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Dosage analysis of Z chromosome genes using microarray in silkworm, *Bombyx mori*

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ABSTRACT

In many organisms, dosage compensation is needed to equalize sex-chromosome gene expression in males and females. Several genes on silkworm Z chromosome were previously detected to show a higher expression level in males and lacked dosage compensation. Whether silkworm lacks global dosage compensation still remains poorly known. Here, we analyzed male:female (M:F) ratios of expression of chromosome-wide Z-linked genes in the silkworm using microarray data. The expression levels of genes on Z chromosome in each tissue were significantly higher in males compared to females, which indicates no global dosage compensation in silkworm. Interestingly, we also found some genes with no bias (M:F ratio: 0.8–1.2) on the Z chromosome. Comparison of male-biased (M:F ratio more than 1.5) and unbiased genes indicated that the two sets of the genes have functional differences. Analysis of gene expression by sex showed that M:F ratios were, to some extent, associated with their expression levels. These results provide useful clues to further understanding roles of dosage of Z chromosome and some Z-linked sexual differences in silkworms.

1. Introduction

Sex chromosomes show striking difference between sexes in many eukaryotes, such as humans, *Drosophila melanogaster* and *Caenorhabditis elegans*. Male-heterogametic species carry a pair of X chromosomes in female and an XY pair in male. Female-heterogametic species have two Z chromosomes in male and a ZW in female. The X and Z chromosomes can carry many genes, but the Y and W chromosomes are often composed mainly of transposable elements and possess few functional genes (Abe et al., 2005; Venter et al., 2001; Adams et al., 2000; International Chicken Genome Sequencing Consortium, 2004). Females and males have a different genomic dosage of sex-chromosome genes. To balance the dosage difference, some species use different molecular mechanisms. Mammals randomly inactivate one of X chromosomes in females and increase the expression of the single active X genes in both sexes to be on par with that of the autosomal genes (Nguyen and Disteche, 2006). *D. melanogaster* increases transcription from the single X in males (Baker et al., 1994; Meller and Kuroda, 2002). In *C. elegans*, hermaphrodites have a pair of sex chromosomes (XX); the males have only one sex chromosome (X0). Hermaphrodites (XX) reduce the level of transcripts from each of their two X chromosomes by half to equal the expression from the single male X (Meyer and Casson, 1986).

The silkworm, *Bombyx mori*, is a female-heterogametic insect. Suzuki et al. (1998) first reported a Z-linked gene, named as *T15.180a*, showed twice as much mRNA transcript from this in males than in females (Suzuki et al., 1998). A second Z-linked gene, *Bmkettin*, had the same expression pattern as *T15.180a* in that it lacked dosage compensation (Suzuki et al., 1999). Recently, expression levels of 13 Z-chromosome-linked genes around the *Bmkettin* locus were detected, and most of them were shown to express more abundant mRNA in males than in females (Koike et al., 2003). In order to determine whether chromosome-wide Z-linked genes in the silkworm are dosage compensated, we tested the mRNA level of Z-chromosome genes between sexes of the silkworm using microarray.

2. Materials and methods

2.1. Animals, tissues and microarray

Gene-expression profiles of silkworm tissues/organs using oligonucleotide microarrays were obtained from our previous study (Xia et al., 2007). Briefly, microarrays were designed based on the draft silkworm genome sequence database, and included probe

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sequences for over 18,000 silkworm genes. The silkworm strain *Dazao* used in this study was provided by the Silkworm Genetic Resource Center of Southwest University, China. In order to investigate gene-expression differences by sex, we prepared male and female samples at the same time. In this research, gene-expression profiles were surveyed in five tissues/organs including anterior/median silk gland (A/MSG), gonads, integument, malpighian tubules and head from silkworm larvae on day 3 of the fifth instar. Biological replicates were done 12 times in A/MSG, 12 times in gonads, 4 times in integument, 8 times malpighian tubules and 4 times in head, respectively.

2.2. Identification of probes located on Z chromosome

A genome-wide microarray with 70-mer oligonucleotide probes used in this study was designed by using the predicted genes from the draft sequence of the silkworm genome and the expressed sequence tags (Xia et al., 2004, 2007). Recently, the complete sequence of the silkworm genome has been finished and newly predicted genes from the complete sequence have also been done (Unpublished). We identified lists of the candidate Z-chromosome probes using BLAST. Z-chromosome genome sequences were used to search the database of the sequences of the probes using BLASTN. Since probes are 70-mer oligonucleotides, all BLAST matches with 100% identity and >35-bp length shows E values from $5E{-}33$ to $1E{-}11$. Thus, we used BLASTN with a relaxed threshold $E < 1e{-}11$. To exclude the hitted probes from all other chromosomes, we did the second BLAST search. High-score probes with ≥99% identity were BLASTNed in the complete sequences of all chromosomes to identify the best hit. If the best hit was the same site of Z chromosome, the probe was confirmed to locate on Z chromosome. Then, the probes were searched to match newly predicted genes from the complete Z-chromosome sequence. If the probes were searched to the same gene, probe IDs were combined to decrease gene redundancy.

