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Tissue-specific gene expression analysis of silkworm (*Bombyx mori*) by quantitative real-time RT-PCR

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The *Bombyx mori* Microarray Database (BmMDB; http://silkworm.swu.edu.cn/microarray) provides information for tissue-specific gene expression by using the whole-genome oligonucleotide microarray in the silkworm. We analyzed the tissue-specific expression patterns in the silk gland, fat body, and midgut five days of fifth instar larvae during the development of *B. mori*. To verify the tissue-specific expression, analysis was conducted using quantitative Real-time RT-PCR and the highly expressed endogenous *Actin* RNA as an intrinsic reference. Finally, we confirmed five genes, (sw15872, sw00692, sw20990, sw05300,and sw2250), out of 18 candidates expressed in two different tissues, which was consistent with the data published by Dr. Xiang's group, thereby supporting the BmMDB. Further studies for promoter regions of candidate genes can be applied in creating transgenic silkworms as biomedical insects for use in producing biomaterials, and to serve as well-characterized models for understanding the mechanism for the genetic regulation of tissue-specific development. [BMB reports 2010; 43(7): 480-484]

INTRODUCTION

The mulberry silkworm, *Bombyx mori*, is a powerful model for the analysis of the genetic regulatory mechanism, not to mention one of the most important commercial insects to the silk industry, since its first introduction to Korea in 10th century B.C. (1). Compared to the traditional value of *B. mori* as a source of silk, its major role in the near future will be as a biomedical insect contributing to the production of proteins and biomaterials. In fact, silk fibroin-based scaffold materials have already been employed for several tissue-engineering applications in place of synthetic polymers (2). The silkworm can also express high levels of foreign genes through the Baculovirus system. These resultant recombinant proteins may be used as vaccine antigens (3) and antibacterial proteins (4), such as the Cholera toxin B subunit (CTB) and Transferrin (Tf), respectively. In 2004, Mita *et al*. completed genomic sequencing of the entire 500 Mb *B. mori* genome (5) and over 11,000 of the expressed sequence tags (EST) were analyzed (6, 7). More than 150,000 ESTs for the silkworm are available in the database (8). Very recently, the Japanese and Chinese groups began to assemble the 28-chromosome sequence data from the *B. mori* genome (1). Interestingly, Dr. Xiang's group used whole-genome oligonucleotide microarray for the survey of tissue-specific gene expression (9). This data can be useful in providing laboratory information concerning use of the transgenic silkworm as a bioreactor.

In this study, we analyzed the tissue-specific expression patterns of the silk gland, fat body, and midgut from five days of fifth instar larvae during the development of *B. mori*. A total of 18 candidates (9 for the silk gland, 1 for the fat body, and 8 for the midgut) were selected from the data published by Dr. Xiang's group (9). Genes from each candidate showed the highest rates of expression in the microarray data (9). To verify the tissue-specific expression, analysis was conducted by quantitative Real-time RT-PCR, using the highly expressed endogenous *Actin* RNA as an intrinsic reference. Finally, we confirmed five genes out of 18 candidates as being expressed in two different tissues, which was consistent with the data published by Dr. Xiang's group.

RESULTS AND DISCUSSION

To verify the tissue-specific expressions of the candidate genes from the silk gland, fat body and midgut, we used the RT-PCR method. The 35 cycles of PCR reactions were carried out for all candidates and control genes. As shown in Fig. 1, the endogenous internal control gene (*Actin*) was detected in all tissues. However, two genes previously reported by Dr. Xiang's group - the silk gland-specific (*Sericin 1B*) and midgut-specific (*Chitin synthase*) genes, were used to verify their tissue-specific expressions in this study (9). The result showed 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 dur-

