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Full Length Research Paper

Differentially expressed genes in white egg 2 mutant of silkworm, *Bombyx mori*, at early embryo development stages

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White egg 2 is one of white egg mutants in silkworm, whose molecular mechanism remains unknown so far. In order to obtain an overall view on gene expression profiles at early embryo development stages, the white egg 2 near-isogenic line was constructed and the whole-genome of silkworm microarray system containing 21375 predicted genes from the silkworm whole genome sequence was employed to investigate gene expression profiles at 0, 24 and 48 h post oviposition between white egg 2 mutant and normal black egg strain. At 24 h post oviposition, 49 genes exhibited at least 2.0 fold differences at expression level, including 24 up-regulated genes and 25 down-regulated genes while at 48 h post oviposition, 52 genes, including 23 up-regulated genes and 29 down-regulated genes were expressed differentially over 2.0 change fold. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that nine differentially expression genes were involved in nine significant ($p<0.05$) pathways at 24 h post oviposition and 24 significant pathways at 48 h post oviposition, respectively. These pathways were related to amino acid metabolism, sugar metabolism, and series of major physiological metabolism. Our results hopefully shed light on the further study of molecular mechanism of white egg 2 mutant.

Key words: *Bombyx mori*, white egg 2 mutant, microarray, embryo, differentially expressed gene.

INTRODUCTION

White egg mutants in silkworm, *Bombyx mori*, exhibit several genotypes defined as white egg 1 (*w-1*), white egg 2 (*w-2*) and white egg 3 (*w-3*), caused by different deficient pigment metabolism *in vivo* (Lu, 1991). The mutant white egg 1 (*w-1*) is characterized by its white eyes and the production of white eggs as a result of its loss of the ninth and tenth exons of kynurenine 3-monooxygenase (KMO, EC1.14.13.9) gene (Quan et al., 2002). The mutant white egg 3 (*w-3*) has white eyes and eggs with translucent larval skin, resulting from a single-base deletion in exon 2 and a premature stop codon at the 5' end of exon 3 (Komoto et al., 2009) while the white egg 2 (*w-2*) has the same phenotypes as white egg 1 and white egg 3 mutants with white egg color, but its mechanism is more complicated than white egg 1 and white egg 3 mutants based on recent report (Tatematsu et al., 2011) which suggest that the silkworm *w-2* locus existed multi-allelic mutations.

As of other insects, the color of the eggs of silkworm mainly depends on the color of the serosa, which is a membrane consisting of flattened polygonal cells, located between the yolk and the shell. The ommochrome pigment production is often accumulated in those specific pigment granules of serosa cells in eggs to produce the color of eggs, a process that involves several enzymes and relevant pathways. The first detected enzymes were kynurenine formamidase (Glassman, 1956) and

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kynureninase (Inagami, 1958) in insect homogenates, which are involved in the kynurenine pathway, and mainly contribute to the ommochrome pigment production. Subsequently, other enzymes such as tryptophan oxygenase (Egelhaaf, 1963a; Baglioni, 1959), kynurenine-3-hydroxylase (Mayer et al., 1968), and kynurenine transaminase (Leibenguth, 1967; Pinamonti et al., 1970) were detected and con-firmed to play essential roles in ommochrome biosynthesis, with any enzyme gene mutated during the evolutionary development in silkworm resulting in blocking or affecting the process of ommochrome bio-synthesis, which finally leads to egg color mutants.

At present, DNA microarray technology is a cost-efficient and high-throughput method for investigating the differentially expressed genes or the gene different expression levels in different samples on the whole-genome scale, such as human (Son et al., 2005; Liu et al., 2009), rat (Walker et al., 2004), fruit fly (Arbeitman et al., 2002), rice (Ma et al., 2005), worm (Jiang et al., 2001), and yeast (DeRisi et al., 1997). It has been employed to successfully investigate gene expression profiles in multiple tissues of the domesticated silkworm using whole-genome oligonucleotide microarray (Xia Q et al., 2007). This microarray is a very efficient tool to investigate the response of the host infected by its pathogen on gene expression level (Luo et al., 2010; Wu et al., 2011). Based on cDNA microarray technology, investigators had recently analyzed gene expression patterns in eggs of silkworm at different stages during embryonic development from 2445 unique ESTs (Hong et al., 2006).In this study, we applied the whole-genome of silkworm microarray to comprehensively screen the differentially expressed genes of white egg 2 mutant compared to normal silkworm strain at early embryo development stages and tried to understand the formation mechanism involved in pathways or the differentially expressed genes undergoing white egg 2 mutation. Hopefully, this will pave the way for clear understanding of the mechanism of the mutant white egg 2 in molecular level and the use of white egg 2 mutant as biomarker in sericulture further research.

MATERIALS AND METHODS

The white egg 2 near-isogenic line construction

The white egg 2 near-isogenic line defined as Jingsong A white was constructed with the black egg sex-limited variety Suluanban as white egg 2 gene donor and the normal black egg variety Jingsong A as back-cross parent. The theoretical homozygous rate between the white egg 2 near-isogenic line and normal black egg back-cross strain was 99% tested by general genetics method after eight backcrosses. All the varieties used in this study were maintained and preserved by close inbreeding in laboratories in Sericultural Research Institute, Chinese Academy of Agricultural Sciences. The silkworm larvae were reared at standard temperature and humidity condition with a photoperiod of 12 h of light and 12 h of dark.

RNA isolation

Egg samples of white egg 2 mutant, Jingsong A white, and normal black egg strain, Jingsong A, were collected at 0, 24 and 48 h time point, respectively after eggs were laid. Total RNA was extracted from eggs using NucleoSpin® RNA II kit (MACHEREY-NAGEL, Germany), according to the manufacturer's protocol. The RNA samples were further purified using DNase (TaKaRa, Japan) to remove potential genomic DNA contamination. Purified RNA was then quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA). The ratio 28 s/ 18 s equal to 1.8 to 2.0 shows the high quality of purified total RNA without degradation. For each experimental condition, three independent samples were collected for microarray analysis.

The 23k silkworm genome array and microarray hybridization

The 23k silkworm genome array used in this study was constructed by Southwest University and CapitalBio Corporation containing 21375 predicted genes from the silkworm whole genome sequence (Xia et al., 2007). 5 µg of RNA for each sample proceeded to the fluorescent dye-labeled cDNA using the mRNA amplification procedure earlier described (Patterson et al., 2006; Guo et al., 2005), then the labeled cDNAs was dissolved in 100 µl of hybridization solution containing 3 × standard saline citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution and 25% formamide, followed by denaturing at 95°C for 3 min before hybridization. The mixed hybridization buffer was loaded onto a microarray slide, and covered with a LifeSlip™ coverslip (Erie Company, Portsmouth, NH, USA). The hybridizations were performed in a hybridization chamber (BioMixerTM, CapitalBio Corp.). After hybridization, slides were washed with washing solution I (0.2% SDS, 2 × SSC) and II (2 × SSC) respectively at 42°C for 5 min. Finally, the arrays were scanned using the Affymetrix GeneChip® Scanner 3000 7G (Affymetrix, Santa Clara, USA).

Detection of differentially expressed genes

After microarray hybridization, sample intensities were quantified using the LuxScan 3.0 image analysis software (CapitalBio). Significant analysis of microarray (SAM) (multiclass, 3.0) was applied to infer the differentially expressed genes between white egg 2 mutant and normal black egg strain. We set fold change >2 or <0.5 and p value <0.05 as cutoff values for differentially expressed genes up-regulated or down-regulated (Tusher et al., 2001).

Gene ontology and pathway analysis of the gene expression

Gene ontology (GO) categories and pathway enrichment analysis were carried out by CapitalBio® MAS software. P value used in a pathway and GO analysis was calculated by a hyper geometric distribution probability formula. P value reflects the importance of GO or the pathway in the experimental results. In the actual analysis, after selection employing the threshold of P value, we acquired the significant pathway and the GO false positive rate, called false discovery rate (FDR). It is more scientific to assign a Q value to each P value to reflect the selection of this P value as the threshold value of the FDR. Pathway and GO in the analysis of statistical results can also be integrated to consider P- and Q-value, according to the specific circumstances of experimental design set differential thresholds for analysis.

RT-PCR based validation

The total RNA for microarray hybridization was also used for RT-

Supplementary Table 1. List of primers of the 15 differentially expressed genes at 24 h post oviposition for RT-PCR validation.

Gene ID	Forward primer	Reverse primer
Sw00132	5'-TCAAGTTCGGTGACCAGTG-3'	5'-GGAAGCGGTCTCCTAACAC-3'
Sw04840	5'-ATCCTCTGACAGCGACTTGA-3'	5'-AGCCACGGGCATAGAAACC-3'
Sw21951	5'-AGTTTACGCCGCTGAGGA-3'	5'-CCACTGGTTGGCCGAGAT-3'
Sw 01081	5'-CTTCCACTCCGACCAAGACT-3'	5'-ATGTAGTAACGGTGGGTGCTT-3'
Sw07899	5'-GCCACCGCAACTGTTATCTC-3'	5'-CAATCCAGCCATCACCAGAC-3'
Sw19836	5'-ATTGCACGCCAGTTAGAAATA-3'	5'-GTTCAAGCGAGACTCCGAT-3'
Sw15246	5'-GAATCCGACTACCGCAAATC-3'	5'-ACAGAAGACGCAGTACCAGA-3'
Sw13395	5'-CGGTTGAGGTAATGGTTT-3'	5'-AAAGGGCAAGGTGAAGTA-3'
Sw14678	5'-CGATTTGACGTACCGCCA-3'	5'-GGACAAAGCTCTTTTTCGC-3'
Sw04534	5'-CGCTTTAATCCAAGAATACG-3'	5'-TCCACTCAATCACCGAATAA-3'
Sw08015	5'-GCCAAAACTCCTAAAATAACA-3'	5'-TGAATATGTGGGCCTTCTA-3'
Sw09721	5'-AAACCTCGTAAACACGTTGT-3'	5'-TTTCAAAAATACTGTCATGGG-3'
Sw10598	5'-TGGCCGACAACGAGAATC-3'	5'-TTAATTGAAGACCTCGCGAC-3'
Sw20824	5'-GTCGTCTAATCAAATGTAGA-3'	5'-GTTATAAACTTACGCAAGTC-3'
Sw20970	5'-AGAAATGAAGGGGTCGC-3'	5'-CTCGCCAATGTCCATGT -3'
*Bm*Actin B	5'-GCGCGGCTACTCGTTCACT-3'	5'-GGATGTCCACGTCGCACTT-3'

