP, BGIBMGA014298 and internal control sw222934. (C) Western blot results of different P2 tissues. These protein samples were detected with antibodies of EGFP and reference GAPDH, respectively. (D) RT-PCR results of different developmental stages of P2. Points 1–16 represent P2 2, 4, 6 and 8 day-old eggs, hatched silkworm, first instar molt, second instar, second instar molt, third instar, third instar molt, fourth instar, fourth instar molt, fifth instar larva, wandering silkworm, 4 day-old, pupa and adult moth, respectively.

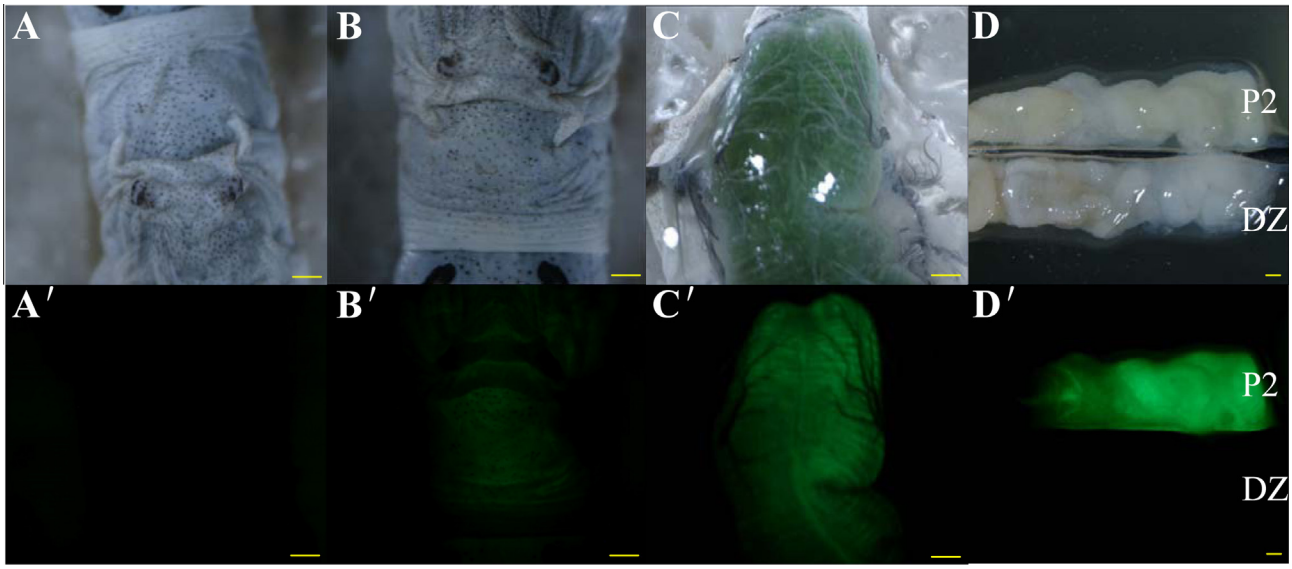


**Fig. 3.** Observation of fluorescence. (A–D) Images taken under white light; A′–D′ images taken under green fluorescence. P2 and DZ typify the transgenic and normal silkworm (*Dazao*), respectively. (A) Image of DZ; (B and C) images of P2 before and after dissection, respectively. (D) Image of P2 and DZ midguts. The yellow space bars represent 1 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

strong it could be seen through the hemocyte and integument in P2 under a fluorescence microscope, indicating P2P had a high level of activity. The results of RT-PCR and Western blot showed that EGFP was expressed only in the anterior midgut of P2 (Fig. 2B and C), suggesting P2P is a midgut-specific promoter. RT-PCR showed that P2P had activity throughout the larval stage (Fig. 2D). The expression pattern of EGFP was the same as that of BGIBMGA014298 in P2 (Fig. 2B and D). These results confirmed we had cloned a silkworm midgut-specific promoter with a high level of activity (GenBank accession number KC573068).

