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Analysis of Hemocyte-specific Gene Expression from *Bombyx mori*

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A previous data was provided information for tissue-specific expression genes by means of whole-genome oligonucleotide microarray in the silkworm. We analyzed the tissue-specific expression patterns in the hemocyte tissue on 5 days of 5th instar larvae during the development of *B. mori*. Total 5 candidates pick out from the *Bombyx mori* Microarray Database (BmMDB; http://silkworm.swu.edu.cn/microarray). To verify the hemocyte-specific expression, we analyzed by semi-quantitative and real-time quantitative RT-PCR using the highly expressed endogenous *Actin* RNA as an intrinsic reference. In this study, we confirmed that one gene-sw17255- out of 5 candidates expressed in the hemocyte tissue, which was consistent with the previous data. Circulating hemocytes in the body fluid of the *B. mori* are most powerful target organ for producing biomaterials. We need further studies to find hemocyte-specific promoter region from sw17255 gene. Finally, this result can be applied in creating transgenic silkworms as a biomedical insect.

Key words: Silkworm, Hemocyte-specific expression, Real-time RT-PCR, Biomedical insect

Introduction

The *Bombyx mori* is the most important commercial insects to the silk industry since its first introduction to Korea in 10th century B.C. (International Silkworm Genome Consortium, 2008). Up to now, the traditional value of *B. mori* was a source of silk. However, its major role in the near future will be as a biomedical insect contributing to the production of biomedical proteins and biomaterials (Park *et al*., 2010). In fact, the silkworm can also express high levels of foreign genes through the baculovirus expression vector system (BEVS). These recombinant proteins may be used as vaccine antigens (Gong *et al*., 2005) and antibacterial proteins (Yun *et al*., 2005), such as the cholera toxin B subunit (CTB) and transferrin (Tf), respectively (Park *et al*., 2010). The Japanese and Chinese groups opened a novel assembly of a 28-chromosome sequence data from the *B. mori* genome (International Silkworm Genome Consortium, 2008; Mita *et al*., 2003, 2004; Ote *et al*., 2004; Parthasarathy *et al*., 2004). Interestingly, a previous study used whole-genome oligonucleotide microarray for the survey of tissue-specific gene expression (Xia *et al*., 2007). Recently, we verified the tissue-specific genes expression by using the BmMDB from three different tissues (Park *et al*., 2010). This data also can be useful to provide the laboratory information concerning transgenic silkworm as a bioreactor (Park *et al*., 2010).

In this study, we analyzed the tissue-specific expression patterns in the hemocyte tissue on five days of fifth instar larvae during the development of *B. mori*. A total of 5 candidates were selected from the BmMDB (Xia *et al*., 2007). Genes from candidate showed the highest rates of expression in the microarray data (Xia *et al*., 2007). To verify the tissue-specific expression, we analyzed by semi-quantitative and real-time quantitative RT-PCR using the highly expressed endogenous *Actin* RNA as an intrinsic reference. Finally, we confirmed a gene out of 5 candidates expressed in the hemocyte tissues.

Materials and Methods

Experimental silkworm and tissue-specific RNA isolation

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Table 1. Oligonucleotide PCR primers for tissue-specific and internal reference genes

Oligonucleotide name	Primer pairs sequence (Forward and Reverse), 5`→3`
Actin	
BmA3	GAAGCTGTGCTACGTCGCTC, CCGATGGTGATGACCTGACC
Ceh-32	
hemocyte	CGCCCAACGACATCCA, CGCACAAGCCGTGGATA
Chitin Synthase	
chitin	GCCATCTAGTATCTTCACGG, ATCCTTCGGGTTGGTGC
Hemocyte	
sw17255	ACGTTCCCACGTCTAAAG, GTAATGAACCGTCGACGT
sw10859	CGAAGCCTGGTATTCATG, GGACGCACATTATCACTC
Sw05834	ATGGAGACAGGTCCCGAG, GTTGCGGTACATGGCGAG
Sw11226	TCAAAGGATACAATTATC, CAATTCTAGAGAAACAAC
Sw14411	ATGAGTGAACATTCCGGA, TTAGGCGACGACGACTTG

In this study, most of methods were followed according to the previous research (Park *et al*., 2010) except tissue sample preparation. The *B. mori* larvae of the racial F_1 hybrid between Jam123 and Jam124 of the Korean silkworm strain were reared on an artificial diet at a stable temperature of 25°C. Day 5 of the 5th instar larvae were used for the experiments. Circulating hemocytes in the body fluid of the F_1 hybrid larvae were collected using the 15 ml conical tube. After centrifuge, total RNAs were isolated from the hemocyte tissue sample using the TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The amounts of RNAs were measured spectrophotometrically by the absorbance of 260 nm. The RNAs were stored at −70°C until use.

