Identification, mRNA expression and functional analysis of several yellow family genes in *Tribolium castaneum*

Yasuyuki Arakane a,*, Neal T. Dittmer b, Yoshinori Tomoyasu b, Karl J. Kramer a, c, Subbaratnam Muthukrishnan a, Richard W. Beeman c, Michael R. Kanost a

a Department of Biochemistry, Kansas State University, 141 Chalmers Hall, Manhattan, KS 66506-3702, USA
b Department of Zoology, Miami University, 212 Pearson Hall, Oxford, OH 445056, USA
c USDA-ARS Center for Grain and Animal Health Research, 1515 College Avenue, Manhattan, KS 66502, USA

**A B S T R A C T**

Querying the genome of the red flour beetle, *Tribolium castaneum*, with the *Drosophila melanogaster* Yellow-y (DmY-y) protein sequence identified 14 Yellow homologs. One of these is an ortholog of DmY-y, which is required for cuticle pigmentation (melanization), and another is an ortholog of DmY-f/f2, which functions as a dopachrome conversion enzyme (DCE). Phylogenetic analysis identified putative *T. castaneum* orthologs for eight of the *D. melanogaster* yellow genes, including *DmY-b*, -c, -e, -f, -g, -g2, -h and -y. However, one clade of five beetle genes, Ty-Y-1-5, has no orthologs in *D. melanogaster*. Expression profiles of all *T. castaneum* yellow genes were determined by RT-PCR of pharate pupal to young adult stages. Ty-Y-b and Ty-Y-c were expressed throughout all developmental stages analyzed, whereas each of the remaining yellow genes had a unique expression pattern, suggestive of distinct physiological functions. Ty-Y-b, -c and -e were all identified by mass spectrometry of elytrial proteins from young adults. Eight of the 14 genes showed differential expression between elytra and hindwings during the last three days of the pupal stage when the adult cuticle is synthesized. Double-stranded RNA (dsRNA)-mediated transcript knockdown revealed that Ty-Y is required for melanin production in the hindwings, particularly in the region of the pterostigma, while Ty-Y-f appears to be required for adult cuticle sclerotization but not pigmentation.

*Insect Biochemistry and Molecular Biology* 40 (2010) 259–266

1. Introduction

Insect cuticle consists of multiple functional layers including a lipophilic waterproofing envelope, a protein-rich epicuticle and a chitinous procuticle (Ostrowski et al., 2002; Tonnig et al., 2006). Shortly after molting, newly formed, soft, light-colored cuticle undergoes a maturation process known as tanning (sclerotization and pigmentation) that is vital for insect growth, development and survival. Tanning is a complex process that involves the oxidative conjugation and cross-linking of cuticular proteins by quinones, which renders the proteins insoluble and also hardens and darkens the exoskeleton (Andersen, 2005, 2010; Arakane et al., 2009; Hopkins and Kramer, 1992).

Pigmentation is an important physiological event in insects and is associated with not only cuticle tanning, but also hardening of the egg chorion, coloration of the eyes and Malpighian tubules, and defensive responses such as wound healing and microbial encapsulation (Li, 1994; Sugumar, 2002; Andersen, 2010). Melanization is one type of pigmentation that generally involves hydroxylation of tyrosine to dihydroxyphenylalanine (dopa), oxidation of dopa to dopaquinone, conversion of dopaquinone to dihydroxyindole (DHI) and/or 5,6-dihydroxyindole-2-carboxylic acid (DHICA), oxidation of DHI and DHICA to DHI-chrome and DHICA-chrome (melanochrome) and then polymerization of melanochrome to form dopa-melanin (Simon et al., 2009). In insect cuticle tanning, tyrosine hydroxylase (TH) converts tyrosine to dopa, dopa decarboxylase (DDC) converts dopa to dopamine, and laccase 2 oxidizes dopa, dopamine and DHI to dopaquinone, dopamine-quinone and melanochrome, respectively (Arakane et al., 2005; Gorman and Arakane, 2010; Andersen, 2010).

The conversion of dopachrome to DHI and/or DHICA is an important step in melanin production. Although the conversion of dopachrome to DHI can occur spontaneously, dopachrome conversion enzyme (DCE), which is encoded by one of the yellow genes in insects, accelerates the conversion of dopachrome and dopaminechrome to DHICA and DHI during melanization of the exoskeleton. In vertebrates, another enzyme, dopachrome tautomerase (Dct), catalyzes the
production of DHICA from dopachrome (Simon et al., 2009). A gene that encodes a Dct-like protein, however, does not appear to be present in the genome of the red flour beetle. Tribolium castaneum, suggesting that the alternative Dct-catalyzed reaction is not functional for melanin production in this coleopteran species. However, we have identified 14 putative yellow family genes in red flour beetle genome and report here the results of a study designed to evaluate the roles of some of those in T. castaneum development.