PCR validation. The concentration of RNA of each sample was adjusted with DEPC H_2O to a final concentration of 500 ng/µL. A total of 800 ng RNA was reverse transcripted in a 20 µl reaction system using the Prime Script™ RT Reagent Kit (TaKaRa). Quantitative real time PCR was performed using 1 µl of 1:10 diluted first-strand cDNA in a 25 µl reaction volume according to the manufacturer's instructions of the SYBR Premix ExTaqTM (TaKaRa). The specific primers of 15 genes and the endogenous control gene *Bm* Actin B are listed in Supplementary Table 1. The final concentration of the primers was 300 nM. PCR reactions were run in triplicates on an Opticon Lightcycler (BioRad) using thermal cycling parameters at 95°C for 10 s followed by 40 cycles of 95°C for 5 s, 54 to 58°C for 30 s, and 72°C for 7 s. Following amplification, melting curves were constructed. Data were analyzed and normalized to *Bm* Actin B transcript level by the Opticon Monitor Analysis software (MJ Research). A relative quantitative method ($^{\Sigma}$Ct) was used to evaluate relative expression differences.

RESULTS

The white egg 2 near-isogenic line construction

Near-isogenic lines have been applied to study the (Figure 1 A and B). The gene expression profiles of each group exhibited significant differences when normal black egg strain was compared to white egg 2 mutant at 0, 24 and 48 h time points post oviposition. In normal black egg strain, the expression level of 825 genes increased sharply from 0 to 24 h, then remained steady from 24 to 48 h. Yet in white egg 2 mutant, only 449 genes exhibited the similar expression tendency. Especially, 104 genes of relationship between specific biological properties and related genes in many plants, such as rice, tomato, wheat, barley, soybean etc (Young and Tanksley, 1989). In our study, before microarray hybridization, the white egg 2 near-isogenic strain (harbored white egg 2 gene, *w-2*) was constructed with the black egg sex-limited variety Suluanban as white egg 2 gene donor, and the normal black egg variety Jingsong A as recurrent parent. Firstly, the hybrid (F1) was raised between the donor parent and the recurrent parent and subsequently the recurrent parent repeatedly backcrossed. At each backcrossed generation, the progeny was selected for the target gene (*w-2*) of the donor parent prior to each backcrossing. After eight backcrosses, the theoretical homozygous rate between the white egg 2 near-isogenic line and the normal black egg back-cross strain was more than 99%; tested by general genetics method.

Gene expression profiles at the early embryonic development stages

The microarray used in this study was constructed using 21375 predicted genes from the silkworm whole genome sequence (Xia et al., 2007). We attempted to determine the difference of the genes expression profiles at 0, 24 and 48 h time point after egg were laid between white egg 2 mutant and normal black egg strain. We selected a cutoff fold change >2.0 for the up-regulated genes at each of time point, and chose fold change <0.5 for the down-regulated genes (Tusher et al., 2001). Out of the 21375 predicted genes in the microarray, 2055 genes in

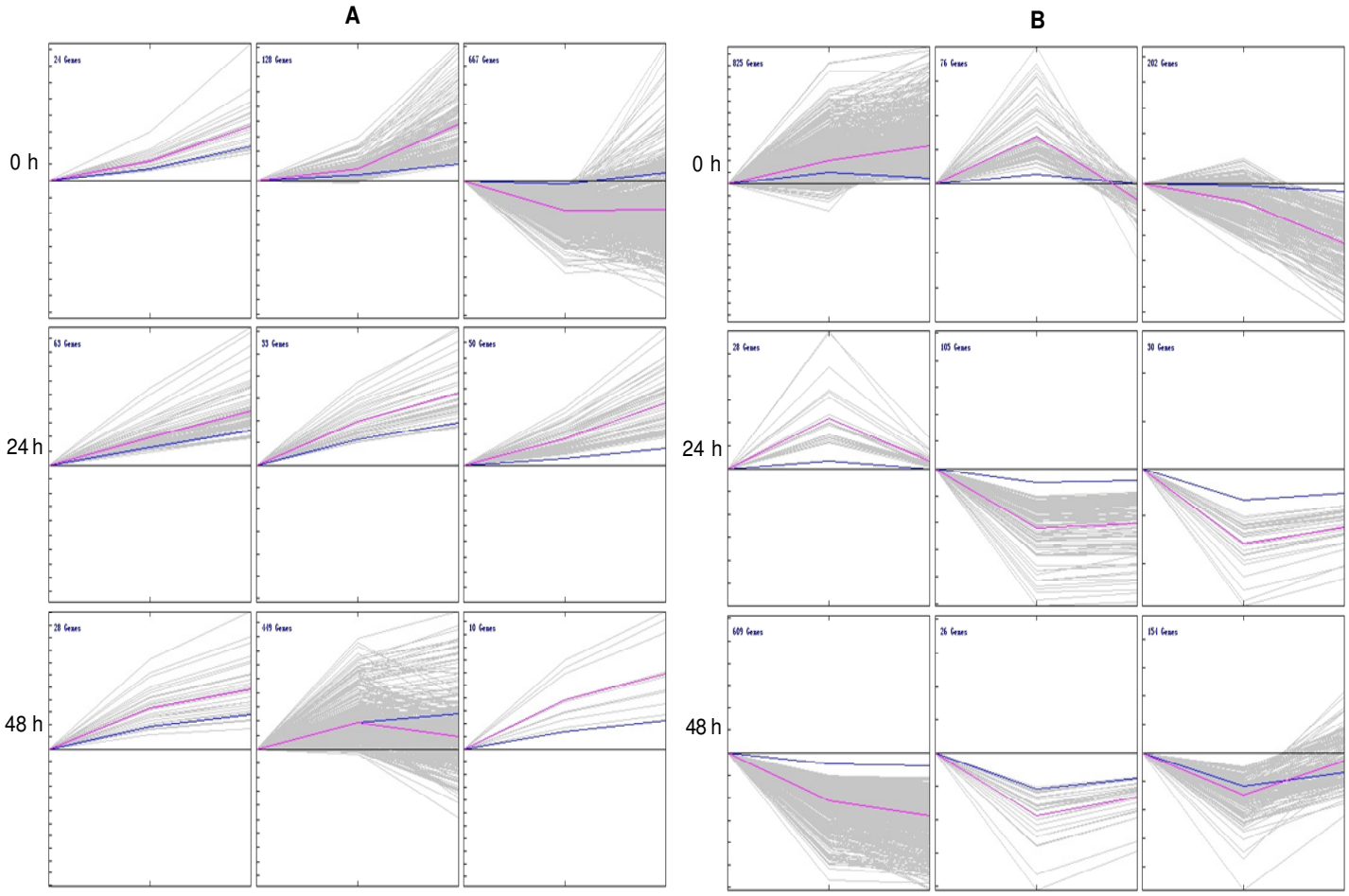

Figure 1. Comparison of genes expression levels in *Bombyx mori* at 0 h, 24 h, and 48 h post oviposition based on SAM analysis. The p value of >2.0 for up-regulated genes and <0.5 for down-regulated genes were used as cutoff standard. (A) White egg 2 mutant; (B) Normal black egg strain.

normal black egg strain and 1452 genes in white egg 2 mutant exhibited significant difference at three time points (data not shown).

Furthermore, 2055 differentially expressed genes of normal black egg strain, and 1452 of white egg 2 mutant were classified to nine groups based on SAM analysis normal black egg strain was expressed increasingly and drastically from 0 to 24 h, but dropped down sharply from 24 to 48 h, in contrast to white egg 2 mutant.

Differentially expressed genes at 24 or 48 h post oviposition between white egg 2 mutant and normal black egg strain

At 24 h post oviposition between normal black egg strain and white egg 2 mutant, the analyses identified a total of 157 genes that were statistically different. Out of them, 80 (50.96%) genes were up-regulated, and 77 (49.04%) genes were down-regulated (Supplementary Table 2). At 48 h time point, 178 genes were expressed differentially between normal black egg strain and white egg 2 mutant. 98 (55.06%) genes were up-regulated and 80 (44.94%) genes were down-regulated respectively (Supplementary Table 3). Among all differentially expressed genes identified by microarray in this study, 40 genes (25.48%) at 24 h time point and 48 genes (26.97%) at 48 h time point were unknown due to the absence of genetic information at present, respectively.

Each data in this study was collected from three repeat samples. The gene expression patterns from three repeat samples at 24 or 48 h each time point between normal black egg strain and white egg 2 mutant were analyzed by Cluster analysis v3.0 software for hierarchical cluster analysis. Figure 2A and B show as a bar diagram the number of genes that were up-regulated or down-regulated at 24 and 48 h time point, respectively. The gene expression patterns of each three repeat samples were quite similar in normal black egg strain or white egg 2 mutant. However, the pattern of gene expression between normal black egg strain and white egg 2 mutant was quite different. On the other hand, there was a good

Supplementary Table 2. List of differentially expressed genes at 24 h post oviposition meared by microarray between white egg 2 mutant and normal black egg strain.

