Int. J. Indust. Entomol. Vol. 25, No. 1, 2012, pp. 111~114

International Journal of *Industrial Entomology*

Hemocyte-specific Promoter for the Development of Transgenic Silkworm, *Bombyx mori*

Seung-Won Park*, Tae-Won Goo, Seong Ryul Kim, and Gwang-Ho Choi

Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Association, Suwon 441-100, South Korea

(Received 13 August 2012; Accepted 5 September 2012)

In previous studies we have shown that a sw17255 gene was expressed in hemocyte-specific tissues of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). It was verified that the sw17255 core promoter region contains elements that regulate the expression of this gene in hemocyte tissue; the selected promoter region spans nucleotides -1 to -2,112 upstream of the start codon. Each of the luciferase reporter gene expression vectors under the control of 4 different kinds of promoter candidates, (-2,112/-1), (-1,640/-1), (-1,169/-1) and (-579/-1), and the control reporter plasmid DNA, were introduced into *B. mori* larval coelom by direct injection using a syringe. The promoter candidate [E] (-579/-1) showed more than 1.67 fold transcriptional activity compared to control promoter activity. Higher productivity of an expressed gene in the transgenic silkworm by this promoter combination could be achieved in the near future. The foreign recombinant protein could be easily harvested from the blood of the transgenic silkworm.

Key words: Tissue-specific expression, Core promoter, Transgenic insect, Biomaterial

Introduction

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae), is a powerful model for research and also an economically important insect to the silk industry (Park *et al.*, 2010; Cong *et al.*, 2011). *B. mori* synthesizes large amounts of silk proteins in its silk glands (Cong *et al.,* 2011). Compared to the traditional value of *B. mori* as source of silk, its major role in the near future will be as a biomedical insect for producing biomaterials (Park *et al.,* 2010). Therefore, the silkworm might be suited as a host for the mass production of recombinant proteins compared to mammalian and non-mammalian cultured cells (Cong *et al.,* 2011). The expressions of target genes in transgenic insects have to regulate in a time- or tissue-specific manner the effective production of recombinant proteins. This goal has driven the successful search for several tissue-specific or inducible promoters capable of driving expression in transgenic insects relevant to recombinant production, including the midgut, hemolymph, fat body and salivary glands (Moreira *et al*., 2000; Abraham *et al.*, 2005; Yoshida and Watanabe 2006; Nirmala *et al.*, 2006; Chen *et al*., 2007; Nolan *et al.*, 2011). In particular, we think that hemolymph-transcribed promoters are ideal candidates to produce the exogenous genes while it completes its life cycle within the silkworm (Nolan *et al.,* 2011). One of the most important events in the control of gene expression is the regulation of transcription initiation (Garcia *et al.,* 2011). The major components of the eukaryotic core promoters are composed of several sequence motifs such as the initiator, TATA box, TFIIB recognition element, downstream core promoter element, motif ten element and downstream core element (Smale and Kadonaga 2003; Juven-Gershon *et al.*, 2008; Juven-Gershon and Kadonaga 2010; Garcia *et al.*, 2011).

In previous data, we analyzed tissue-specific expression patterns in hemocyte tissue from five days of fifth instar larvae during the development of *B. mori* (Park *et al.,* 2011). A total of 5 candidates were selected from the Bombyx mori microarray database (BmMDB; http://silkworm.swu.edu.cn/microarray) (Xia *et al*., 2007; Park *et al*., 2011). To verify

*To whom the correspondence addressed
Sericultural & Apicultural Materials Division, National Academy of Agricultural Science, RDA, Suwon 441-100, Republic of Korea.
Tel: +82-31-290-8493; Fax +82-31-290-8503;
E-mail: microsw@korea.kr
http://dx.doi.org/10.7852/ijie.2012.25.1.111

Table 1. Oligonucleotide PCR primers for hemocyte-specific promoter candidates PCR amplification. 1) *Mlu*I restriction enzyme site, 2) *Hind*III restriction enzyme site