In the dipteran species Drosophila melanogaster, the yellow gene DmYellow (Dmy-yellow) (to avoid any confusion, we denote the yellow gene as “Y-y” in this paper) and a second, unrelated gene, N-[β-ala-nyllopinamine synthase (ebony), are required for proper pigment patterning and intensity because mutations of Dmy-yellow or ebony genes lead to the formation of a yellower or blacker cuticle, respectively, in the body wall and wing than that of wild type (Wittkopp and Beldade, 2009). Immuno localization of DmY-y is also consistent with the pattern of black pigment development in D. melanogaster (Gompel et al., 2005; Wittkopp et al., 2002a, 2003, 2009). In two lepidopteran species, Papilio xuthus and Bombyx mori, mRNA expression patterns of the Pxy-yellow and BmY-yellow, respectively, are also consistent with the development of black-pigmented regions (Futahashi and Fujiwara, 2007; Futahashi et al., 2008). Han et al. (2002) expressed D. melanogaster yellow genes including DmY-y as well as c, c+, y-f and f2 using the baculovirus/insect cell gene expression system and demonstrated that recombinant DmY-f and DmY-f2 were enzymatically active DCE-like proteins, whereas neither recombinant DmY-y-b nor c exhibited this enzymatic activity. These authors also reported differences in temporal expression patterns of DmY-f and DmY-f2, suggesting that DmY-f is probably involved in larval and pupal pigmentation, whereas DmY-f2 appears to play a critical role in pigmentation during late pupal and adult stages. Although DmY-y itself apparently is not a DCE, it may influence the expression of DmY-f, f2 or other genes involved in cuticle pigmentation.

Recent studies have suggested that yellow is a rapidly evolving gene family generating functionally diverse paralogs in D. melanogaster, 14 genes have been annotated as members of the yellow gene family (Maleszka and Kucharski, 2000). DmY-y catalyzes melanin production (Drapeau, 2001; Drapeau et al., 2006a; Maleszka and Kucharski, 2000; Prud’homme et al., 2007; Wittkopp et al., 2002a, 2003, 2009). DmY-y also affects male courtship behavior (Drapeau et al., 2003). DmY-g and DmY-g2 in D. melanogaster are critical for follicle cell function and act to cross-link the vitelline membrane, which is critical for rigidity of the egg (Claycomb et al., 2004). Proteins with sequences related to that of the Yellow protein are also major components of honey bee royal jelly (Major Royal Jelly Protein, MRJP), where they are believed to play a nutritional role due to their high content of essential amino acids (Schmitzova et al., 1998). In addition, some of the mrjp genes in Apis mellifera are expressed not only in the hypopharyngeal gland but also in the brain and venom gland, suggesting that mrjp genes possess diverse physiological functions (Drapeau et al., 2006b).

In this study we have investigated whether yellow-like genes participate in cuticle pigmentation and sclerotization in a coleopteran species. The red flour beetle, T. castaneum, is an excellent experimental model for functional genomic analysis because systemic, gene- and transcript-specific RNA interference (RNAi) experiments can be performed at any life stage by injecting gene-specific, double-stranded RNA (dsRNA) (Arakane et al., 2008; Tomoyasu and Denell, 2004; Tomoyasu et al., 2008). To study the functions and roles of several yellow genes in beetle cuticle pigmentation and sclerotization, we first identified all 14 genes that encode Yellow-like proteins based on similarity to DmY-y. We then analyzed mRNA expression profiles of all of these genes during the late post-embryonic developmental stages. Finally, we determined phenotypic changes produced by injection of dsRNAs for TcY-y and TcY-f, which are orthologs of the previously well-characterized DmY-y and Dmy-f genes.

2. Materials and methods

2.1. Insects

The wild-type GA-1 strain of T. castaneum (Haliscak and Beeman, 1983) and a black body color mutant strain, b^chy^b^hy^, were reared at 30 °C under standard conditions (Arakane et al., 2009; Beeman and Stuart, 1990).

2.2. Cloning of the T. castaneum Yellow cDNAs

By searching Beetlebase (http://www.bioinformatics.ksu.edu/BeetleBase/) with the tblastn program using the annotated amino acid sequence for D. melanogaster Yellow-yellow protein (Dmy-yellow) as query, 14 genes including the previously reported TcY-y (FJ647799) (Tomoyasu et al., 2009) were identified. To clone full- or partial-length cDNAs of all of the T. castaneum yellow-like genes, template cDNAs were synthesized from total RNA isolated from pupae using primer pairs listed in Table 1. PCR fragments were cloned in the pGEM-T vector (Promega) and sequenced. DNA sequences were deposited in GenBank. Accession numbers of T. castaneum yellow genes are listed in Table 1.