Oligo ID	BGI Gene	Fold Change	q-value (%)	Description	Organism
sw14879	Bmb028436	33.0000	0	High affinity nuclear juvenile hormone binding protein	*Bombyx mori*
sw18857	Bmb031444	15.5682	0	CU18A_LOCMI Cuticle protein 18.6, isoform A (LM-18.6A) (LM-ACP 18.6A)	*B. mori*
sw21951	swu00925	13.6061	0	muscle LIM protein	*B. mori*
sw07571	Bmb002543	11.1555	0	Similar to heterogeneous nuclear ribonucleoprotein methyltransferase-like 4	*Bos taurus*
sw17388	Bmb012614; Bmb027728; Bmb012212	6.7107	0	Actin 6	*Aedes aegypti*
sw05379	Bmb007812	6.4169	0	Unknown	*unknown*
sw19457	Bmb043349	5.9540	0	Unknown	*unknown*
sw18951	Bmb018409	5.7202	0	Unknown	*unknown*
sw12780	Bmb014333	5.5765	0	Unknown	*unknown*
sw22687	Bmb027726; Bmb029431	5.1283	0	Similar to putative alcohol dehydrogenase	*Apis mellifera*
sw14332	Bmb024486	4.6344	0	cAMP responsive element binding protein	*B. mori*
sw14657	Bmb026771	4.2569	0.5662	Unknown	*Unknown*
sw14678	Bmb026943	3.9397	0	Multiple inositol polyphosphate phosphatase 2; MIPP2	*D.melanogaster*
sw19836	Bmb024025	3.8766	0	Nuclear pore complex protein Nup155	*Pan troglodytes*
sw04336	Bmb031948	3.8516	0	Acid-sensitive two pore domain K+ channel dTASK-6	*D. melanogaster*
sw13395	Bmb018121	3.6836	0	Microsomal glutathione transferase GSTMIC1	*Anopheles gambiae*
sw20084	Bmb024309	3.5977	0	Unknown	*Unknown*
sw05952	Bmb017793	3.3972	0	Thioredoxin domain containing 4 (endoplasmic reticulum) (predicted)	*R. norvegicus*
sw18518	Bmb038909	3.3839	0	Glycine rich protein	*B. mori*
sw10697	Bmb001143	3.3569	0	Unknown	*Unknown*
sw17848	Bmb031447	3.3566	0	Similar to splicing factor 3b, subunit 1 isoform 1 isoform 15	*C. familiaris*
sw02199	Bmb035313	3.2333	0	Unknown	*Unknown*
sw14290	Bmb024169	3.1963	0.5662	Similar to male sterility domain containing 1	*C. familiaris*
sw05620	Bmb011740	3.1597	3.1894	Ataxia telangiectasia mutated	*Xenopus laevis*
sw18245	Bmb010729	3.0938	0	Serpin-5B [*Manduca sexta*] serpin-5A	*M. sexta*
sw19898	Bmb033824	3.0125	0.5662	Polyprotein	*D. melanogaster*
sw09940	Bmb038994	2.9302	0.5662	ENSANGP00000026588	*A. gambiae*
sw18643	Bmb034121	2.8913	0	Unknown	*Unknown*
sw21809	swu15685	2.8845	1.3786	Unknown	*Unknown*
sw13994	Bmb022091	2.7672	1.4383	Tropomyosin	*C. kiiensis*
sw07399	Bmb000017	2.7038	2.4524	Unknown	*Unknown*
sw15246	Bmb031311	2.6971	0	Pre-mRNA processing factor 31 homolog	*X. tropicalis*
sw08356	Bmb013598	2.6513	0	Carbonyl Reductase Sniffer Of	*D. Melanogaster*
sw22190	Bmb019140	2.6342	0.5662	SEC63-like	*M. musculus*
sw12528	Bmb012775	2.6247	1.4383	Interferon, gamma-inducible protein 30	*X. tropicalis*
sw12472	Bmb012443	2.6221	0	Similar to polymerase (RNA) II (DNA directed) polypeptide H	*S. purpuratus*
sw18910	Bmb005190	2.5814	0.5662	Transposase homolog	*H. contortus*
sw17430	Bmb024074	2.5618	0	Serine proteinase-like protein 2	*M. sexta*
sw11257	Bmb004667	2.5460	0	Unknown	*Unknown*
sw17412	Bmb019228	2.5058	0	LP20363p	*D. melanogaster*
sw06412	Bmb026625	2.3922	0.5662	Similar to GA10180-PA	*A. mellifera*
sw01065	Bmb015655	2.3847	0.5662	RE24382p	*D. melanogaster*

Supplementary Table 2. Continue

sw06538	Bmb029492	2.3226	0	Similar to zinc finger protein 91 (HPF7, HTF10)	*D. rerio*
sw22223	Bmb021795	2.3133	0	Unknown	*Unknown*
sw14575	Bmb026300	2.3069	0.5662	Antennal esterase	*M. brassicae*
sw14966	Bmb029076	2.3045	0	Eukaryotic translation initiation factor 3 subunit 6	*B. mori*
sw00467	Bmb006688	2.2901	0	Similar to EF-hand domain (C-terminal) containing 1 isoform 1	*B. Taurus*
sw12536	Bmb012815	2.2876	0.9576	CG3790-PA	*D. melanogaster*
sw14012	Bmb022191	2.2769	1.1890	DNA polymerase zeta catalytic subunit	*D. melanogaster*
sw09976	Bmb039484	2.2668	0	Similar to ribosomal protein S6 kinase, 90kDa, polypeptide 5 isoform a	*S. purpuratus*
sw06806	Bmb035128	2.2653	0.5662	Similar to Nuclear pore glycoprotein p62 (62 kDa nucleoporin)	*G. gallus*
sw10798	Bmb001707	2.2590	0.5662	GASP precursor	*D. melanogaster*
sw17215	swu10880	2.2581	0	Unknown	*Unknown*
sw02911	Bmb000374	2.2530	0.5662	Similar to RUN and SH3 domain containing protein 2	*B. Taurus*
sw20362	Bmb036027	2.2378	0.5662	Similar to zinc finger protein 236	*S. purpuratus*
sw15513	Bmb033312	2.2332	0	Unknown	*Unknown*
sw05067	Bmb002365	2.2169	0.5662	Unknown	*Unknown*
sw05717	Bmb013611	2.2058	0	ENSANGP00000004146	*A. gambiae*
sw15861	Bmb035955	2.2050	0.9576	Similar to ENSANGP00000010111	*A. mellifera*
sw17812	Bmb020198	2.1909	0.5662	Solute carrier family 22 (organic cation transporter), member 15	*H. sapiens*
sw00485	Bmb006977	2.1714	0.5662	ENSANGP00000012541	*A. gambiae*
sw11578	Bmb006684	2.1681	2.4524	General transcription factor IIF polypeptide 1 (74kD subunit)	*M. musculus*
sw01720	Bmb026821	2.1629	0.5662	Mo-molybdopterin cofactor sulfurase	*B. mori*
sw07792	Bmb005416	2.1440	0.5662	Unknown	*Unknown*
sw17365	Bmb007487	2.1421	0.5662	Chitinase	*A. aegypti*
sw20668	Bmb039216	2.1004	0	Peroxiredoxin 3	*R. norvegicus*
sw21438	Bmb016644	2.0975	0.5662	Putative ATP synthase beta subunit	*A. pisum*
sw10598	Bmb000514	2.0829	1.1890	Unknown	*Unknown*
sw05448	Bmb008940	2.0829	1.1890	Eukaryotic translation initiation factor 3 subunit 9	*G. gallus*
sw15134	Bmb030451	2.0800	0.5662	Similar to fucosidase, alpha-L- 2, plasma	*S. purpuratus*
sw09451	Bmb030165	2.0769	2.4524	Similar to Hydroxyacid oxidase 1 (HAOX1) (Glycolate oxidase) (GOX) isoform 1	*C. familiaris*
sw18644	Bmb034399	2.0760	4.2835	Urbain	*B. mori*
sw08031	Bmb008736	2.0731	3.1894	GM10395p	*D. melanogaster*
sw11189	Bmb004223	2.0619	0.5662	Unknown	*Unknown*
sw03280	Bmb007620	2.0530	0.5662	Similar to phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	*S. purpuratus*
sw00873	Bmb012612	2.0527	0.5662	Unknown	*Unknown*
sw04534	Bmb037816	2.0410	0.5662	Carbonyl reductase	*P. altivelis*
sw17774	Bmb009456	2.0394	0.5662	Similar to GA17982-PA	*A. mellifera*
sw20001	Bmb009519	2.0362	0.5662	Similar to alanine-glyoxylate aminotransferase 2-like 1	*G. gallus*
sw05071	Bmb002410	2.0134	1.7835	Similar to integral membrane protein 1	*R. norvegicus*
sw02000	Bmb031366	0.4951	0.8917	Cell wall surface anchor family protein	*S. agalactiae*
sw04230	Bmb029407	0.4927	1.3786	COMMD4 protein	*B. mori*
sw11579	Bmb006686	0.4916	0.8917	Dally-like protein	*D. melanogaster*
sw01399	Bmb021146	0.4894	0.8917	Protein phosphatase 1, regulatory subunit 15A	*A. entomopoxvirus*
sw19873	Bmb030383	0.4837	1.7835	Mitochondrial phosphoenolpyruvate carboxykinase 2 isoform 1	*B. Taurus*
sw19262	Bmb006986	0.4811	1.5577	Beta-glucosidase precursor	*S. frugiperda*
sw20246	Bmb014616	0.4794	1.3786	Unknown	*Unknown*
sw07407	Bmb000171	0.4791	0.8917	Ultrabithorax	*J. coenia*