Oligonucleotide name	Primer pairs sequence (5`3`)	Fragment size, bp
sw17255 (B) Forward	TA*ACGCGT*[1)]AGACAATAAATACTCCGTCCTA,	2, 112
sw17255 (C) Forward	TA*ACGCGT*[1)]CATACCTTACTAAACTGTTTACG	1,640
sw17255 (D) Forward	TA*ACGCGT*[1)]AAGTTAGGCAATTTAAAGGTGG	1,169
sw17255 (E) Forward	TA*ACGCGT*[1)]TGAAGTTGATAAATTTGCGACTA	579
sw17255 Reverse	GT*AAGCTT*[2)]TTATAGTTGCTTATATTTCAACT	

tissue-specific expression, we analyzed real-time quantitative RT-PCR using the highly expressed endogenous *Actin* RNA as an intrinsic reference. We confirmed that a sw17255 gene was expressed in the hemocyte-specific tissues (Park *et al.,* 2011). Here to determine the core promoter region, 4 different partial mutant clones were tested using the luciferase assay in the silkworm by direct injection of target plasmid DNAs. Finally, we demonstrate that the 579 bp fragment (-579/-1) sw17255 hemolymph-specific promoter shows higher luciferase activity than the constitutive *B. mori Actin3* (BmA3) promoter. Development of this promoter directly or indirectly specifically inducible in the tissue is very useful to make recombinant proteins in the transgenic silkworm.

Materials and Methods

Experimental silkworm and genomic DNA isolation

The *B. mori* larvae of the race F1 hybrid between Jam123 and Jam124 of the Korean silkworm strain were reared at a temperature of 25°C. Day 5 of the 5th instar larvae were used for the experiments. Circulating hemocytes in the body fluid of the F1 hybrid larvae were collected into 15 ml conical tubes. After centrifugation, genomic DNA was isolated from the hemocyte tissue sample using the TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The amount of genomic DNA was measured spectrophotometrically by the absorbance at 260 nm and stored at –20ºC until use.

Subcloning of candidates sw17255 promoter sequences

A 2,112 bp PCR fragment (sw17255 Pro [B]) of the sw17255 hemocyte-specific gene was amplified employing genomic DNA derived from hemolymph tissue of the racial F1 hybrid between Jam123 and Jam124 of the Korean silkworm strain as a template and the sw17255 [B] forward and reverse primer pair (primer pairs are given in Table 1). *Mlu*I and *Hind*III restriction enzyme sites were inserted at the 5`- end of forward and reverse primers, respectively. A 5 ul aliquot of genomic DNA was amplified using the Top-*Taq* PreMix (CoreBioSystem Co., Ltd., Republic of Korea) according to the manufacturer's instructions. The PCR protocol was performed using 20 sec denaturation at 94ºC, 20 sec annealing at 52ºC, and 2 min elongation at 72ºC for 25 cycles. A ~2.1 kb PCR product was gel purified and subcloned into pGEM-T Easy plasmid vector (Promega, WI) and sequenced at Bioneer Co., Ltd., Republic of Korea. The resulting plasmid was called pGEMT-sw17255 Pro [B].

Three different candidates of expected promoter sequences were amplified employing pGEMT-sw17255 Pro [B] plasmid DNA with specific primer pairs (primer pairs are given in Table 1). Each PCR products were inserted into the pGEM-T Easy plasmid vector (Promega) and were called pGEMT-sw17255 Pro [C], [D], and [E], respectively.

Plasmid DNAs construction

All recombinant DNA manipulations were performed using standard techniques (Park *et al.,* 2010). To generate the luciferase reporter gene expression vector under the control of 4 different kinds of promoter candidates, the candidate genes were excised following *Mlu*I and *Hind*III double digestion from the pGEMT-sw17255 Pro [B], [C], [D], and [E] plasmid DNAs. These fragments were inserted into the *Mlu*I / *Hind*III sites of the pGL3-Basic vector (Promega) to generate pGL3-sw17255 Pro [B], [C], [D], and [E] plasmid DNA vectors, respectively (Fig. 1).