2.3. RT-PCR analysis of T. castaneum yellow gene expression

To analyze the expression patterns of the 14 putative T. castaneum yellow genes, total RNA isolation, first strand cDNA synthesis and RT-PCR were carried out as described previously (Arakane et al., 2005) using the same primer sets used for cDNA cloning (see Table 1). Total RNA was isolated from whole insects (n = 5) at late post-embryonic developmental stages (pharate pupae to young adults) by using the RNeasy Mini Kit (Qiagen), and first strand cDNA was synthesized by using SuperScript III (Invitrogen). The PCR reaction conditions were as followed: denaturation at 94 °C for 30 s, annealing at 52–56 °C for 30 s and extension at 72 °C for 1.5 min for 28 cycles. The PCR products were visualized after electrophoresis in a 1% agarose gel containing ethidium bromide. The primer pair 5′-AGATATATGGAAGCATCATGA for the yellow-y gene and 5′-CGCTGCTTTCTTTGCTCAATTG for the yellow-f gene were used to amplify TcY-f (ribosomal protein S6) that served as the internal control for RT-PCR.

2.4. Phylogenetic analysis of Yellow family proteins

ClustalW software was used to align multiple sequences of Yellow family proteins from D. melanogaster and T. castaneum. The MEGA 4.0 program (Tamura et al., 2007) was used to construct the phylogenetic tree using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). To evaluate the branch length of the phylogenetic tree, a bootstrap analysis of 5000 replications was performed.

2.5. Synthesis and injection of double-stranded RNAs

dsRNA for the T. castaneum yellow-y gene (dsTcY-y) was synthesized as described previously (Tomoyasu et al., 2009). For synthesis of the dsRNA for TcY-f (dsTcY-f), we used 5′-(T7)-CCGATCTCAAATGCTTCTCTGCTC-3′ and 5′-(T7)-ACTCCGATTTCGCTC-3′ to amplify the dsRNA target region, where T7 indicates the T7 RNA polymerase recognition sequence. dsTcY-y (1057 bp) and dsTcY-f (456 bp) were injected into late-stage larva (a mixture of penultimate instar and last instar larvae) and/or 1 d-old pupae (200 ng per insect). dsRNA
for the *T. castaneum* *vermilion* (dsTcVer) gene was injected as a negative control (Arakane et al., 2009).

### 2.6. Proteomic analysis of *T. castaneum* elytra and hindwings

Elytra and hindwings were dissected from 185 newly molted adults (0–2 h) and stored at −80 °C until all samples had been collected. The samples were homogenized in a buffer consisting of 5% SDS, 4 M urea, 10% glycerol, 50 mM acetic acid, and 10 mM boric acid (Hopkins et al., 2000) and incubated for 22 h at room temperature on a rotary mixer. The extracts were centrifuged twice for 5 min at maximum speed in a microcentrifuge and the supernatants were saved. Protein concentration was determined using the BCA Protein Assay Kit (Pierce). Two-dimensional (2D) gel electrophoresis was performed by Kendrick Labs Inc. (Madison, WI). Peptide mass fingerprinting was performed by the Biotechnology/Proteomics Core Facility in the Department of Biochemistry at Kansas State University. Protein spots picked for analysis were digested with trypsin and the resulting peptides were analyzed using a Bruker Daltonics Ultraflex III MALDI TOF/TOF Mass Spectrometer in MS mode. Data were analyzed using Mascot software v 2.2.04 (Matrix Science Ltd.).

### 2.7. Microarray analysis of *T. castaneum* elytra and hindwings

Microarray analysis was performed using a custom designed 8 × 15 k array (Agilent). The array consisted of 60-mer probes for 15,208 *T. castaneum* gene models (93% coverage) (*Tribolium Genome Sequencing Consortium, 2008*). Having eight arrays on one chip was determined using the BCA Protein Assay Kit (Pierce). Two-dimensional (2D) gel electrophoresis was performed by Kendrick Labs Inc. (Madison, WI). Peptide mass fingerprinting was performed by the Biotechnology/Proteomics Core Facility in the Department of Biochemistry at Kansas State University. Protein spots picked for analysis were digested with trypsin and the resulting peptides were analyzed using a Bruker Daltonics Ultraflex III MALDI TOF/TOF Mass Spectrometer in MS mode. Data were analyzed using Mascot software v 2.2.04 (Matrix Science Ltd.).

### 3. Results and discussion

#### 3.1. Identification and phylogenetic analysis of *T. castaneum* Yellow family proteins

The gene family encoding Yellow-like proteins has been identified previously in several insect species including *D. melanogaster*, *A. mellifera* (Albert and Klaudiny, 2004; Drapeau, 2001, 2006a, b; Xia et al., 2006). The completion of both sequencing and high-quality annotation of the *T. castaneum* genome provided us with the opportunity to search for genes encoding Yellow proteins in a species from the order Coleoptera for the first time.