Supplementary Table 2. Continue

sw09563	Bmb031815	0.4768	0	WD repeat and FYVE domain containing 3	*M. musculus*
sw01567	Bmb024065	0.4719	1.2816	Unknown	*Unknown*
sw01551	Bmb023791	0.4715	0.8917	Homologue of Sarcophaga 26,29kDa proteinase	*P. Americana*
sw00121	Bmb001792	0.4714	1.3786	Unknown	*Unknown*
sw21390	Bmb004850	0.4710	0.8917	Unknown	*Unknown*
sw04354	Bmb032271	0.4657	1.2816	Unknown	*Unknown*
sw01808	Bmb028240	0.4640	1.5577	ENSANGP00000019924	*A. gambiae*
sw03787	Bmb019416	0.4633	1.2816	similar to carbonic anhydrase 15	*C. familiaris*
sw22152	Bmb015872	0.4625	0.8917	unknown	*Unknown*
sw01712	Bmb026708	0.4584	0.8917	Inhba	*M. musculus*
sw05622	Bmb011759	0.4576	0.8917	Unknown	*Unknown*
sw20310	Bmb025565	0.4559	1.7835	Soluble guanylyl cyclase beta-3	*M. sexta*
sw03831	Bmb020371	0.4458	2.4524	Similar to molting fluid carboxypeptidase A	*A. mellifera*
sw01523	Bmb023279	0.4457	1.5577	ENSANGP00000014394	*A. gambiae*
sw00391	Bmb005508	0.4404	1.2816	CG14497-PA	*D. melanogaster*
sw01032	Bmb015059	0.4359	1.3786	RE64894p	*D. melanogaster*
sw00579	Bmb008363; Bmb008362	0.4320	0	ENSANGP00000025956	*A. gambiae*
sw12033	Bmb009712	0.4299	0.9576	RE69223p	*D. melanogaster*
sw00812	Bmb011876	0.4297	0	ENSANGP00000010837	*A. gambiae*
sw00203	Bmb002882	0.4249	1.3786	Troponin C 73F	*D. virilis*
sw01798	Bmb028109	0.4206	0.9576	Unknown	*Unknown*
sw07787	Bmb005346	0.4187	0.8917	Hypothetical protein	*M. sexta*
sw08313	Bmb012895	0.4048	1.5577	Similar to thyroid adenoma associated isoform 1	*S. purpuratus*
sw21403	Bmb008644	0.3998	1.3786	Unknown	*Unknown*
sw15202	Bmb030969	0.3953	0.8917	ENSANGP00000008377	*A. gambiae*
sw01721	Bmb026864	0.3892	0	Unknown	*Unknown*
sw19463	Bmb000383	0.3873	0	Ubiquitously transcribed tetratricopeptide repeat, X chromosome, partial	*B. Taurus*
sw22319	Bmb030913	0.3827	0.8917	Unknown	*Unknown*
sw12271	Bmb011179	0.3787	0.8917	CHROMOSOME 6 open reading frame 71	*H. sapiens*
sw01589	Bmb024438	0.3769	1.5577	Mucus-like protein	*G. cydonium*
sw10422	swu10068	0.3767	0	Mitochondrial ribosomal protein L10	*L. testaceipes*
sw05450	Bmb008974	0.3755	1.7835	Neutral endopeptidase 24.11	*B. mori*
sw04660	Bmb041587	0.3702	0.8917	Serine/threonine protein kinase BRAF	*D. rerio*
sw04333	Bmb031898	0.3612	0.8917	Projectin	*P. clarkia*
sw13661	Bmb019926	0.3567	0	Unknown	*Unknown*
sw00288	Bmb004081	0.3525	1.7835	LP05231p	*D. melanogaster*
sw16560	Bmb043324	0.3523	3.3355	AT01548p	*D. melanogaster*
sw07899	Bmb006922	0.3513	0.9576	ENSANGP00000014874	*A. gambiae*
sw12787	Bmb014393	0.3483	0	Proteasome (prosome, macropain) activator subunit 4	*S. purpuratus*
sw04500	Bmb036395	0.3424	0	CG5543-PA	*D. melanogaster*
sw14730	Bmb027341	0.3403	0	Unknown	*Unknown*
sw01043	Bmb015232	0.3374	0.8917	Wing cuticle protein	*L. migratoria*
sw01811	Bmb028272	0.3351	1.3786	Unknown	*Unknown*
sw19333	Bmb031441	0.3269	4.2835	Chondrocyte-derived ezrin-like protein	*P. troglodytes*
sw16327	Bmb040403	0.3244	0.8917	cdk-binding protein	*G. gallus*
sw00916	Bmb013327	0.3151	0	ENSANGP00000026666	*A. gambiae*
sw00092	Bmb001446	0.3035	0	KIAA1736 protein	*H. sapiens*
sw15126	Bmb030382	0.2988	0	Serologically defined colon cancer antigen 13	*S. purpuratus*
sw00132	Bmb001949	0.2959	1.7835	ALdehyde deHydrogenase family member (alh-9)	*C. elegans*
sw20240	Bmb014222	0.2941	0.8917	Unknown	*Unknown*

Supplementary Table 2. Continue

sw11812	Bmb008256	0.2918	0.8917	Unknown	*Unknown*
sw22513	Bmb019594; Bmb003381	0.2834	0	Venom proteinase (EC 3.4.21.-)	*A. mellifera*
sw20970	Bmb015873	0.2825	0	ARM_MUSDO Armadillo segment polarity protein armadillo protein	*B. mori*
sw01851	Bmb028809	0.2540	0	C-type lysozyme	*G. morsitans*
sw10080	Bmb042171	0.2398	0	Adrenodoxin reductase	*A. aegypti*
sw22044	Bmb006297	0.2343	0	ENSANGP00000011385	*A. gambiae*
sw08326	Bmb013051	0.2282	4.2835	Unknown	*Unknown*
sw14002	Bmb022136	0.1872	0	Proboscipedia ortholog	*T. castaneum*
sw15636	Bmb034219	0.1813	1.5577	La related protein	*D. melanogaster*
sw18073	Bmb032136	0.1801	0.8917	chromosome 10 open reading frame 42	*G. gallus*
sw13432	Bmb018358	0.1717	0	cDNA clone E_EL_fcP8_19A01_R_0 3'	*B. mori*
sw17991	Bmb019426	0.1596	0	cDNA clone E_EL_fcP8_04G06_R_0 3'	*B. mori*
sw08438	Bmb014684	0.1447	0	Unknown	*Unknown*
sw01593	Bmb024458	0.1434	0	Origin recognition complex subunit 4	*D. melanogaster*
sw21758	swu05701	0.1433	1.7835	Unknown	*Unknown*
sw06613	Bmb030914	0.1363	0	RE22242p	*D. melanogaster*
sw19840	Bmb024593	0.1193	0	CG5621-PA	*D. melanogaster*
sw13551	Bmb019155	0.1101	0	Unknown	*Unknown*
sw12154	Bmb010503	0.1004	0	A disintegrin and metalloproteinase with thrombospondin motifs like	*A. mellifera*

Table 1. Gene categories of differentially expressed genes at 24 h post oviposition.

Gene category	Number of genes	Percent (%)
Molecular function		
Catalytic activity	19	24.17
Binding	13	16.54
Transcription regulator activity	4	5.09
Biological process		
Physiological process	16	13.45
Cellular process	15	12.61
Metabolism	15	12.61
Biological regulation	3	2.52
Regulation of biological process	3	2.52
Negative regulation of biological process	1	0.84
Biological adhesion	1	0.84
Cellular component	9	6.62

reproducibility between samples, and the genes were successfully distinguished between white egg 2 mutant and normal black egg strain.

Analysis of differentially expressed gene ontology

To investigate the global differences at gene level, the gene ontology (GO) hierarchy analysis was carried out on the differentially expressed genes. The genes were categorized according to CapitalBio Molecule Annotation System (MAS) software (MAS 3.0) (http://bioinfo.capitalbio.com/mas3). The MAS software was developed to integrate all differential gene expression datasets, and defines those GO categories that share a similar functional role from the differentially expressed genes according to a gene enrichment principle. Three GO terms (biological process, cell component, and molecular function) were evaluated in our study. P value <0.05 was considered statistically significant.

At 24 h post oviposition, 102 differentially expressed genes had annotations according to MAS analysis (Table

Supplementary Table 3. List of differentially expressed genes at 48 h post oviposition meared by microarray between white egg 2 mutant and normal black egg strain.

Oligo ID	BGI Gene	Fold Change	q-value (%)	Description	Organism
sw22666	Bmb026840	14.5167	0	Hypothetical protein XP_397032	*A. mellifera*
sw14816	Bmb027992	12.4449	0	CG1561-PA, isoform A	*D. melanogaster*
sw11607	Bmb006921	11.3448	0	Chemokine-like factor super family 4	*M. musculus*
sw15620	Bmb034101	10.2182	0	CG13813-PA	*D. melanogaster*
sw18046	Bmb027675	9.6848	0	Putative protein, with at least 6 transmembrane Domains, of ancient origin (58.5 kD) (3N884)	*S. purpuratus*
sw19457	Bmb043349	8.8177	0	Unknown	*unknown*
sw05363	Bmb007606	8.6616	0	Unknown	*unknown*
sw14669	Bmb026839	8.4499	0	Putative protein, with at least 6 transmembrane Domains, of ancient origin (58.5 kD) (3N884)	*S. purpuratus*
sw05379	Bmb007812	8.1543	0	Unknown	*unknown*
sw17455	Bmb029210	7.8514	0	Unknown	*unknown*
sw03311	Bmb008122	7.1548	0	Antitrypsin precursor	*B. mori*
sw19859	Bmb028204	6.2172	0	Hemicentin 1	*M. musculus]*
sw17796	Bmb016373	5.8355	0	Unknown	*unknown*
sw01040	Bmb015193	5.5572	0	Unknown	*unknown*
sw10603	Bmb000545	5.1777	0	Unknown	*unknown*
sw17602	Bmb013104	4.4970	0	Unknown	*unknown*
sw10441	swu11399	4.3924	0	Unknown	*unknown*
sw03212	Bmb006210	4.2639	0	Unknown	*unknown*
sw03565	Bmb014157	4.1932	0	Unknown	*unknown*
sw03504	Bmb012669	4.0851	0	Novel protein	*X. tropicalis*
sw12920	Bmb015191	4.0735	0	120-kDa protein	*S. peregrina*
sw19849	Bmb026530	3.9738	0	Polyprotein	*B. mori*
sw20070	Bmb021738	3.5816	0	ENSANGP00000015204	*A. mellifera*
sw02199	Bmb035313	3.5592	0	Unknown	*unknown*
sw14252	Bmb023817	3.5553	0	Zinc finger MYND domain containing protein 10 (BLu protein)	*S. purpuratus*
sw21980	Bmb001284	3.2173	0	Reverse transcriptase family member (XS300), partial	*S. purpuratus*
sw04534	Bmb037816	3.2168	0.8726	Carbonyl reductase	*P. altivelis*
sw17812	Bmb020198	3.1408	0	Solute carrier family 22 (organic cation transporter), member 15	*H. sapiens*
sw17130	swu07146	3.1099	1.2529	Unknown	*unknown*
sw15491	Bmb033104	3.0691	0	ENSANGP00000015052	*A. gambiae*
sw09612	Bmb032783	3.0336	0.8726	Unknown	*unknown*
sw11643	Bmb007234	2.9878	0	DGP-1 protein	*D. melanogaster*
sw15855	Bmb035939	2.9850	0.6108	Unknown	*unknown*
sw14331	Bmb024480	2.9384	0.8726	OMBP_MANSE Ommochrome-binding protein precursor (OBP) (YCP) ommochrome-binding protein	
sw01506	Bmb022944	2.9368	0	VACUOLAR protein sorting 11	*A. mellifera*
sw13775	Bmb020674	2.9194	0	Putative alcohol dehydrogenase	*A. mellifera*
sw13900	Bmb021430	2.9177	0	CG33290-PA	*D. melanogaster*
sw22124	Bmb008872	2.8649	0	Chromosome 2, BAC clone 081P21	*B. mori*
sw18473	Bmb035079	2.8380	4.1102	Rotatin	*M. musculus*
sw11130	Bmb003777	2.8311	0	Putative anticoagulant peptide AP1	*B. martensii*
sw16854	Bmb029919	2.8165	0	Glycerophosphoryl diester phosphodiesterase	*B. mori*
sw08648	Bmb017403	2.7945	1.2529	Glucose oxidase [Apis mellifera] glucose oxidase	*A. mellifera*
sw06553	Bmb029756	2.7931	0.8726	Whn	*G. gallus*
sw06806	Bmb035128	2.7038	0.8726	Nuclear pore glycoprotein p62 (62 kDa nucleoporin)	*G. gallus*
sw09118	Bmb024723	2.6716	0.8726	ENSANGP00000014129	*A. gambiae*