The critical region of P2P was analyzed by a truncation experiment. The transcription factor binding sites (TFBSs) were predicted using TFSEARCH, which showed there might be some conserved TFBSs in P2P, including GATA-binding factor 1 (GATA-1) [16], octamer factor 1 (Oct-1) [17], homeodomain factor Nkx-2.5/Csx (Nkx-2.5) [18], CdxA [19], Broad-Complex Z4 (BR-C) [20], CCAAT/enhancer-binding protein (C/EBP) [21], cAMP-responsive element-binding protein (CREB) [22] and POU-factor Tst-1/Oct-6 (Tst-1) [23] (Fig. 1). Using bioinformatics analysis, we found the coding sequence (CDS) of BGIBMGA003326 was very similar to that of BGIBMGA014298 in SilkDB [10]; so, we compared the promoter of BGIBMGA003326 (2000 bp sequence upstream of ATG) with that of P2P and found there was a similar sequence between the promoters at region −305 to +181 of P2P. We presumed that this region would be important for P2P activity and set it as the first truncated promoter P2-T1 (Fig. 4). By average truncation, the P2-T2 and P2-T3 promoters were located at regions −213 to +181 and −121 to +181, respectively, P2P was truncated at+107 to +181 and +32 to +106 to generate the P2-T4 and P2-T5 promoters, respectively (Fig. 4). Luciferase assays showed that P2P and five truncated promoters did not have activity in the BmN, BmE, Sf21 or Spli-221 cell lines (data not shown). We had to construct five transgenic vectors in which EGFP were driven by truncated promoters (Supplementary Fig. S1A). Five transgenic lines (P2-T1–T5) had a single copy inserted into an intergenic region (Supplementary 3). The qPCR results showed that there was

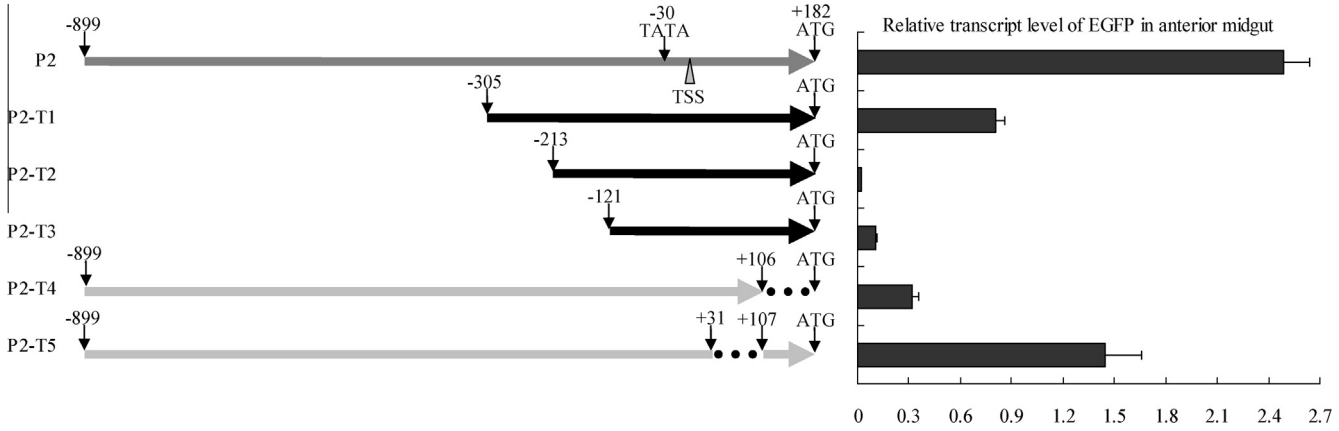


**Fig. 4.** Analysis of the critical region of the P2 promoter. P2, P2 promoter; P2-T1–T5 represent the five truncated promoters. A schematic of truncated promoters is shown at the left and the relative transcript level of EGFP in the anterior midgut of the corresponding transgenic line is shown at the right. TSS, transcript start site, numbers indicate the position of each arrow. The level of expression of EGFP in the anterior midgut was analyzed by qPCR.

near-zero expression of EGFP in the posterior midgut, silk gland, hemocyte or fat body of all transgenic lines detected (Supplementary Fig. S1B), and the EGFP expressions in the anterior midguts of P2-T2–T4 were much lower than that in P2 compared to P2-T1 and -T5 (Fig. 4), suggesting regions −305 to −214 and +107 to +181 were the crucial regions affecting P2P activity. There were four predicted TFBSs in the region of −305 to −214 (Fig. 1) and we presumed that BR-C [20] might be the most critical of the four TFBSs. The 5′ untranslated region (5′ UTR) is very important for promoter activity. The PCR results showed there was a 112 bp 5′ UTR in P2P (Fig. 1), in which there was a TFBS Tst-1 [23] that could be crucial. The insertion site might have an impact on the results, which would be surmounted by site-specific integration in *B. mori*. Further truncation experiments will be analyzed when *B. mori* midgut-derived cell lines are generated.