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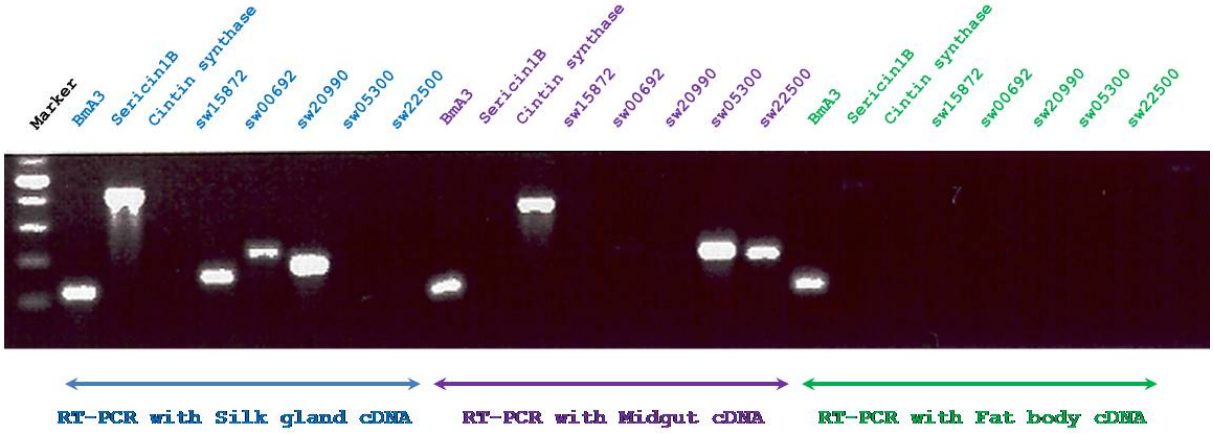

Fig. 1. RT-PCR expression patterns of *B. mori.* in the silk gland, fat body and midgut on day 5 at 5th instar larvae. We used the RT-PCR method to amplify tissue-specific and internal reference genes from the silk gland, fat body, and midgut. Endogenous internal control gene (*Actin*) was detected in all the tissues. However, silk gland specific (*Sericin 1B*) and midgut specific (*Chitin synthase*) genes were used to verify their tissue-specific expression in this study. A total of 5 genes were tissue specifically expressed in two tissues. The EtBr staining of PCR products are shown as a control for equal loading.

ing this experiment. Next, we analyzed 18 candidate genes, which were selected from previously published data (9). This data was partially consistent with data from Dr. Xiang's group (Table 1). Those candidates that were not, however, were detected in all tissues, two or not.

Among 9 silk gland-specific candidates, 3 targets (33.3%) were determined to be tissue-specific phenotypes (Table 2). Six candidates were undetermined for silk gland specificity. Two candidates (25.0%) were identified to be the midgut-specific gene. Therefore, sw15872, sw00692, sw20990, sw05300, and sw22500 were revealed to be tissue-specific. Unfortunately, the target from fat body tissue did not show specificity. We detected only one candidate as the target for fat body specificity; we are certain, however, that if the number were larger, specificity would be found in the fat body as well. However, fat body-specific candidates occurred in numbers too low to compare with other tissues (9). Dr. Xiang's study designed and constructed a microarray using 22,987 oligonucleotides covering the presently known and predicted genes in the silkworm genome. 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 (9). Among 1,642 tissue-specific genes, 53 genes (1.2%) were determined to be both A/MSG and PSG-specific, while 216 genes (3.87%) were identified as midgut-specific genes (9). Because we detected the highest expression level of target genes, the number of selected genes in this study was very much limited, (9/53 genes in the silk gland, 8/216 genes in the midgut and 1/6 genes in the fat body). The electrophoresis bands of RT-PCR products are shown in Fig. 1. Five tissue-specific genes were detected in each specific tissue.

Table 1. Summarized data for tissue-specific expression in this study

	Tissue	Probe number	Silk gland cDNA RT-PCR	Midgut cDNA RT-PCR	Fat body cDNA RT-PCR	Specificity
1	Silk gland	sw134441	***	***	N.D	X
2		sw15872	**	N.D	N.D	O
3		sw19571	***	*	N.D	X
4		sw00692	*	N.D	N.D	O
5		sw01222	N.D	N.D	N.D	X
6		sw20990	***	N.D	N.D	O
7		sw19374	***	*	N.D	X
8		sw20836	***	***	***	X
9		sw22099	N.D	N.D	N.D	X
10	Fat body	sw05710	**	**	**	X
11	Midgut	sw05300	N.D	***	N.D	O
12		sw14336	***	***	***	X
13		sw04538	***	***	***	X
14		sw04624	N.D	***	*	X
15		sw07617	**	N.D	***	X
16		sw19250	N.D	**	**	X
17		sw22500	N.D	**	N.D	O
18		sw22899	***	***	***	X
19	Internal control	BmA3	***	***	***	O
20	Silk Gland	Sericin 1B	***	N.D	N.D	O
21	Midgut	Chitin synthase	N.D	***	N.D	O

RT-PCR band intensity, *(weak) < **(medium) <***(strong). N.D; None Detect.