Supplementary Table 3. Continue

sw17818	Bmb022496	2.6291	1.2529	Unknown	*unknown*
sw02266	Bmb036883	2.6183	0.6108	Unknown	*unknown*
sw03770	Bmb019103	2.6071	1.2529	5,10-Methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase)	*H. sapiens*
sw11471	Bmb006018	2.6057	0.8726	Cytochrome b561 (Cytochrome b-561)	*D. rerio*
sw21414	Bmb010186	2.5945	1.2529	Unknown	*unknown*
sw08900	Bmb021285	2.5879	3.6781	CG14321-PA	*D. melanogaster*
sw20571	Bmb023821	2.5826	4.1102	Endonuclease and reverse transcriptase-like protein	*B. mori*
sw03040	Bmb002768	2.5065	0.8726	Novel protein	*R. norvegicus*
sw10995	Bmb002885	2.4918	0	GTP binding protein 2	*G. gallus*
sw08562	Bmb016310	2.4868	0.8726	Unknown	*unknown*
sw08949	Bmb022058	2.4738	2.2728	CG17127-PA	*D. melanogaster*
sw14829	Bmb028107	2.4450	0	Transmembrane transporter	*B. mori*
sw00467	Bmb006688	2.4450	4.1102	EF-hand domain (C-terminal) containing 1 isoform 1	*B. taurus*
sw09376	Bmb028849	2.4132	1.2529	Similar to arrow, partial	*A. mellifera*
sw12528	Bmb012775	2.3634	0.8726	Interferon, gamma-inducible protein 30	*X. tropicalis*
sw08305	Bmb012773	2.3513	3.6781	Unknown	*unknown*
sw19836	Bmb024025	2.3386	0.8726	Nuclear pore complex protein Nup155	*P. troglodytes*
sw11637	Bmb007188	2.3365	1.2529	Beta-glucosidase	*N. koshunensis*
sw20527	Bmb016879	2.3326	1.2529	Unknown	*unknown*
sw22151	Bmb015744	2.3165	1.2529	Unknown	*unknown*
sw17816	Bmb021741	2.3116	0.8726	CCR4-NOT transcription complex, subunit 1 isoform a	*H. sapiens*
sw06242	Bmb023470	2.3031	0.8726	Glutathione S-transferase 1	*Bombyx mori*
sw12254	Bmb011068	2.2875	3.6781	Unknown	*unknown*
sw04192	Bmb028430	2.2682	4.1102	Carboxypeptidase Vitellogenic carboxypeptidase precursor	*B. mori*
sw03148	Bmb004849	2.2646	0.8726	Odorant receptor 83b, partial	*A. mellifera*
sw13847	Bmb021130	2.2373	1.2529	LYS_BOMMO Lysozyme precursor (1,4-beta-N-acetylmuramidase) lysozyme	*B. mori*
sw14339	Bmb024531	2.2363	2.2728	Cell wall surface anchor family protein	*S. pneumoniae*
sw17630	Bmb018507	2.2288	2.2728	CF105_MESAU Protein C6orf105 homolog	*B. mori*
sw17679	Bmb032274	2.2116	1.2529	Similar to phosphoglucomutase 2	*B. taurus*
sw09921	Bmb038653	2.2077	3.6781	Glutathione S-transferase 1	*B. mori*
sw14500	Bmb025771	2.2013	1.2529	Unknown	*unknown*
sw06727	Bmb033245	2.1999	4.1102	Unknown	*unknown*
sw00700	Bmb010188	2.1909	4.1102	Unknown	*unknown*
sw05034	Bmb001719	2.1864	2.2728	cDNA clone E_EL_ovS0_23G10_F_0 5'	*D. simulans*
sw11152	Bmb003983	2.1777	0.8726	MBF2	*B. mori*
sw03396	Bmb010184	2.1751	1.2529	Unknown	*unknown*
sw22244	Bmb023779	2.1687	4.1102	D4, zinc and double PHD fingers family 2	*X. tropicalis*
sw06509	Bmb028730	2.1678	0.8726	Unknown	*unknown*
sw14725	Bmb027295	2.1406	1.2529	Unknown	*unknown*
sw08454	Bmb014882	2.1137	2.2728	Solute carrier family 26, member 11	*B. taurus*
sw12948	Bmb015359	2.1024	1.2529	Unknown	*unknown*
sw06173	Bmb022240	2.1011	1.2529	Similar to ENSANGP00000015052	*A. mellifera*
sw22467	swu16037	2.1010	1.2529	Contains weak similarity to GATA-6 DNA-binding protein	*A. thaliana*
sw15731	Bmb035060	2.0919	3.6781	Similar to transcriptional co-repressor Sin3A	*S. purpuratus*
sw08469	Bmb015032	2.0866	3.6781	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 2, partial	*B. taurus*
sw20327	Bmb028549	2.0795	3.6781	Prophenoloxidase activating proteinase-2	*M. sexta*
sw07486	Bmb001246	2.0563	3.6781	Unknown	*unknown*
sw19098	Bmb020004	2.0502	1.2529	Hypothetical protein GLP_170_76141_77547	*G. lamblia*

Supplementary Table 3. Continue

sw03875	Bmb021300	2.0464	1.2529	Unknown	*unknown*
sw10581	Bmb000368	2.0435	0.8726	Unknown	*unknown*
sw04558	Bmb038315	2.0431	0.8726	Heterogeneous nuclear ribonucleoprotein F	*M. musculus*
sw13878	Bmb021312	2.0331	0.8726	Similar to Eyes absent homolog 4 isoform 2	*B. taurus*
sw00225	Bmb003317	2.0280	2.2728	mKIAA0979 protein	*M. musculus*
sw21144	Bmb025384	0.4983	1.6706	DRPE65	*D. melanogaster*
sw01653	Bmb025563	0.4962	1.6706	Solute carrier family 2 (facilitated glucose transporter), member 1	*G. gallus*
sw20747	Bmb003575	0.4961	3.6781	Histone H2A-like protein	*B. mori*
sw12880	Bmb014924	0.4918	4.1102	Similar to lipidosin	*C. familiaris*
sw20824	Bmb007456	0.4860	2.2728	Hypothetical protein MCAP_0861	*M. capricolum*
sw04041	Bmb025306	0.4799	3.6781	ENSANGP00000019864	*A. gambiae*
sw11768	Bmb007935	0.4727	1.0739	ENSANGP00000020967	*A. gambiae*
sw03530	Bmb013207	0.4675	2.2728	Aquaporin-1	*G. gallus*
sw01378	Bmb020850	0.4652	4.1102	Similar to WD repeat domain 34	*A. mellifera*
sw18578	Bmb020390	0.4533	1.6706	Clb2p: G2/Mitotic-specific cyclin 2 (Swiss Prot. accession number P24869)	*S. cerevisiae*
sw00642	Bmb009381	0.4514	1.6706	Similar to glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1	*G. gallus*
sw22239	Bmb023386	0.4495	3.6781	HRPX_PLALO Histidine-rich glycoprotein precursor histidine-rich protein	*P. lophurae*
sw01851	Bmb028809	0.4486	3.6781	C-type lysozyme	*G. morsitans*
sw16325	Bmb040396	0.4423	1.6706	Unknown	*unknown*
sw18898	Bmb002839	0.4414	1.6706	Heat shock protein hsp20.8	*B. mori*
sw10080	Bmb042171	0.4390	1.6706	Adrenodoxin reductase	*A. aegypti*
sw20551	Bmb020417	0.4369	3.6781	Reverse transcriptase	*B. mori*
sw04343	Bmb032134	0.4313	3.6781	24-Dehydrocholesterol reductase	*H. sapiens*
sw12464	Bmb012391	0.4308	1.6706	Unknown	*unknown*
sw18670	Bmb002840	0.4268	1.0739	Heat shock protein hsp 19.9	*B. mori*
sw13661	Bmb019926	0.4260	1.0739	Unknown	*unknown*
sw09379	Bmb028908	0.4249	1.0739	Similar to tubulointerstitial nephritis antigen	*G. gallus*
sw09693	Bmb034140	0.4221	3.6781	Histone H1	*B. mori*
sw01856	Bmb028866	0.4217	1.6706	SD27140p [Wolbachia endosymbiont of *Drosophila ananassae*]	*D. ananassae*
sw08084	Bmb009643	0.4192	1.6706	Unknown	*unknown*
sw14235	Bmb023706	0.4134	1.6706	LP09268p	*D. melanogaster*
sw12541	Bmb012836	0.4124	1.6706	Glycine rich protein	*B. mori*
sw22859	Bmb040666	0.4116	1.6706	Similar to mitochondrial ribosomal protein L2	*S. purpuratus*
sw00461	Bmb006574	0.4081	0	LOC496020 protein	*X. laevis*
sw12615	Bmb013262	0.4049	1.0739	Pyruvate dehydrogenase phosphatase regulatory subunit precursor	*B. taurus*
sw22513	Bmb019594	0.4004	1.0739	Similar to venom proteinase (EC 3.4.21.-)	*A. mellifera*
sw10983	Bmb002809	0.3957	1.6706	ENSANGP00000009437	*A. gambiae*
sw22044	Bmb006297	0.3934	0	ENSANGP00000011385	*A. gambiae*
sw09721	Bmb034697	0.3927	2.2728	Unknown	*unknown*
sw05319	Bmb006900	0.3901	1.6706	Similar to ENSANGP00000015190	*A. mellifera*
sw11189	Bmb004223	0.3853	1.6706	Unknown	*unknown*
sw21900	swu16328	0.3794	1.0739	similar to carcinoma related gene	*S. purpuratus*
sw05002	Bmb000881	0.3721	0	GA15131-PA	*D. pseudoobscura*
sw11662	Bmb007296	0.3703	3.6781	BSDC1 protein	*H. sapiens*
sw09712	Bmb034569	0.3697	0.6108	Mitochondrial aldehyde dehydrogenase	*B. mori*
sw15254	Bmb031389	0.3695	1.0739	Unknown	*unknown*
sw13432	Bmb018358	0.3684	1.0739	cDNA clone E_EL_fcP8_19A01_R_0 3'	*B. mori*