### Table 1

Members of the gene family that encodes yellow-like proteins in *T. castaneum.*

| Yellow genes | Accession number | GLEAN number | Linkage group | Map position | Scaffold | Primer set for cloning and gene expression analysis (5’ to 3’)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TcY-b</td>
<td>GU111762</td>
<td>05480</td>
<td>8</td>
<td>43.2</td>
<td>Contig6965_Contig294</td>
<td>F:ATGCGACACCTGGCTGGC</td>
</tr>
<tr>
<td>TcY-c</td>
<td>GU111763</td>
<td>12699</td>
<td>9</td>
<td>40.6</td>
<td>Contig5571_Contig829</td>
<td>F:CGACAGATGAAATGATGTC</td>
</tr>
<tr>
<td>TcY-e</td>
<td>GU111765</td>
<td>06227</td>
<td>8</td>
<td>9.4</td>
<td>Contig2833_Contig6624</td>
<td>F:ATGTTATGGCGACCTTCCTTC</td>
</tr>
<tr>
<td>TcY-e3</td>
<td>GU111764</td>
<td>06229</td>
<td>8</td>
<td>9.4</td>
<td>Contig2833_Contig6624</td>
<td>F:ATGTTATGGCGACCTTCCTTC</td>
</tr>
<tr>
<td>TcY-f</td>
<td>GU111766</td>
<td>05565</td>
<td>8</td>
<td>34.8</td>
<td>Contig1938_Contig5361</td>
<td>F:GATCCTCTCCTTGTTGCC</td>
</tr>
<tr>
<td>TcY-g</td>
<td>GU111767</td>
<td>06226</td>
<td>8</td>
<td>9.4</td>
<td>Contig2833_Contig6624</td>
<td>F:AAATGGCGAACATTCGTGC</td>
</tr>
<tr>
<td>TcY-g2</td>
<td>GU111768</td>
<td>05927</td>
<td>8</td>
<td>9.4</td>
<td>Contig2833_Contig6624</td>
<td>F:AAATGGCGAACATTCGTGC</td>
</tr>
<tr>
<td>TcY-h</td>
<td>GU111769</td>
<td>06230</td>
<td>8</td>
<td>9.4</td>
<td>Contig2833_Contig6624</td>
<td>F:AAATGGCGAACATTCGTGC</td>
</tr>
<tr>
<td>TcY-y</td>
<td>GU111770</td>
<td>00802</td>
<td>2</td>
<td>8.5</td>
<td>Contig798_Contig4094</td>
<td>F:ATAAACACTCCTAATAACTA</td>
</tr>
<tr>
<td>TcY-1</td>
<td>GU111771</td>
<td>05444</td>
<td>8</td>
<td>44.4</td>
<td>Contig4095_Contig2533</td>
<td>F:CACGATACACTCTCTAC</td>
</tr>
<tr>
<td>TcY-2</td>
<td>GU111772</td>
<td>03539</td>
<td>3</td>
<td>14.8</td>
<td>Contig2778_Contig2095</td>
<td>F:CGGCTTGTTCCTTGGAAAA</td>
</tr>
<tr>
<td>TcY-3</td>
<td>GU111773</td>
<td>03898</td>
<td>3</td>
<td>67</td>
<td>Contig4979_Contig4226</td>
<td>F:CGGCTTGTTCCTTGGAAAA</td>
</tr>
<tr>
<td>TcY-4</td>
<td>GU111774</td>
<td>02509</td>
<td>3</td>
<td>67</td>
<td>Contig4979_Contig4226</td>
<td>F:CGGCTTGTTCCTTGGAAAA</td>
</tr>
<tr>
<td>TcY-5</td>
<td>GU111775</td>
<td>02508</td>
<td>3</td>
<td>67</td>
<td>Contig4979_Contig4226</td>
<td>F:CGGCTTGTTCCTTGGAAAA</td>
</tr>
</tbody>
</table>

* A: forward primer, R: reverse primer.
We searched the *T. castaneum* genome database via BLAST (blastn program) using the *D. melanogaster* Yellow-y protein (DmY-y) as query and identified 14 genes that encode proteins homologous to *D. melanogaster* Yellow proteins (Table 1). Full- or partial-length cDNAs of all *T. castaneum* yellow genes including TcY-y (GLEAN_00802) whose partial cDNA was cloned previously (Tomoyasu et al., 2009) were cloned and sequenced. Although all 14 genes encode a conserved, ~300 amino acid-long “major royal jelly protein” (MRJP) domain (Schmitzova et al., 1998), the Yellow protein family overall has a rather low sequence identity with the exception of TcY-4 and TcY-5 that have 80.4% similarity (Supplementary Table 1), indicating that the biochemical properties of these proteins may vary substantially. Each Yellow protein has from 1 to 4 putative exon/intron organization amongst themselves, suggesting that these genes are single-copy genes in both species. Unlike *D. melanogaster*, the exon/intron structure of 9 mrjp genes is highly conserved, while that of 10 yellow genes is generally unconserved (Drapeau et al., 2006b). The exon/intron organization of each TcY gene was determined by comparison of the TcY cDNAs (coding region) with the corresponding genomic sequences (Supplementary Fig. 2). Like the yellow genes from *A. mellifera*, the exon/intron organization of TcY gene family is not well conserved. TcY-1-5, which are apparently unique to *T. castaneum* (see below), exhibit a similar exon/intron organization amongst themselves, suggesting that these five genes are the result of rapid, lineage-specific gene duplications.