These data were somewhat different from those of Dr. Xiang's study, the reason for which we are unsure. It is our hypothesis

that it may be attributable to a number of factors. For instance, for our study, total RNAs were isolated from each specific tissue of the racial F_1 hybrid larvae between Jam123 and Jam124 of the Korean silkworm strain on day 5 of fifth instar larvae during the development of *B. mori*, therefore causing the silkworm strain and developmental stage to be different from those of Dr. Xiang's study, where the Chinese silkworm strain, *Dazao*, was used on the 3 day of 5th instar larvae.

The analysis above, using different tissue for tissue-specific RNAs, was validated by quantitative real-time RT-PCR amplifications. The expressions of five candidates and two known tissue-specific genes (*Sericin 1B* and *Chitin synthase*) were normalized to the expression levels of the silkworm cytoplasmic *Actin* gene as an internal reference. The expression rate of each 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 five candidate genes, indicated their expression in the tissue-specific genes that were examined. We observed that the expression rates of all five candidates (sw15872, sw00692, sw20990, sw05300 and sw22500) were much lower than those of *Sericin 1B* and *Chitin synthase*. This data indicated that all the candidate transcript expression rates were much less compared to the *Sericin 1B* and *Chitin synthase* transcripts.

The successful engineering of genetically transformed insects for the production of biomaterials depends on multiple variables. As we know, spatial and temporal expression pattern of transgenes in a transgenic organism is a very important factor. Over-expression strategies, utilizing global regulatory promoters, may lead to undesirable pleiotropic effects in the developmental stages (10). Therefore, the identification of tissue-specific gene promoters is important for the development of novel transgenic organisms. *B. mori* synthesizes vast amounts of silk protein in its silk glands and is therefore a good candidate host for the production of recombinant proteins at an industrial scale (11). For the same reason, sericin-1 gene promoter is used for the transgenic silkworm. However, we need more tissue-specific promoter excavation for the production of a larger variety of biomaterials. Because the spatial and temporal expression pattern of transgenes for production of high quality recombinant proteins is an important factor it can be used to increase the successful engineering of genetically transformed insects. To identify novel specific promoters, we need to investigate specific gene expression rates from specific organ samples.

In the present study, we report the confirmation of five genes expressed in two different tissues in the silkworm: sw 15872, sw00692, sw20990, sw05300 and sw22500. For its validation, we used the quantitative real-time RT-PCR method. This method is ideal for studies when there is only a limited amount of tissue (12). Xiang *et al*. provides information for expression of tissue-specific genes by means of whole-genome oligonucleotide microarray in the silkworm. They have constructed a *B. mori* Microarray Database and a web browser to

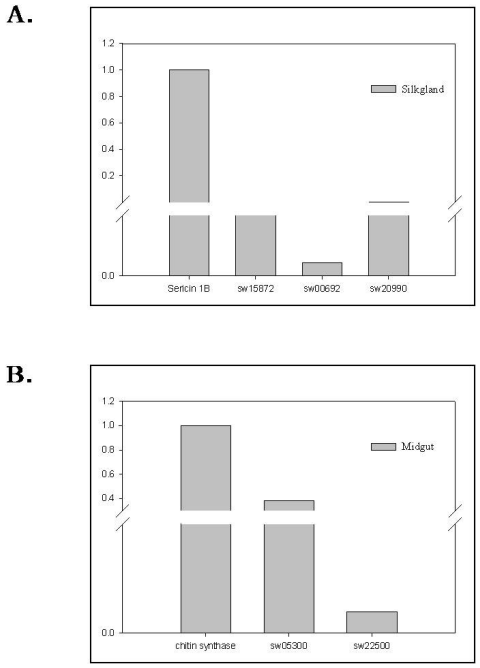

Fig. 2. Quantitative Real-time RT-PCR analysis of tissue-specific genes. To validate tissue-specific expression data, we performed real-time RT-PCR. The expressions of five candidates and two known tissue-specific genes (*Sericin 1B and Chitin synthase*) were normalized to the expression levels of silkworm cytoplasmic Actin gene as an internal reference. The expression rate of each target gene was compared with known specific genes. Expression rates of all five candidates (sw15872, sw00692, sw20990, sw05300 and sw22500) were much lower than those of than *Sericin 1B* (A) and *Chitin synthase* (B).

open the silkworm gene expression data. Huge numbers of transcriptomes were analyzed at the whole-genome level by means of microarray; while RT-PCR assay was also performed simultaneously to confirm the microarray data. 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 creating transgenic silkworms as a biomedical insect for producing biomaterials, while providing a well characterized model for understanding the mechanism for the genetic regulation of tissue-specific development. Further studies are needed to elucidate the promoter regions of five candidates to produce a transgenic silkworm for production of biomaterials.