P2P provides a tool for functional research and application of silkworm genes. There are 216 midgut-specific genes in the silkworm [5], some of which are predicted to have important biological functions we could explore those via transgenic RNAi or overexpression using P2P. The midgut is the digestive apparatus responsible for digestion of food and assimilation of nutrition in *B. mori* larvae. Mulberry leaves are natural diet of *B. mori*; some molecules, such as urease [24–26] cannot be synthesized in *B. mori* and must be absorbed from food. Urease absorbed by the midgut is involved in converting urea into ammonia for use as a nitrogen source in *B. mori* [24–26]. We are overexpressing urease with P2P in the transgenic silkworm to promote nitrogen metabolism in *B. mori* feeding on mulberry leaves especially an artificial diet. Digestion is usually controlled by digestive enzymes, so it is assumed that food conversion efficiency can be increased by P2P-controlled overexpression of digestive enzymes in the silkworm. The midgut is also an immune organ responsible for synthesis and secretion of antiviral proteins [6–8]. P2P-controlled overexpression of antiviral genes would be an ideal method for enhancing the resistance of the silkworm to pathogens [27]. Using P2P would lead to inhibition of its multiplication in the silkworm because BmCPV and BmDNV infect only the midgut of *B. mori*, RNAi of the viral genes [12,28]. *B. mori* is a good candidate host for the production of recombinant proteins [29] and we might be able to generate a midgut bioreactor for P2P-controlled synthesis of numerous important proteins.

In summary, we have identified a silkworm midgut-specific promoter with a high level of activity by transgenic technology. This promoter will be a good research tool for *B. mori*, aiding future research and application of silkworm genes. This study provides a reference for similar studies in other members of the Lepidoptera.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.019.

## References

[1] Q.Y. Xia, Z.Y. Zhou, C. Lu, et al., A draft sequence for the genome of the domesticated silkworm (*Bombyx mori*), Science 306 (2004) 1937–1940.

[2] K. Mita, M. Kasahara, S. Sasaki, et al., The genome sequence of silkworm, *Bombyx mori*, DNA Res. 11 (2004) 27–35.

[3] Q.Y. Xia, J. Wang, Z.Y. Zhou, et al., The genome of a lepidopteran model insect, the silkworm *Bombyx mori*, Insect Biochem. Mol. Biol. 38 (2008) 1036–1045.

[4] Q.Y. Xia, Y.R. Guo, Z. Zhang, et al., Complete resequencing of 40 genomes reveals domestication events and genes in silkworm (*Bombyx*), Science 326 (2009) 433–436.

[5] Q.Y. Xia, D.J. Cheng, J. Duan, et al., Microarray-based gene expression profiles in multiple tissues of the domesticated silkworm, *Bombyx mori*, Genome Biol. 8 (2007).

[6] K.M. Ponnuvel, H. Nakazawa, S. Furukawa, et al., A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus, J. Virol. 77 (2003) 10725–10729.

[7] H. Nakazawa, E. Tsuneishi, K.M. Ponnuvel, et al., Antiviral activity of a serine protease from the digestive juice of *Bombyx mori* larvae against nucleopolyhedrovirus, Virology 321 (2004) 154–162.

[8] R. Selot, V. Kumar, S. Shukla, et al., Identification of a soluble NADPH oxidoreductase (BmNOX) with antiviral activities in the gut juice of *Bombyx mori*, Biosci. Biotechnol. Biochem. 71 (2007) 200–205.

[9] L. Jiang, G. Wang, T. Cheng, et al., Resistance to *Bombyx mori* nucleopolyhedrovirus via overexpression of an endogenous antiviral gene in transgenic silkworms, Arch. Virol. 157 (2012) 1323–1328.

[10] J. Duan, R. Li, D. Cheng, et al., SilkDB v2.0: a platform for silkworm (*Bombyx mori*) genome biology, Nucleic Acids Res. 38 (2010) D453–D456.