Phylogenetic analysis revealed that putative *T. castaneum* orthologs were identified for most of the *D. melanogaster* Yellow proteins, including DmY-b, -c, -e, -e3, -f, -g, -g2, -h, and -y (Fig. 1). The *T. castaneum* genes TcY-b, -c, -h, -e, -g, and -g2 all have 1:1 orthologs in *D. melanogaster*, i.e. they are single-copy genes in both species. However, the ortholog of the single-copy TcY-f gene is duplicated in the dipteran genome, and the single-copy TcY-e3 gene is most similar to a rather divergent group of 4 dipteran genes, DmY-e3, -d, -d2 and -e2. Multiple genes most closely related to DmY-f were also identified in the *B. mori* genome (Futahashi et al., 2008; Xia et al., 2006). Interestingly, one *T. castaneum* clade of five genes, TcY-1-5, has no orthologs in *D. melanogaster*. Drapeau et al. (2006b) reported that in *A. mellifera*, two genes encoding Yellow-like proteins, AmY-x1 and AmY-x2, have no *D. melanogaster* orthologs, whereas seven Yellow-like proteins in *B. mori* all have corresponding *D. melanogaster* orthologs (Drapeau et al., 2006b; Futahashi et al., 2008; Xia et al., 2006). Eight of the 14 *T. castaneum* yellow genes are arranged in two separate clusters of closely linked genes (Table 1). The larger cluster occurs on the 8th linkage group and contains a tandem array of five yellow genes (-g, -g2, -e, -e3 and -h, listed in sequential order in the cluster) that are interrupted by three unrelated genes. Within this cluster, TcY-g and -g2 are tightly linked in an head-to-head orientation with their start codons separated by only 259 nucleotides. The smaller cluster on the 3rd linkage group contains an uninterrupted tandem array of three yellow genes (-3, -4 and -5, listed in sequential order). None of the remaining six yellow genes are clustered (Table 1).

### 3.2. Developmental patterns of expression of *T. castaneum* yellow genes

Prior to analyzing the functions of individual yellow genes in adult cuticle pigmentation and sclerotization by RNAi, it was necessary to determine the developmental pattern of expression of mRNA for each yellow gene to pinpoint the optimal timing for injection of dsRNAs and also to help anticipate what biochemical and morphological changes might occur afterwards. Expression patterns of *T. castaneum* yellow genes were first analyzed by RT-PCR from pharate pupal through young adult stages. As shown in Fig. 2, TcY-b, TcY-c, TcY-e3 and TcY-4 genes are expressed throughout all of the developmental stages analyzed, whereas the remaining *T. castaneum* yellow genes show variations in patterns of expression within the pupal stage. Most striking is the observation that several yellow genes that have low or undetectable levels of transcripts during the early pupal stages are highly induced on pupal day 3–4 and decline thereafter (e.g. TcY-h, TcY-y, TcY-3 and TcY-5). Trace amounts of TcY-g and TcY-g2 transcripts were observed throughout the stages analyzed. The mRNA levels of these two genes, however, were drastically increased at the mature adult stage (data not shown), suggesting that TcY-g and TcY-g2 have roles similar to those of DmY-g and DmY-g2 (see section 3.3.2). TcY-b and TcY-e have two peaks of expression, one at the prepupal stage and the second during later pupal stages (pupal day 4 or later) with the lowest expression on pupal day 1–2. Transcript levels for the two genes TcY-2 and TcY-4 peak on pupal day 1–2. Thus, there are at least four patterns of expression among the *T. castaneum* genes during the prepupal–pupal interval, including either a nearly constant level of...
expression, an early peak level of expression, a late peak level, or two peak levels with lower expression in the middle pupal stages.