***In-vivo* dual-luciferase reporter assay**

The racial F1 hybrid between Jam123 and Jam124 of day 2 of the 5th instars larvae were used for the experiments. 50 ng of pRL-A3 plasmid DNA was mixed with each 2 ug of candidate plasmid DNAs or pGL3-BmA3, a plasmid containing constitutive *B. mori Actin3* promoter sequences as an internal control. After incubation for 10 min, 2 ug of FuGENE HD Transfection Reagent (Roche, Germany) was added to the DNA mixtures, respectively. Doubly distilled water was added up to 50 ul volume. After additional incubation for 15 min, 50 ul volume of DNA and Lipofectamine

reagent mixture were injected into the silkworm body fluids using the syringe. After 72 hrs post-transfection, circulating hemocytes in the body fluid were collected using the 1.5 ml micro tube. Collected hemocyte cells were washed with PBS, lysed with 100 ul 1X passive lysis buffer and then subsequently assayed for luciferase activity in cell lysate using the Dual-Luciferase Reporter Assay Kit (Promega). Assays in 96-wells plates were read with the Tecan GENios (MTX Lab Systems, VA) Microplate Reader, 0.5 second integration/well.

Results and Discussion

Previous studies from our group reported that the sw17255 gene in the hemocyte tissue on day 5 of fifth instar larvae was expressed in hemocyte-specific tissues (Park *et al.,* 2011). To verify that the sw17255 core promoter region contains elements that regulate the expression of this gene in hemocytes, we have first determined the nucleotides -1 to -2,112 upstream of the start codon as maximum promoter candidate region. The largest 2,112 bp PCR fragment of the sw17255 hemocyte-specific gene was amplified employing genomic DNA derived from hemolymph tissue of the race F1 hybrid between Jam123 and Jam124 as a template (Fig. 1A). Next, the PCR product was cloned into pGEM-T Easy vector to generate plasmid pGEMT-sw17255 Pro [B]. The three different 5`-deleted PCR products, pGEMT-sw17255 Pro [C], [D], and [E], were amplified with plasmid pGEMT-sw17255 Pro [B] as the template (Fig. 1B). As explained above in Materials and Methods, the candidate genes were excised following *Mlu*I and *Hind*III double digestion from the pGEMT-sw17255 Pro [B], [C], [D], and [E] plasmid DNAs and inserted into the *Mlu*I / *Hind*III sites of the pGL3-Basic vector to generate pGL3-sw17255 Pro [B], [C], [D], and [E] plasmids (Fig. 1C).

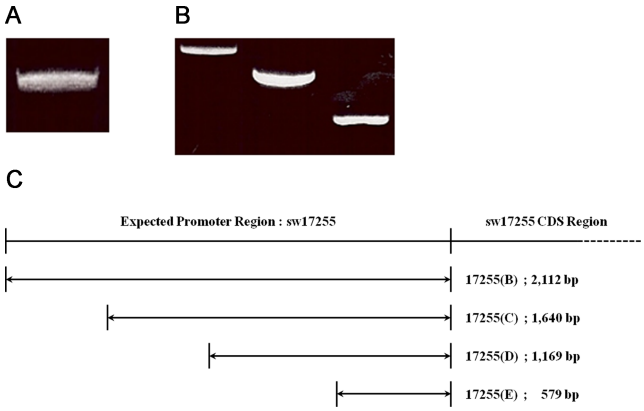

Fig. 1. Expected sw17255 gene promoter and diagrams of the analyzed constructs. (A). The largest 2,112 bp PCR fragment of the sw17255 hemocyte-specific gene was amplified employing genomic DNA derived from hemolymph tissue. (B). The three different 5`-deleted PCR products were amplified with plasmid DNA containing the largest fragment. (C). Partially deleted promoter genes were inserted into the *Mlu*I / *Hind*III sites of the pGL3-Basic vector to generate pGL3-sw17255 Pro [B], [C], [D], and [E] plasmids.