Supplementary Table 3. Continue

sw08094	Bmb009770	0.3679	1.6706	Similar to Myosin, light polypeptide kinase, telokin isoform	*R. norvegicus*
sw01324	Bmb019960	0.3645	0.6108	Putative dodecenoylCoA deltaisomerase	*A. gambiae*
sw08015	Bmb008533	0.3639	0	RE70136p	*D. melanogaster*
sw04656	Bmb041509	0.3487	0	Myb-MuvB complex subunit Lin-52	*B. mori*
sw04354	Bmb032271	0.3440	1.0739	Unknown	*unknown*
sw22659	Bmb026637	0.3341	0	Unknown	*unknown*
sw07613	Bmb003176	0.3299	1.6706	Unknown	*unknown*
sw17736	Bmb020390	0.3181	0	Clb2p: G2/Mitotic-specific cyclin 2 (Swiss Prot. accession number P24869)	*S. cerevisiae*
sw15548	Bmb033549	0.3102	0	Similar to 1-acylglycerol-3-phosphate O-acyltransferase 1	*S. purpuratus*
sw12787	Bmb014393	0.3099	0	Similar to proteasome (prosome, macropain) activator subunit 4	*S. purpuratus*
sw14567	Bmb026253	0.2982	0	Myb-MuvB complex subunit Lin-52	*B. mori*
sw14802	Bmb027878	0.2926	0	Unknown	*unknown*
sw09365	Bmb028558	0.2860	0	H3/o protein	*H. sapiens*
sw15056	Bmb029785	0.2831	0	ARPP-21 protein	*H. sapiens*
sw09089	Bmb024185	0.2817	0	Similar to lipase, member I	*M. musculus*
sw00969	Bmb014154	0.2785	0	Unknown	*unknown*
sw20134	Bmb036012	0.2652	0	GBB1_HOMAM Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1	*H. americanus*
sw11837	Bmb008373	0.2459	1.0739	CG5953-PB, isoform B	*D. melanogaster*
sw20970	Bmb015873	0.2400	0	ARM_MUSDO Armadillo segment polarity protein armadillo protein	*B. mori*
sw18806	Bmb039008	0.2253	0	ENSANGP00000020978	*A. gambiae*
sw08314	Bmb012904	0.2173	0	RE74861p	*D. melanogaster*
sw22291	Bmb028604	0.2146	0	arylalkylamine N-acetyltransferase	*P. americana*
sw19170	swu07281	0.1898	0	Unknown	*unknown*
sw19840	Bmb024593	0.1845	0	CG5621-PA	*D. melanogaster*
sw12540	Bmb012821	0.1818	0	Wdr22 protein	*M. musculus*
sw15828	Bmb035727	0.1811	0	Unknown	*unknown*
sw01081	Bmb015839	0.1805	0	RE67575p	*D. melanogaster*
sw00132	Bmb001949	0.1738	0	ALdehyde deHydrogenase family member (alh-9)	*C. elegans*
sw01581	Bmb024372	0.1645	0	Cytochrome P450	*B. mori*
sw11055	Bmb003256	0.1509	0	Cellular repressor of E1A-stimulated genes	*H. sapiens*
sw06613	Bmb030914	0.1462	0	RE22242p	*D. melanogaster*
sw14730	Bmb027341	0.1267	0	Unknown	*unknown*
sw12154	Bmb010503	0.1062	0	Similar to A disintegrin and metalloproteinase with thrombospondin motifs like	*A. mellifera*
sw09321	Bmb027794	0.0876	0	Alpha trypsin	*D. erecta*
sw22208	Bmb020773	0.0866	0	Similar to integrase, catalytic domain containing protein family member (2N547)	*S. purpuratus*
sw01593	Bmb024458	0.0835	0	Origin recognition complex subunit 4	*D. melanogaster*
sw07448	Bmb000676	0.0827	0	ORF61 protein	*M. musculus*
sw04017	Bmb024762	0.0658	0	General odorant binding protein 1	*B. mori*

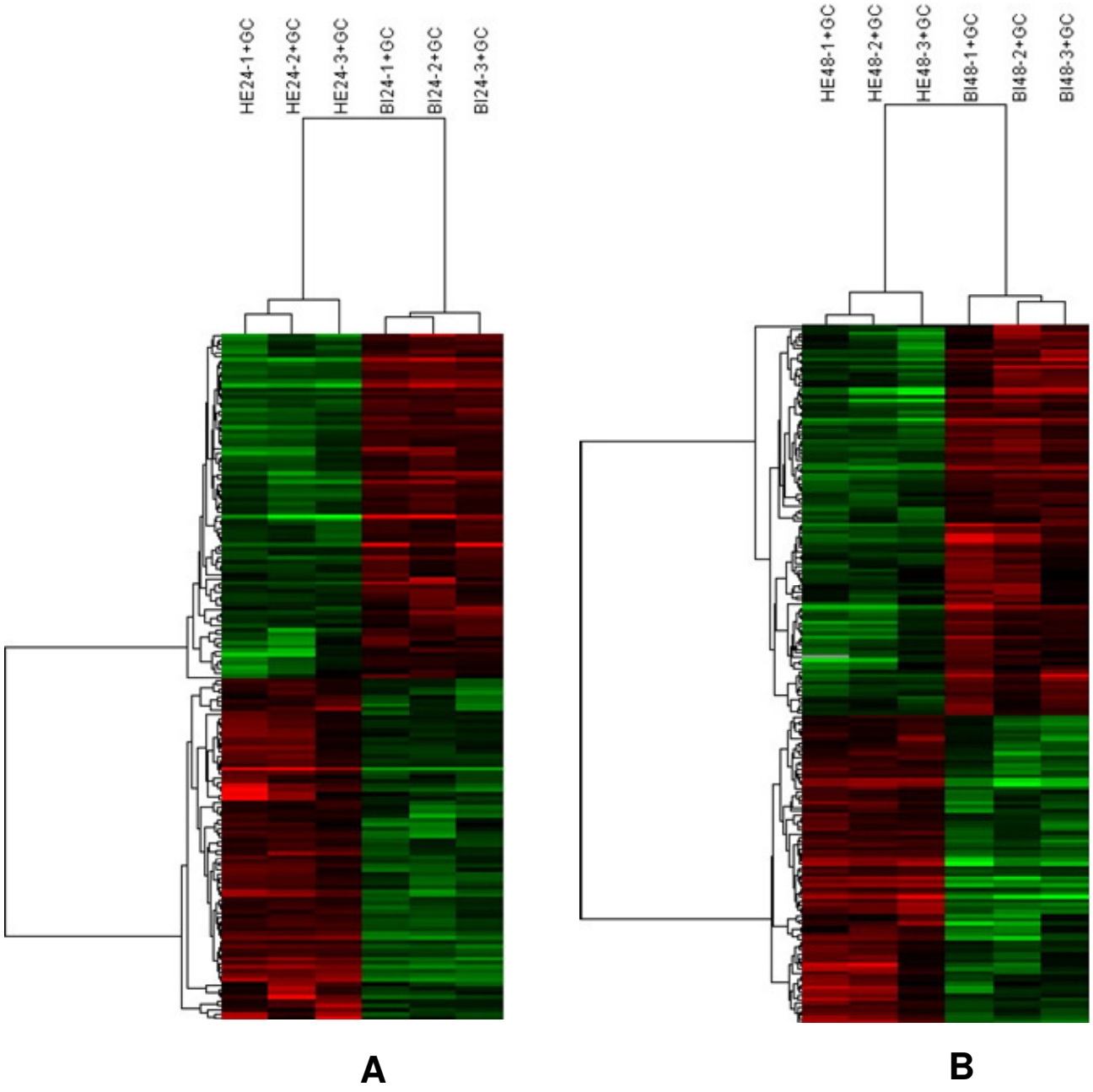

Figure 2. Hierarchical cluster analysis of genes characteristically expressed at 24 h and 48 h post oviposition with three repeats. Columns represent three repeats at each time point, rows present gene expression profile. A, 24 h; B, 48 h. (HE presents normal black egg strain; BI presents white egg 2 mutant).