Futahashi and Fujiwara (2007) reported that the expression of the yellow-y gene (PxY-y) in the swallowtail butterfly, P. xuthus, was induced by high titers of 20-hydroxyecdysone (20HE), whereas a decline in 20HE titer was required for the expression of the genes involved in the cuticle tanning pathway such as tyrosine hydroxylase (TH), dopa decarboxylase (DDC) and N-β-alanyldopamine synthase (ebony). Similar developmental expression profiles of yellow-y and ebony were observed in B. mori (Futahashi et al., 2008). The B. mori yellow-y gene (BmY-y) was highly expressed at stage E1, while a high expression of the ebony gene was observed slightly later at stage E2 after the 20HE titer declined between the two stages (Kiguchi and Agui, 1981). The developmental expression patterns of TcY-y, TH, DDC and ebony observed in T. castaneum are consistent with those reported for the lepidopteran species. The highest expression level of TcY-y and several other yellow genes occurred on pupal day 3 and declined substantially by the time of adult apolysis, whereas high levels of expression of TcTH, TcDDC and Tcebony were observed on pupal day 4–5 (Arakane et al., 2009; Gorman et al., 2007; Gorman and Arakane, 2010). T. castaneum pupal day 4–5 is the stage corresponding to the time for pigmentation in P. xuthus (Futahashi and Fujiwara, 2007) when the 20HE titer must be very low and adult cuticle tanning was observed under the pupal cuticle. While the titers of 20HE have not been precisely determined during pupal stages of T. castaneum, it is likely that the differences in patterns of expression of yellow genes are due to differences in their response to the 20HE titer during pupal/adult development. Our results obtained with a coleopteran species and results reported by others obtained in studies of dipteran and lepidopteran species suggest comparable, specialized physiological functions of yellow genes in species from the three different Orders.

3.2.1. Microarray and proteomic analyses of yellow genes/proteins in elytra and hindwings

Among cuticle-forming tissues of T. castaneum that undergo tanning reactions, the elytron and hindwing are two of the easiest to obtain in pure form for analysis. We used a microarray analysis to examine the expression of the TcY genes in those two appendages, which possess distinct physical properties. The former has a highly sclerotized and pigmented cuticle, whereas the latter has a translucent and membranous one. We chose to analyze these tissues on pupal days 3–5 when the adult cuticle is synthesized because of the relative ease of obtaining these tissues at this time point, and because all yellow genes are expressed at this time. TcY-e, e3, f, -h, -y, -1, -3 and -5 display their highest level of expression during this time (Fig. 2). However, higher levels of expression were found in the elytron for some of these genes and in the hindwing for others. Most striking is TcY-y, with a 57-fold higher expression in the hindwing, and TcY-5, with a 168-fold higher expression in the elytron. In all, eight of the 14 TcY genes were differentially expressed between the elytron and hindwing (Table 2). The other six genes showed less than a 2-fold difference including TcY-h, which is expressed at high levels in pharate adult stages.

We also employed peptide mass fingerprinting mass spectrometry to uniquely identify some of the Yellow proteins in extracts prepared from elytra and hindwings of newly enclosed adults. The elytral cuticle is not heavily sclerotized at this stage, allowing extraction of proteins that are not yet heavily cross-linked. TcY-b and -e were detected only in elytra, while TcY-c was detected in both the elytra and hindwings (Table 3). The failure to find other Yellow proteins in extracts of elytra or hindwings may be due to an inability to extract them sufficiently or to identify unique tryptic peptides derived from those proteins. Also, it should be noted that the developmental RT-PCR was performed using cDNA generated from total RNA extracted from whole bodies. Therefore, tissues other than the elytra or hindwing may have contributed to the expression levels observed. Because only a single probe was present on the microarray for each gene, no conclusions can be made as to their absolute transcript level.

3.3. Role of yellow proteins in pigmentation

Dopachrome conversion enzyme (DCE, Yellow), one of the key enzymes in the melanin biosynthetic pathway, accelerates the conversion of dopachrome to dihydroxyindole. In D. melanogaster, 14 genes have been annotated as members of the yellow gene family. Recent studies indicate that DmY-y is involved in melanin production. In addition, the distribution of the Yellow-y protein coincides with species-unique pigmentation patterns in D. melanogaster.

Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold difference</th>
<th>p-value</th>
<th>Tissue with higher expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcY-c</td>
<td>4.9</td>
<td>4.0 × 10⁻⁶</td>
<td>Hindwing</td>
</tr>
<tr>
<td>TcY-f</td>
<td>4.4</td>
<td>1.5 × 10⁻⁴</td>
<td>Elytron</td>
</tr>
<tr>
<td>TcY-h</td>
<td>3.2</td>
<td>1.6 × 10⁻⁴</td>
<td>Elytron</td>
</tr>
<tr>
<td>TcY-y</td>
<td>57</td>
<td>1.7 × 10⁻³</td>
<td>Hindwing</td>
</tr>
<tr>
<td>TcY-1</td>
<td>8.4</td>
<td>5.5 × 10⁻⁵</td>
<td>Hindwing</td>
</tr>
<tr>
<td>TcY-2</td>
<td>12</td>
<td>1.4 × 10⁻⁴</td>
<td>Elytron</td>
</tr>
<tr>
<td>TcY-3</td>
<td>22</td>
<td>3.5 × 10⁻⁶</td>
<td>Elytron</td>
</tr>
<tr>
<td>TcY-5</td>
<td>168</td>
<td>9.2 × 10⁻⁴</td>
<td>Elytron</td>
</tr>
</tbody>
</table>
Gene is expressed at a 57-fold higher level in hindwing over the elytron. The expression profile of melanin pathway required for the black pigmentation in the pigmentation, including that in the pterostigmata of the hindwings pupae caused a significant defect in the pterostigma in the hindwings. dsRNA for T. castaneum vermilion (dsVer) was injected as the negative control. Black and yellow arrows indicate pterostigma of the dsVer and dsTcY-y-treated insects, respectively. (For interpretation of the references to colour in figure legends, the reader is referred to the web version of this article.)