To investigate the tissue-specific promoter activity of sw17255 gene, we need a cell line derived from hemocyte-specific tissue of *B. mori*. Unfortunately, we do not have hemocyte cell line yet. Several strategies have been proposed as an alternative to the development of hemocyte cell line. The direct plasmid DNA injection into living bodies of *B. mori* was the most efficient way. The control reporter contained the *B. mori* actin A3 gene promoter (Park *et al*., 2011) and the luciferase coding region. Each the luciferase reporter gene expression vectors under

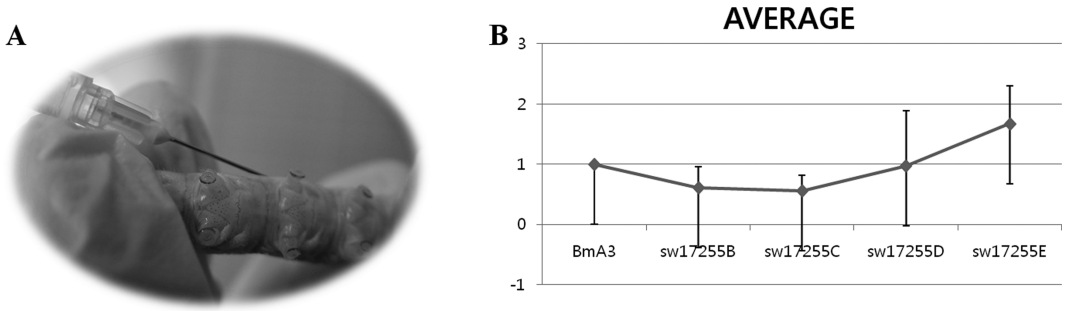

Fig. 2. The direct plasmid DNA injection into living bodies of *B. mori* and luciferase activity driven by the hemocyte-specific promoter. (A) To investigate the tissue-specific promoter activity of sw17255 gene, each the luciferase reporter gene expression vectors under the control of 4 different kinds of promoter candidates and the control reporter plasmid DNA were introduced into *B. mori* larval coelom by direct injection method using a syringe. (B) In hemocyte cells introduced with the (-1,169/-1) and (-579/-1) were expressed luciferase protein better than control promoter. Especially, promoter candidate [E] (-579/-1) shows more than 1.67 fold transcriptional activity compared to control promoter activity.

the control of 4 different kinds of promoter candidates and the control reporter plasmid DNA were introduced into *B. mori* larval coelom by direct injection using a syringe (Fig. 2A). As shown in Fig. 2B, among 4 hemocyte-specific core promoter candidates, one candidate E was determined to have tissue-specific phenotypes. The analysis of the lines introduced with the constructs: (-2,112/-1), (-1,640/-1), (-1,169/-1) and (-579/-1), reveals luciferase gene expression in larval hemocyte cells. The reporter activity in this tissue was detected and repeated at least three times. In hemocyte cells introduced with the (-2,112/-1) and (-1,640/-1) constructs, the activity of the reporter gene showed lower than control promoter activity. However, another two candidates, (-1,169/-1) and (-579/-1) expressed luciferase protein better than the control promoter. The promoter candidate [E] (-579/-1) shows more than 1.67 fold transcriptional activity compared to control promoter activity. The small size of promoter is useful in a transgenic insect. The recombinant DNA cassette for transgene expression is composed of the promoter and gene of interest. A main problem of transgenic insect approaches is how to introduce the foreign gene cassette into the host genomic DNA. The probability of achieving transgenesis is higher than promoter containing large sequences. Therefore, a smaller promoter can be inserted containing more genes of interest.

Here, we describe a novel promoter engineered to obtain high levels of hemocyte-specific expression. We also defined the core promoter sequences of them not exceeding 600 nucleotides. This promoter is strong enough to achieve in hemocyte cells transcriptional levels higher than those obtained with the very strong and universal *B. mori* actin A3 promoter region, still retaining their hemocyte-specificity. To get higher productivity of expressed gene in the transgenic silkworm by this promoter combination in the near future, we can easily harvest the foreign recombinant protein from the blood of the transgenic silkworm.

Acknowledgement

We are grateful to Hee-Yeon Cho for work of luciferase assay and for technical support. This study was supported by the Rural Development Administration, Republic of Korea (grant no. PJ008475022012).

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