MATERIALS AND METHODS

Experimental silkworm and tissue-specific RNA isolation

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^{o}C. Day 5 of the 5th

Table 2. Oligonucleotide PCR primers for tissue-specific and internal reference genes

Oligonucleotide name	Primer pairs sequence (forward and reverse), 5'→3'	Fragment size, bp
Actin		
BmA3	GAAGCTGTGCTACGTCGCTC,CCGATGGTGATGACCTGACC	111
Sericin 1B		
Sericin	TGCACTTTGATTGCGTTGG, ATGTTGGCGGATCGGTTT	418
Chitin synthase		
Chitin	GCCATCTAGTATCTTCACGG, ATCCTTCGGGTTGGTGC	367
Silk gland		
sw13441	TCAGTGATGGGAGTGCT, TGATTCTCCCTGGCTCTG	203
sw15872	GCATATGGCCGTTGAAAACG, TCATCGTCTAGGTTTCAAAGC	148
sw19571	TGACAACGAGGATCTTAGC, CAAAAAGCTCTGTCAAGAGTC	182
sw00692	CAGAACATTCCTCCATACG, CGAAGCAGCATAGGATGC	207
sw01222	GCATTATGTGGAGTATCGTTC, GGAGTATTAAACCGGAAGC	193
sw20990	GAACTCAGTTCACCAGTTTTC, TCGAATTTTAAGTTCTGTAATG	178
sw19374	GTGTCTTTGCAACAGTCTTC, AGCAGTTGTCCAAGAGTC	188
sw20836	GTCTGGCACAATGGTTTG, GAATATCGTTGACGAATAAGCTA	205
sw22099	CAATCTGGATAGTGAAGATGC, GTAACAACTACAACCTTACC	207
Fat body		
sw05710	ACTTGCGATCATAGTTTGC, GTTTGGAAGATCCTTATTGTC	202
Midgut		
sw05300	TTCGACGTTAGCGTTGG, GTAGTAGTAATCGTTGAATCAG	207
sw14336	CTGTGGATGTAGACAATAAGAT, TCATCGGTGAGTTGCATG	193
sw04538	ATGTTCCTTCACACAGAACC, GGTTCTGTGTGAAGGAACAT	204
sw04624	CTACGCATCGCTGTTTC, ACGAGACCAATGGTATCC	206
sw07617	GCTGCAATTAAAGTAACTCTA, CATTGTCACTATCGAAAGC	205
sw19250	GAACATCGCTCAATCTGG, TCTCTCATTTTCCAGGAATGA	205
sw22500	AGTTCAGTCACACTGACG, CTGATAATCCTCGTTTCTG	196
sw22899	GAGTTCCATTCAACAGCATC, TAGTAGCCTTCGAATAAGTC	202

instar larvae were used for the experiments. The dissected tissues were snap-frozen and held in liquid nitrogen for total RNA extraction. Total RNAs were isolated from each tissue sample using the TRIZOL reagent (Invitrogen, CA) according to the manufacturer's instructions. The amounts of RNAs were measured spectrophotometrically by the absorbance at 260 nm. The RNAs were stored at −70°C until use.

Reverse transcription

To detect the specific expression patterns of the candidate genes in other tissues of the silkworm, total RNAs were treated with DN*a*se I for 15 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, CA). 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 2). 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 a 25 ul system containing 12.5 ul of SYBR Premix Ex Taq (Applied Biosystems), 9.5 ul ddH_2O, 0.5 ul/10 pmol primers each, and 2 ul of cDNA. The PCR protocol was conducted using 20-second denaturation at 94°C, 20- second annealing at 52°C, and 60-second elongation at 72°C for 40 cycles. Fluorescence was detected at the end of every 72°Cextension 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 with SYBR Green using ABI7300 Real-time PCR Instrument (Applied Biosystems). The fold changes of gene expressions were determined by comparative C_T method as described in the ABI Prism 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems).

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