3.3.1. Effect of RNAi for TcY-y on adult cuticle pigmentation

The diversity of insect yellow genes involved in cuticle tanning probably reflects functional specializations among these genes. To identify the functions of some of the T. castaneum yellow genes by RNAi, we focused on TcY-y and TcY-f genes that are orthologs of the more fully characterized DmY-y and DmY-f2 genes, respectively. We recently demonstrated that injection of dsRNA for TcY-y (dsTcY-y) caused a defect in the hindwing, particularly in the anterior portion that includes the pterostigma (Tomoyasu et al., 2009). However, dsTcY-y injection into last instar larvae or young pupae had no effect on pupal development or adult eclosion (Fig. 3). Adult cuticle tanning in the head, mandibles and legs proceeded normally, and mature adults had normal pigmentation. Progressive tanning of adult cuticle was visible even in pupae one day before adult molting, since pupal cuticle is translucent and mostly devoid of pigment in T. castaneum (top left panel in Fig. 3). Unlike yellow-y mutant strains of D. melanogaster or B. mori (Drapeau, 2001; Futahashi et al., 2008; Wittkopp et al., 2002a, 2003, 2009), RNAi-induced TcY-y knockdowns in T. castaneum had normal adult body cuticle pigmentation. However, the black pigmentation of the T. castaneum hindwing, including the pterostigma and nearby surrounding regions, was specifically reduced by injection of dsTcY-y (Fig. 3, bottom panel).

In D. melanogaster, Yellow-y (DmY-y) may be involved in both the dopa- and dopamine-melanin synthetic pathways (Gibert et al., 2007). In P. xuthus, a dopa decarboxylase (DDC) inhibitor prevented the black pigmentation of the larval cuticle, suggesting that Yellow-y (PxY-y) acts in the dopamine-melanin pathway (Futahashi and Fujiwara, 2005). A similar defect was observed after injection of dsRNA for TcDDC (dsTcDDC). Injection of dsTcDDC into 1-d-old pupae caused a significant delay and reduction in adult cuticle pigmentation, including that in the pterostigmata of the hindwings (Supplementary Fig. 3) (Arakane et al., 2009). All of these results suggest that TcY-y plays an important role in the dopamine-melanin pathway required for the black pigmentation in the hindwing, but it is not critical for pigmentation of the body wall or elytron. The expression profile of TcY-y, which indicates that this gene is expressed at a 57-fold higher level in hindwing over the level in the elytron as determined by microarray analysis, is consistent with this argument.

Glossy, a mutant strain of T. castaneum, has a body color that is a light yellowish-brown instead of the rust-redish brown color of the wild-type strain, similar to the body color of yellow-y mutant strains of D. melanogaster and B. mori (Supplementary Fig. 4). The glossy mutation has been mapped to a location near the TcY-y gene (Beeman, unpublished data). However, we do not suspect that the glossy phenotype reflects a defect in the gene encoding TcY-y, because RNAi for TcY-y in the wild-type strain had no effect on adult body pigmentation. Thus, there appears to be an as yet unidentified gene regulating body pigmentation in this region of the 2nd linkage group.

We investigated further whether TcY-y is involved in production of black pigment in the black body color mutant, B^B/h^B, in which aspartate 1-decarboxylase mRNA is suppressed, resulting in a deficiency of B-quinone and an abnormally high level of dopamine for cuticle melanization (Arakane et al., 2009; Kramer et al., 1984). dsTcY-y injection had no effect on adult cuticle pigmentation in B^B/h^B beetles, with the above-mentioned exception in the hindwing (Fig. 4). Similar results were obtained after co-injection of dsRNAs for TcY-y and TcEchony (Tomoyasu et al., 2009). These results suggest that TcY-y is not critical for the black body pigmentation in the B^B/h^B strain. It is possible that another yellow gene(s) is involved in melanin production or that an unusually high level of dopamine/dopamine-quinone may lead to their spontaneous conversion to 5,6-dihydroxyindole (DHI), resulting in production of sufficient dopamine-melanin to change the body color from rust-redish brown to black.