Reverse transcription

To detect the specific expression patterns of the candidate genes in hemocyte tissues of the silkworm, total RNAs were treated with DN*ase* I for 30 minutes at 37°C to remove the genomic DNA. After purification, oligo dT-primed cDNAs were made from 20 μg of total RNAs using the High-Capacity cDNA Archive kit (Applied Biosystems, USA). The reaction was allowed to proceed for 2 hours at 37°C. Tissue-specific primers were used for RT-PCR (primer pairs are given in Table 1). The Top-*Taq* PreMix (CoreBioSystems, Korea) polymerase was used. The annealing temperature was 52°C for 35 cycles.

Real-time PCR

PCR was conducted in the 25 μl system containing 12.5 μl of SYBR Premix Ex Taq (Takara, Japan), 9.5 μl ddH_2O, 0.5 ul/10 pmol primers each, and 2 μl of cDNA. The PCR protocol was done by 5-second denaturation at 94°C, 20-second annealing at 52°C, and 15-second elongation at 72°C for 40 cycles. Fluorescence was detected at the end of every 72°C extension phase. The *Actin* gene was used as an internal reference for normalizing the quality of total RNAs purified from each sample (silk gland, fat body, and midgut). Real-time PCR was performed using the SYBR Green PCR master mix in an ABI7300 Real-time PCR Instrument (Applied Biosystems). The fold changes of gene expressions were determined by comparative C_T method as described in ABI Prism 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems).

Results and Discussion

In the previous data, we confirmed five genes, (sw15872, sw00692, sw20990, sw05300, and sw2250), out of 18 candidates expressed in two different tissues (silk gland and midgut), which was consistent with the data by BmMDB (Park *et al*., 2010; Xia *et al*., 2007). To verify the tissue-specific expressions of the candidate genes from the circulating hemocytes, we used the same methods with a previous research (Park *et al*., 2010). Briefly, the 35 cycles of PCR reactions were carried out for all candidates and control genes. As shown in Fig. 1A, endogenous internal control gene (*Actin*) was not detected in RNA samples treatment with DN*ase* I enzyme. The hemocyte-specific (*Ceh-32*) and midgut-specific (*Chitin synthase*) genes were used to verify their tissue-specific expressions in this work (Xia *et al*., 2007). This result shows that isolated RNA samples from specific tissues in combination with these data can validate the accuracy of our tissue dissections, and that there were no cross-contaminations during this experiment (Fig. 1B). Next, we analyzed 5 candidate genes, which were picked out from previously published data (Xia *et al*., 2007). This data was

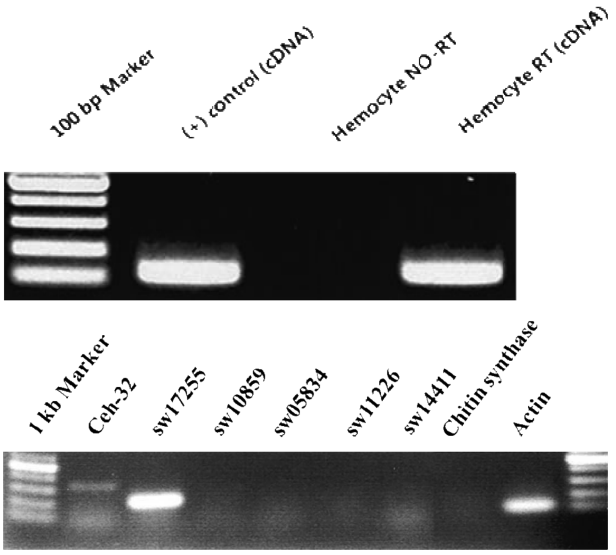

Fig. 1. RT-PCR expression patterns of *B. mori.* in the hemocyte on day 5 at 5th instar larvae. Total RNAs were treated with DN*ase* I for 30 minutes at 37°C to remove the genomic DNA (A). We used RT-PCR method to amplify tissue-specific and internal reference genes from the hemocyte tissue. Endogenous internal control gene (*Actin*) was detected in the tissues. However, midgut specific (*Chitin synthase*) gene was used to verify their tissue-specific expression in this work. One gene tissue specifically expressed in the hemocyte tissue (B). The EtBr staining of PCR products are shown as a control for equal loading.