1), and 37 genes were classified into four subgroups of molecular function, described as catalytic activity (19, 24.17%), binding (13, 16.54%), and transcription regulator activity (4, 5.09%). 56 genes were classified into eight subgroups of biological process, described as physiological process (16, 13.45%), cellular process (15, 12.61%), metabolism (15, 12.61%), biological regulation (3, 2.52%), regulation of biological process (3, 2.52%), negative regulation of biological process (1, 0.84%), andbiological adhesion (1, 0.84%). Nine genes were classified into cellular component (6.62%) (Figure 3A). Moreover, at 48 h post oviposition, 136 differentially expressed genes had annotations and were classified into five subgroups of molecular function, seven subgroups of biological process, and ten genes were cellular component (Table 2). Five subgroups were described as catalytic activity (22, 20.95%), binding (17, 16.19%), transporter activity (5, 4.76%), molecular transducer activity (2, 1.90%) and enzyme regulator activity (2, 1.90%). Seven subgroups of biological process were

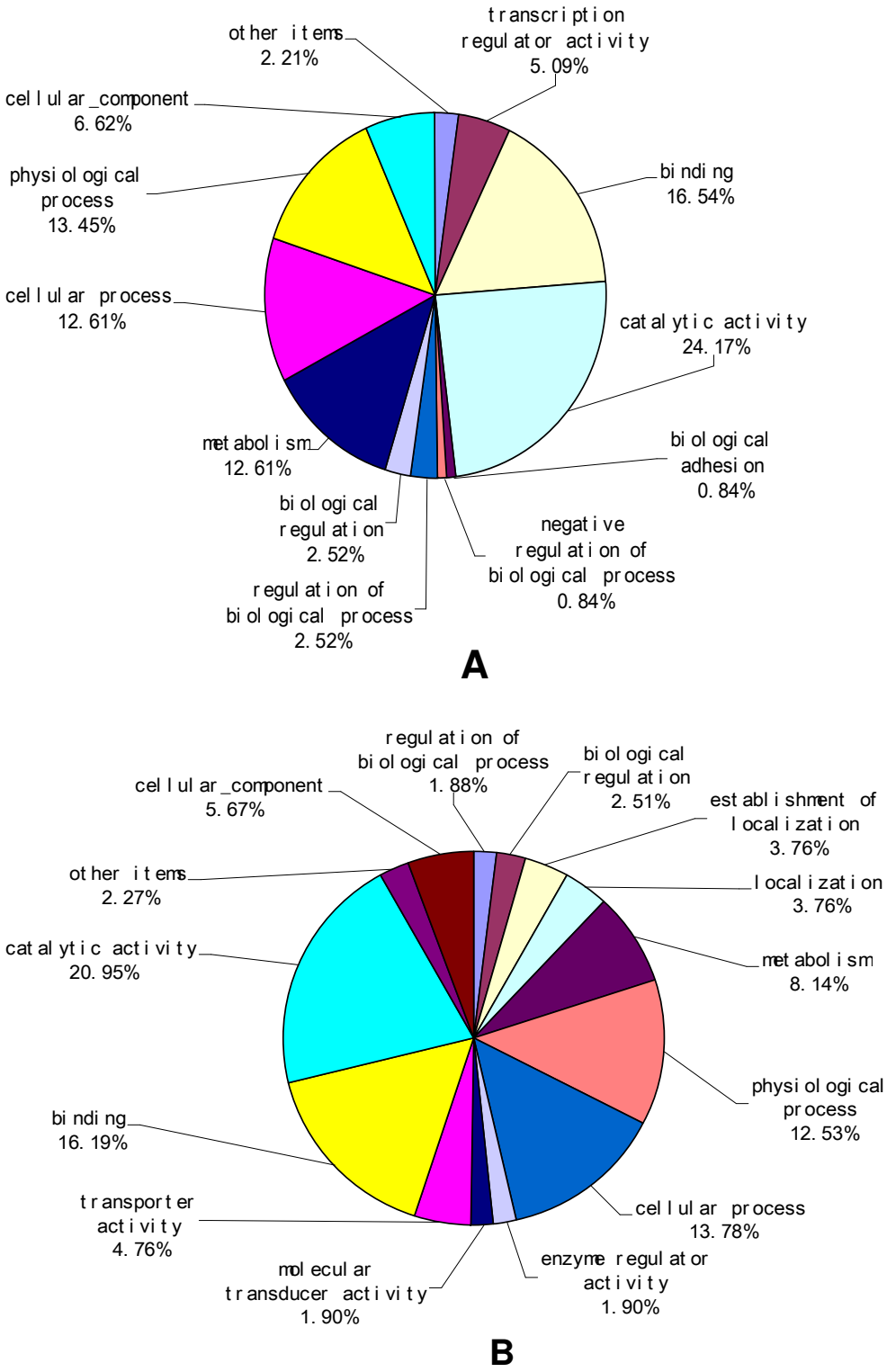

Figure 3. Gene categories of differentially expressed genes at 24 h and 48 h post oviposition according to GO (http://www/geneontology.org/) analysis. A, Gene categories at 24 h post oviposition. Among those genes, 37 genes were classified into four subgroups of molecular function, 56 genes were classified into eight subgroups of biological process, nine genes were classified into cellular component. B, Gene categories of differentially expressed genes at 48 h post oviposition. 136 differentially expressed genes had annotations and classified intof ive subgroups of molecular function, seven subgroups of biological process, and ten genes were cellular component.

Table 2. Gene categories of differentially expressed genes at 48 h post oviposition.

Gene category	Number of genes	Percent (%)
Molecular function		
Catalytic activity	22	20.95
Binding	17	16.19
Transporter activity	5	4.76
Molecular transducer activity	2	1.90
Enzyme regulator activity	2	1.90
Biological process		
Cellular process	22	13.78
Physiological process	20	12.53
Metabolism	13	8.14
Localization	6	3.76
Establishment of localization	6	3.76
Biological regulation	4	2.51
Regulation of biological process	3	1.88
Cellular component	10	5.67

Table 3. Significant KEGG pathways at 24 h post oviposition.

KEGG Pathway	P-value	Number of genes
Glycosaminoglycan degradation	0.001166	1
Phenylalanine, tyrosine and tryptophan biosynthesis	0.001166	1
Pentose and glucuronate interconversions	0.001632	1
Homologous recombination	0.001632	1
Drug metabolism - other enzymes	0.002098	1
Porphyrin and chlorophyll metabolism	0.002564	1
Starch and sucrose metabolism	0.003729	1
Aminoacyl-tRNA biosynthesis	0.004892	1
Pyrimidine metabolism	0.007447	1
Metabolism	0.092528	1
Total		10

included: cellular process (22, 13.78%), transducer activity, enzyme regulator activity, localization, and establishment of localization, were only detected in the ones at 48 h post oviposition.

Analysis of differentially expressed gene pathway

The differentially expressed genes at 24 and 48 h post oviposition were analyzed by CapitalBio MAS software for a pathway-based analysis to identify known pathways such as those in the KEGG (http://www.genome.jp/kegg), Biocarta (http://www.biocarta.com), and GenMAPP. A total of nine differentially expressed genes at 24 h post oviposition based on the KEGG database analysis were involved in nine pathways with a P-value cutoff of less than 0.05, including glycosaminoglycan degradation, phenylalanine, tyrosine and tryptophan biosynthesis, pentose and glucuronate interconversions, homologous recombination, drug metabolism-other enzymes, porphyrin and chlorophyll metabolism, starch and sucrose metabolism, and aminoacyl-tRNA biosynthesis, except metabolism pathway with the p value 0.092528 (Table 3). Compared with differentially expressed genes at 24 h post oviposition, more than 26 differentially expressed genes were involved in 24 significant pathways with a P-value less than 0.05 at 48 h post oviposition. These pathways include metabolism, synthesis and degradation of ketone bodies, benzoate degradation via CoA ligation, pantothenate and CoA biosynthesis, drug metabolism-other enzymes, TGF-beta signaling pathway, galactose metabolism, inositol phosphate metabolism, starch and sucrose metabolism, fructose and mannose metabolism, amino-sugars metabolism, beta-alanine metabolism,

Table 4. Significant KEGG pathways at 48 h post oviposition.

KEGG pathway	P-value	Number of genes
Metabolism	0.0001320	3
Synthesis and degradation of ketone bodies	0.0011664	1
Benzoate degradation via CoA ligation	0.0017492	1
Pantothenate and CoA biosynthesis	0.0017492	1
Drug metabolism - other enzymes	0.0026229	1
TGF-beta signaling pathway	0.0034960	1
Galactose metabolism	0.0034960	1
Inositol phosphate metabolism	0.0037869	1
Starch and sucrose metabolism	0.0046592	1
Fructose and mannose metabolism	0.0052404	1
Aminosugars metabolism	0.0055309	1
beta-Alanine metabolism	0.0055309	1
Propanoate metabolism	0.0058213	1
Fatty acid metabolism	0.0061117	1
Lysine degradation	0.0061117	1
Phosphatidylinositol signaling system	0.0061117	1
Tryptophan metabolism	0.0066922	1
Butanoate metabolism	0.0072724	1
Valine, leucine and isoleucine degradation	0.0078524	1
VEGF signaling pathway	0.0087218	1
Pyrimidine metabolism	0.0093011	1
Pyruvate metabolism	0.0095907	1
Glycolysis / Gluconeogenesis	0.0098801	1
Wnt signaling pathway	0.0113265	1
Total		26

propanoate metabolism, fatty acid metabolism, lysine degradation, phosphatidylinositol signaling system, tryptophan metabolism, butanoate metabolism, valine, leucine and isoleucine degradation, VEGF signaling pathway, pyrimidine metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, and Wnt signaling pathway (Table 4).