3.3.2. Effect of RNAi for TcY-f in cuticle pigmentation

Because recombinant proteins DmTcY-f and DmY-f2 and M. sexta Yellow protein exhibit DCE activity, we injected dsRNA for TcY-f (dsTcY-f) into late-stage larvae to determine whether TcY-f is involved in cuticle pigmentation. dsTcY-f injection had no effect on larval-pupal molting, and the resulting pupae developed normally (Fig. 5). The

### Table 3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptides matched</th>
<th>Percent coverage</th>
<th>Scorea</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcY-b</td>
<td>19</td>
<td>40</td>
<td>195</td>
<td>Elytron</td>
</tr>
<tr>
<td>TcY-c</td>
<td>16</td>
<td>36</td>
<td>106</td>
<td>Elytron</td>
</tr>
<tr>
<td>TcY-e</td>
<td>22</td>
<td>57</td>
<td>201</td>
<td>Hindwing</td>
</tr>
<tr>
<td>TcY-f</td>
<td>25</td>
<td>69</td>
<td>313</td>
<td>Elytron</td>
</tr>
</tbody>
</table>

a Scores above 55 are significant (p < 0.05).
pupae—adult molt, however, was adversely affected. Although apolysis and slippage were evident, adults could not shed the pupal cuticle and died entrapped in their pupal cuticle. A similar pharate adult terminal phenotype was also obtained by co-injection of dsTcY-y and dsTcY-f. However, larvae treated with a mixture of these two dsRNAs (200 ng each per insect) did molt to pupae and the resulting pupae appeared to develop normally. Adult cuticle pigmentation except for that in the hindwings was initiated at the proper timing and proceeded to the same extent as in controls, but these insects failed to molt to adults (data not shown). Claycomb et al. (2004) proposed that DmY-g and DmY-g2 in D. melanogaster act to cross-link the vitelline membrane and are necessary for rigidity of the egg. Eggs from DmY-g mutant mothers spontaneously collapse, probably because of defects in the vitelline membrane. It is possible that TcY-f plays a critical role in adult cuticle sclerotization in a manner similar to that of DmY-g and -g2 for stabilization of the vitelline membrane, but it does not appear to be essential for pigmentation.

To date no gene that encodes a Yellow-like protein has been found in a non-insect metazoan, including two non-insect arthropods whose genomes have been sequenced, the crustacean water flea, Daphnia pulex, and the arachnid deer tick, Ixodes scapularis. However, some microbes, such as the red pigmented bacterium Deinococcus radiodurans, do possess Yellow-like proteins, suggesting that pigmentation and/or melanization is their ancestral function, and that recent paralogs evolved to provide novel, diverse function(s) in higher organisms (Drapeau et al., 2006b; White et al., 1999).

We have conducted a functional analysis of only two of the 14 yellow genes of T. castaneum and have demonstrated that these two genes have different roles in development and/or pigmentation. It is likely that different yellow genes affect pigmentation of different cuticular structures and/or different parts of the same cuticle. In the future we plan to further investigate other Yellow family members and also to analyze the mechanical properties of exoskeletons from TcY-family-treated insects using dynamic mechanical analysis to help determine their functions in T. castaneum development (Arakane et al., 2009).

Acknowledgements

We thank the Kendrick Labs for performing the 2D gel electrophoresis, Dr. Yasuaki Hiromasa for the peptide mass fingerprinting analysis, Dr. Yoosoon Park for design of the microarray, Dr. Nigel Cooper and Ms Xiaohong Li for the microarray hybridization, and Ms. Nanyan Lu for assisting with the microarray statistical analysis. This work was supported by Arthropod Genomics Seed Grant NOBO2-83015 and National Science Foundation Grant IOS 0726425. This is publication is solely for the purpose of providing specific information and does not imply a recommendation or endorsement by the U.S. Department of Agriculture.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2010.01.012.

References

Arakane, Y., Tomonagasa, K., Moriguchi, R., Uchikawa, S., 2008. Effects of in-
creased ecdysteroid levels on cuticle pigmentation in the silkworm

Bettridge, T.J., Thomas, A.L., Cooper, A., Gomes, A., 2006. A new family of
lipid-binding proteins involved in photoreception and opsin coac-

Buchanan, R.B., Gruissem, W., Jones, R.L., editors. 2000. The Arabidopsis

Cali, D., 2001. The molecular basis of insect eclosion and ecdysteroid reg-


Carré, B., 2006. Insect cuticle: morphology, biochemistry and molecular bi-

Rev. Enzymol. 28, 11–43.

Carré, B., Bui, A., Thévenot, K., 1998. Ecdysteroid regulation of cuticle pro-

Carré, B., Bui, A., Thévenot, K., 1997. Ecdysteroid regulation of cuticle pro-

Chen, A., Xiao, J., Li, W., Guo, Y., Wang, X., 2006. Structural and func-
tional characterization of a novel cuticle protein in the silkworm,

Physiol. 52, 1027–1034.

protein (IPL) encoded by a novel cuticle gene in the silkworm,

protein (IPL) encoded by a novel cuticle gene in the silkworm,