partly consistent in comparison with the previous data (Table 2). Among 5 hemocyte-specific candidates, one target (20.0%) were determined to be tissue-specific phenotypes (Table 2). Therefore, sw17255 was revealed to be hemocyte-specific. We picked up only five candidates had been selected as the target for hemocyte specificity; we are certain that if the number were larger, specificity would be found more specificity in the hemocyte as well. However, hemocyte-specific candidate too low numbers compare with other tissues (Xia *et al*., 2007). A previous study shows that they designed and constructed a microarray using 22,987 oligonucleotides covering the presently known and predicted genes in the silkworm genome (Xia *et al*., 2007). A total of 1,642 tissue-specific genes were identified from the microarray data. The number of genes specifically expressed in each selected tissue displayed a remarkable variation ranging from 6 in the fat body to 1,104 in the testis (Xia *et al*., 2007). Among 1,642 tissue-specific genes, 24 genes (1.46%) were identified as hemocyte-specific genes (Xia *et al*., 2007). Because we picked up the highest expression level of target genes, the number of selected genes was very much limited (5/24 genes in the hemocyte tissue) in this study. The electrophoresis bands of RT-PCR products are shown in Fig. 1B. One tissue-specific gene was detected in the specific tissue. As we already mentioned (Park *et al*., 2010), this data was somewhat different from those of a previous data, the reason for which we are unsure of; it is our hypothesis that it is attributable to a number of differences (for example, total RNAs were isolated from each specific tissue of the larvae of the racial F_1 hybrid between Jam123 and Jam124 of the Korean silkworm strain on day 5 of fifth instar larvae during the development of *B. mori* were used for this study, therefore causing the silkworm strain and developmental stage to be different from those of the research performed with the previous data, where the Chinese silkworm strain, *Dazao*, was used on the 3 day of 5th instar larvae).

The analysis above with hemocyte tissue for tissue-specific RNAs was validated by quantitative real-time RT-PCR amplifications. The expression of a candidate and

Table 2. Summarized data tissue-specific expression in this study

	5th instar 5days				
	Tissue	Probe Number	Hemocyte cDNA RT-PCR	Midgut cDNA RT-PCR	Specificity
1	Hemocyte	sw17255	***[1)]	ND[2)]	O
2	Hemocyte	sw10859	N.D	N.D	X
3	Hemocyte	sw05834	N.D	N.D	X
4	Hemocyte	sw11226	N.D	N.D	X
5	Hemocyte	sw14411	N.D	N.D	X
6	(-) Control	BmA3	***	***	
7	(+) Control	ceh-32	***	N.D	

[1)] RT-PCR band intensity; * (weak) < ** (medium) <*** (strong).
[2)] N.D; None Detect.

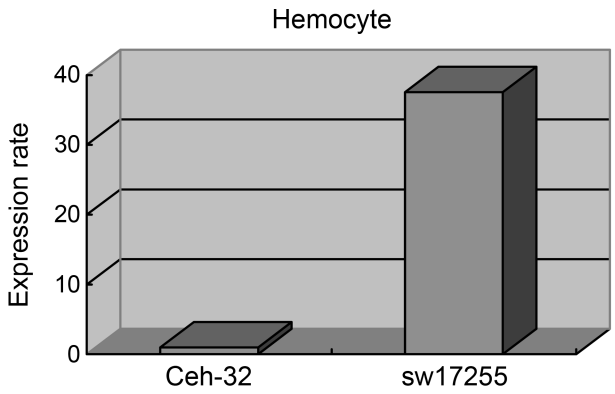

Fig. 2. Quantitative real-time RT-PCR analysis of tissue-specific genes. To validation of tissue-specific expression data, we performed real-time RT-PCR. The expressions of a candidate and known tissue-specific gene (*Ceh-32*) were normalized to the expression levels of silkworm cytoplasmic *Actin* gene as an internal reference. The expression rate of each target genes were compared with known specific genes. Expression rate of a candidate (sw17255) was much more than *Ceh-32*.

known tissue-specific genes (*Ceh-32* and *Chitin synthase*) were normalized to the expression levels of the silkworm cytoplasmic *Actin* gene as an internal reference. The expression rate of target gene was compared with specific genes that were already known (Fig. 2). The C_T values for mRNA expression of *Actin*, two of which were known, and a candidate gene indicated their expression in the tissue-specific genes that were examined. We observed that the expression rates of a candidate (sw17255) was much higher than *Ceh-32*. Interestingly, this data matched the results for the semi-quantitative RT-PCR. This data indicated that candidate transcript was much more compared to the *Ceh-32* transcript.

In this study, we report the confirmation of one gene expressed in hemocyte tissues in the silkworm: sw17255. For its validation, we used the quantitative real-time RT-PCR method. This method is ideal for studies for which there is only a limited amount of tissue (Castello *et al*., 2002; Park *et al*., 2010). A previous data provides information for expression of tissue-specific genes by means of whole-genome oligonucleotide microarray in the silkworm (Park *et al*., 2010; Xia *et al*., 2007) They have constructed a *B. mori* Microarray Database and a web browser to open the silkworm gene expression data (Park *et al*., 2010; Xia *et al*., 2007). Huge numbers of transcriptomes were analyzed at the whole-genome level by means of microarray; partly, RT-PCR assay was also performed simultaneously, in order to confirm the microarray data (Park *et al*., 2010). Though the data is consistent on most occasions, expressions in specific tissues should be confirmed not only because these data are very useful to researchers working with silkworms, but also because they can be used for the creating transgenic silkworms as a biomedical insect for producing biomaterials, not to mention as well-characterized models for understanding the mechanism for the genetic regulation of tissue-specific development (Park *et al*., 2010).

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