2.3. Expression data analysis

We analyzed the male:female gene-expression ratio on five silkworm tissues/organs as mentioned above. We scanned each array with a confocal LuxScan scanner and evaluated the raw intensity value based on the approach of median of pixel intensity at each channel subtract background using LuxScan 3.0 software. Raw data for silkworm genes were background-subtracted before calculating M:F ratios. Weak spots with signal intensities below 400 units and uneven signal were filtered out. To determine which genes showed a sex difference in expression, hybridization to each spot was normalized relative to the mean hybridization of four autosomal house-keeping genes (those encoding proteasome beta subunit, eIF-3 subunit 4, eIF-3A subunit 5, and eIF 4A), and the M:F ratio of gene expression was calculated for each gene and tissue . Considering that global gene-expression changes may exist across different silkworm tissues, we applied a linear normalization method to normalize individual channel data instead of the prevalent LOWESS normalization method for dual-channel microarrays. Three categories were arbitrarily developed in this study: (1) M:F ratio ≥1.5, a ratio of greater than or equal to 1.5 used as a cut-off indicating higher expression in males, which supports no dosage compensation (Craig et al., 2004); (2) M:F ratio between 1.2 and 0.8, indicating relatively equal expression in males and females; and (3) M:F ratio <0.5, indicating higher expression in females. To compare expression levels of Z genes in males and those in females, One-Sample T Test was used to compare the mean score of M:F ratios of Z genes to the population mean of 1. The distribution of M:F ratios on the Z chromosome was analyzed by computing the running average of M:F ratios with a mean length of 30 genes.

2.4. Annotation of gene function

We annotated genes systematically using the following bioinformatics tools. The annotations include homologous searches using BLASTX and functional classifications using Gene Ontologies (GO) (Ashburner et al., 2000). Gene sequences were BLASTXed to nr database in GenBank. GO annotations were assigned using the program Blast2GO (Conesa et al., 2005). After BLASTX, the default settings were used to assign GO terms to gene sequences. From these annotations, analysis was made using 2nd level GO terms based on biological process, molecular function, and cellular component (Ye et al., 2006).

3. Results

3.1. Expression of silkworm genes on Z chromosome

By using Z-chromosome sequences as described above, on microarrays, we identified 697 probes on the Z chromosome. The lists of these probes and their signal intensity after normalization are showed in Table S1. The total of 697 probes represented 579 genes on silkworm Z chromosome owing to some probes recognizing the same gene. We calculated male:female (M:F) gene-expression ratio change in each tissue as shown in Table S2. Microarray detection revealed that active genes are most in the gonads and least in A/MSG, and that majority (65–90%) of genes exhibit M:F ratio higher than 1 in these tissues/organs (Table 1). In addition, the expression ratios of 55% genes are greater than 2 in the gonads while only 3% do in head. To determine whether genes on silkworm Z chromosome in general are expressed at a significantly higher levels in males, we performed T test on expression data. The results were shown in Fig. 1. In each of five samples, the mean of the $\log_2$ M:F is significantly greater than zero (T test: $p = 4.8e{-}28$ for integument, $p = 4.1e{-}28$ for head, $p = 7.5e{-}7$ for malpighian tubules, $p = 0.004$ for A/MSG and $p = 5.7e{-}32$ for gonads), which clearly demonstrates Z-linked genes are expressed at significantly higher levels in males compared to females, indicating silkworm lacks global dosage compensation.

Table 1
Distribution of genes that are differentially expressed between males and females in integument, head, malpighian tubule, A/MSG and gonad.

Tissue/organ	Active genes	Genes with M:F ratio (%)			
		>1.0	>1.2	>1.5	>2.0
Integument	180	162 (90%)	138 (77%)	74 (41%)	20 (11%)
Head	186	158 (85%)	128 (69%)	48 (26%)	5 (3%)
Malpighian tubule	155	115 (74%)	93 (60%)	56 (36%)	26 (17%)
A/MSG	132	86 (65%)	57 (43%)	28 (21%)	10 (8%)
Gonad	314	246 (78%)	222 (71%)	204 (65%)	172 (55%)