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) validation of differentially expressed genes

We confirmed the gene expression level collected from microarray based on quantitative RT-PCR; 15 genes were selected randomly as test genes, while *Bm* Actin B was selected as the control to perform quantitative RT-PCR to validate the gene expression level. The results indicate that the change tendency of the expression levels of the 15 genes (normalized to *Bm* Actin B) had similar patterns with the changes measured by the microarray analysis at 24 h time point. The coefficient of determination was 0.7575 between the two sets of data (Figure 4).

DISCUSSION

The white egg mutants have important biological properties in silkworm with potential application and economic value in sericulture, such as sex-limited application in sericulture, and screening marker in transgenic silkworm study (Klemenz et al., 1987; Loukeris et al., 1995; Zwiebel et al., 1995). White egg 2 mutant characterized by its white eggs and white eyes during its life cycle, is more convenient and easily used as biomarker for transgenesis screening and gene functional study. In this study, to investigate the differentially expressed genes between white egg 2 mutant and normal black egg strain, near-isogenic line of white egg 2 was constructed with the black egg sex-limited variety Suluanban as white egg 2 gene (*w-2*) donor, and the normal black egg variety Jingsong A as recurrent parent. Eight generations proceeded by the selection procedure of the white egg as selecting phenotype. After eight generations, the recurrent parent recovery is assumed to be about 99.95%, retaining all normal black egg traits, while the donor parent genome is reduced to less than 0.05%, eliminating all the undesirable traits except the white egg 2 related genes (Ashwath et al., 2010). Theoretically, the near-

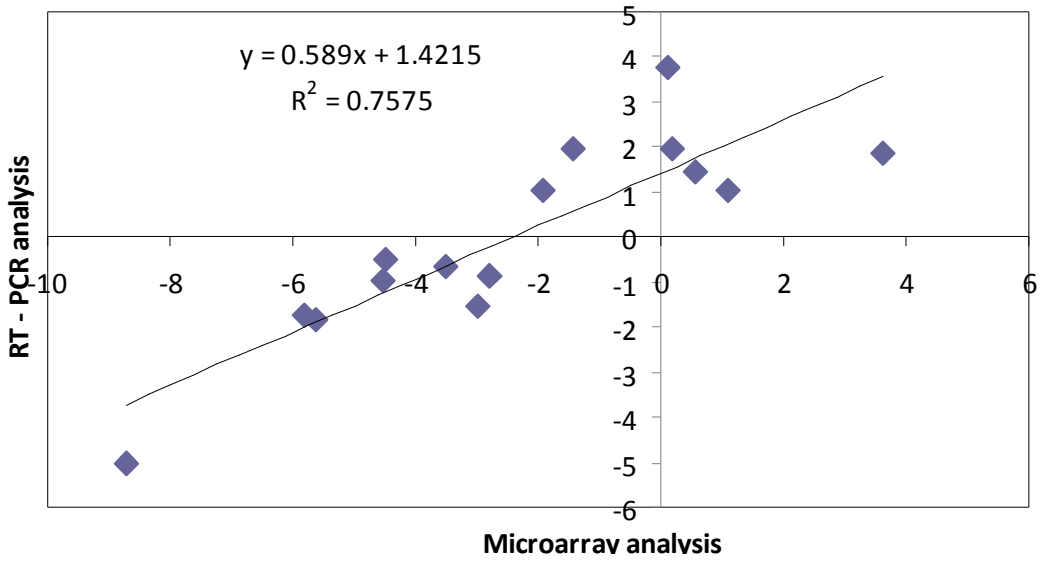

Figure 4. Quantitative RT-PCR analysis was used to validate the differentially expresses genes collected by microarray. A total of 15 genes at 24 h post oviposition were selected randomly; *Bm* Actin B was the control. The change tendency of the expression levels of the 15 differentially expressed genes (normalized to *Bm* Actin B) has similar patterns.

isogenic line of white egg 2 mutant construction paves a high-efficiency way to investigate the differentially expressed genes between white egg 2 mutant and normal black egg strain joint with microarray analysis.

The morphogenesis of the silkworm egg, which has three distinct phases: spheric, ellipsoidal, and plattened-ellipsoid, is an important procedure for its reproduction by strict transmission of genetic information and energy stores to the next generation (Tazima, 1964). As known, the color of the eggs of silkworms depends on three factors: yolk color, shell color, and the color of the serosa. The yolk and shell color of eggs are derived from the silkworm's blood; the pigments passing from the haemolymph of the mother's body into the eggs. However, serosa color is produced by granules which are formed in the serosa cells themselves (Tirelli, 1946). Normally, the color observed apparently concerns mainly the serosa pigment, than that of the yolk or/and shell. It is a fact that the serosa cell pigmentation may therefore be considered as the result of an enzymatic process involved in genes related to pigmentation.

Furthermore, when eggs are newly laid, all silkworm eggs appear yellow, taking their color only from the yolk and shell, since the serosa pigment has not yet developed. As the serosa develops, granules of melanic pigment appear in its cells, yellowish at first, following pink, red, dark red and eventually dark brown. Notably, this dark brown serosa pigment, as modified by the pigment of the translucent shell, is what gives the normal egg its gray color. The time course of this procedure is basically around 24 h post oviposition (Kikkawa, 1941), so our hypothetical genes related white egg 2 phenotype may have been expressed before pigmentogenesis was complete in the serosa at 24 h post oviposition. In this study, we found that 157 genes were expressed differentially, including 80 up-regulated genes and 77 down-regulated genes at 24 h post oviposition, and 178 genes including 98 up-regulated genes and 80 down-regulated genes at 48 h post oviposition.

The GO functional categories for these genes exhibited significant differences at both time points. It was noted that a gene encoding high affinity nuclear juvenile hormone binding protein was expressed over notable 33-fold change at 24 h post oviposition, inferring that this gene was involved in juvenile hormone signal transduction in the morphogenesis of the silkworm egg. Juvenile hormone exerts pleiotropic functions during insect life cycles and it primes the ecdysteroid response of developing follicles (Hartfelder, 2000). The fat body of pre-diapausing, early diapausing and mid-diapausing larvae was found to release a high affinity juvenile hormone binding protein in the southwestern corn borer, *Diatraea grandiosella* (Dillwith et al., 1985). We know that the diapause hormone, which is secreted from the suboesophageal ganglion is mainly responsible for the induction of diapause eggs, acts to control the metabolism of 3-hydroxykynurenine and carbohydrate in silkworm pupae. This hormone accelerates the 3-hydroxykynurenine and glycogen accumulation in pupae ovaries of silkworm (Yamashita et al., 1966). In silkworm, the interaction of diapause hormone and juvenile hormone regulate the diapause event, although we hope to sequentially trace the upstream or downstream genes of this high affinity nuclear juvenile hormone binding protein in further study.

Tryptophan metabolites are the source of pigment

produced by granules accumulated in serosa of the eggs (Bernt Linzen, 1974). Bonse (1969) reported that one mutant of *Drosophila* was unable to accumulate tryptophan in the Malpighian tubules leading to its white color. In our study, the transcript level of gene charged for phenylalanine, tyrosine and tryptophan biosynthesis detected 1.27-fold change. Down-regulation shows that the low tryptophan content at early embryo development stage probably was assumed to be insufficient, so that pigments in serosa led to the color deficiency phenotype of the eggs observed as the white egg. At 48 h post oviposition, one gene encoding reverse transcriptase involved in tryptophan metabolism was 3.2173 fold up-regulated. We therefore inferred that the source of pigment production was from tryptophan metabolites at very early stage after oviposition.

The results of KEGG analysis indicate that the pathways involved in differentially expressed genes were quite different at 24 h post oviposition and at 48 h post oviposition; only two common pathways; starch and sucrose metabolism and pyrimidine metabolism existed at both time points. The genes involved in starch and sucrose metabolism were both up-regulated at two time points. We conjectured that the genes involved in starch and sucrose metabolism were over expressed to fill the needs of the embryo development at non-diapause phase or merely survived at diapause phase. Embryogenesis is an extremely energy-intensive activity, requiring the rapid mobilization of energy source at the early embryo development stages. On the contrary, the gene involved in pyrimidine metabolism at 24 h post oviposition was up-regulated, while the one at 48 h was down-regulated. Pyrimidine nucleotides play a critical role in cellular metabolism serving as activated precursors of RNA and DNA (Evans and Guy, 2004). A gene involved in pyrimidine metabolism was up-regulated leading to more synthesized nucleic acids to meet the need of cell proliferation from newly laid to 24 h. However, at 48 h post oviposition, the eggs used in this experiment were in diapause stage and as the process of cellular morphogenesis was almost completed, the gene expression level dropped down correspondingly.

In order to demonstrate the accuracy of the microarray analysis results, we confirmed the differential expression of several randomly selected genes via RT-PCR validation, as is shown in Figure 4. The coefficient of determination was 0.7575 and the result of microarray analysis was reasonably consistent with those of the RT-PCR analysis. The microarray analysis is based on the hybridization technology; actually there is no strict linear relationship between signal strength and transcript abundance for different genes (Luo et al., 2005). In some cases, the cross-hybridization among the homologous sequences may cause the variance. Therefore, we conclude that our results are reliable, and can be used for further research of the white egg 2 mutant.

In summary, we report herein genes differentially expressed in white egg 2 mutant at 24 and 48 h after oviposition compared to normal black egg strain, as detected by DNA microarray analysis. The aim was to shed more light on any cues of genetic information of white egg 2 mutant. Recent study (Tatematsu et al., 2011) showed that in silkorm *w-2* there exist several multi-allelic mutations encoding the ortholog of *Drosophila scarlet*, which is responsible for the formation of a white/scarlet heterodimer and involved in the transport of ommochrome precursors. In our laboratory, we also found other new allelic mutation (data not shown). Hence, results in this study provide new clues for the exploration of the molecular mechanism of white egg 2 mutant. Furthermore, in subsequent studies, close attention will be focused on those genes with unknown function due to the limitation of the silkworm database.

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