[11] L. Jiang, T. Cheng, P. Zhao, et al., Resistance to BmNPV via overexpression of an exogenous gene controlled by an inducible promoter and enhancer in transgenic silkworm, *Bombyx mori*, PLoS One 7 (2012) e41838.

[12] L. Jiang, P. Zhao, G. Wang, et al., Comparison of factors that may affect the inhibitory efficacy of transgenic RNAi targeting of baculoviral genes in silkworm, *Bombyx mori*, Antiviral Res. 97 (2013) 255–263.

[13] L.A. Moreira, M.J. Edwards, F. Adhami, et al., Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes, Proc. Natl. Acad. Sci. USA 97 (2000) 10895–10898.

[14] E.G. Abraham, M. Donnelly-Doman, H. Fujioka, et al., Driving midgut-specific expression and secretion of a foreign protein in transgenic mosquitoes with *AgAper1* regulatory elements, Insect Mol. Biol. 14 (2005) 271–279.
[15] T. Nolan, E. Petris, H.M. Muller, et al., Analysis of two novel midgut-specific promoters driving transgene expression in *Anopheles stephensi* mosquitoes, PLoS One 6 (2011) e16471.
[16] D.J. Whyatt, E. deBoer, F. Grosveld, The two zinc finger-like domains of GATA-1 have different DNA binding specificities, EMBO J. 12 (1993) 4993–5005.
[17] C.P. Verrijzer, M.J. Alkema, W.W. Vanweperen, et al., The DNA-binding specificity of the bipartite pou-domain and its subdomains, EMBO J. 11 (1992) 4993–5003.
[18] C.Y. Chen, R.J. Schwartz, Identification of novel DNA binding targets and regulatory domains of a murine *tinman* homeodomain factor, *nkx-2.5*, J. Biol. Chem. 270 (1995) 15628–15633.
[19] Y. Margalit, S. Yarus, E. Shapira, et al., Isolation and characterization of target sequences of the chicken *CdxA* homeobox gene, Nucleic Acids Res. 21 (1993) 4915–4922.
[20] L. von Kalm, K. Crossgrove, D. Von Seggern, et al., The *Broad-Complex* directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila* metamorphosis, EMBO J. 13 (1994) 3505–3516.
[21] T. Grange, J. Roux, G. Rigaud, et al., Cell-type specific activity of two glucocorticoid responsive units of rat tyrosine aminotransferase gene is associated with multiple binding sites for C/EBP and a novel liver-specific nuclear factor, Nucleic Acids Res. 19 (1991) 131–139.
[22] D.M. Benbrook, N.C. Jones, Different binding specificities and transactivation of variant CRE′s by CREB complexes, Nucleic Acids Res. 22 (1994) 1463–1469.
[23] X. He, R. Gerrero, D.M. Simmons, et al., Tst-1, a member of the POU domain gene family, binds the promoter of the gene encoding the cell surface adhesion molecule P0, Mol. Cell. Biol. 11 (1991) 1739–1744.
[24] C. Hirayama, M. Sugimura, H. Shinbo, Recycling of urea associated with the host plant urease in the silkworm larvae, *Bombyx mori*, J. Insect Physiol. 45 (1999) 15–20.
[25] M. Sugimura, C. Hirayama, M. Nakamura, Selective transport of the mulberry leaf urease from the midgut into the larval hemolymph of the silkworm, *Bombyx mori*, J. Insect Physiol. 47 (2001) 1133–1138.
[26] H. Kurahashi, P. Atiwetin, S. Nagaoka, et al., Absorption of mulberry root urease to the hemolymph of the silkworm, *Bombyx mori*, J. Insect Physiol. 51 (2005) 1055–1061.
[27] L. Jiang, P. Zhao, T. Cheng, et al., A transgenic animal with antiviral properties that might inhibit multiple stages of infection, Antiviral Res. (2013), http://dx.doi.org/10.1016/j.antiviral.2013.02.015.
[28] A. Fire, S. Xu, M.K. Montgomery, et al., Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis* elegans, Nature 391 (1998) 806–811.
[29] M. Tomita, H. Munetsuna, T. Sato, et al., Transgenic silkworms produce recombinant human type III procollagen in cocoons, Nat. Biotechnol. 21 (2003) 52–56.