



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

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ABSTRACT

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, *TcY-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. *TcY-b* and *TcY-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. *TcY-b*, *-c* and *-e* were all identified by mass spectrometry of elytral 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 *TcY-y* is required for melanin production in the hindwings, particularly in the region of the pterostigma, while *TcY-f* appears to be required for adult cuticle sclerotization but not pigmentation.

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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; Tønning 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; Sugumaran, 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

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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 *Dmyyellow* (*DmY-y*) (to avoid any confusion, we denote the yellow gene as “Y-y” in this paper) and a second, unrelated gene, *N-β*-alanyl-dopamine synthase (*ebony*), are required for proper pigment patterning and intensity because mutations of *DmY-y* 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). Immunolocalization 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 *DmY-y* orthologs, *PxY-y* and *BmY-y*, 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 *-b*, *-c*, *-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^{Chr}/bSt*, 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-y protein (*DmY-y*) 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 AGC-3' and 5'-CGTCGCTTCTTTGCTCAAATTG-3' was used to amplify *TcP56* (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 strength 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)-CCGATGT-TAAATCTG-3' and 5'-(T7)-ACTCGCATTTTGTGC-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 larvae (a mixture of penultimate instar and last instar larvae) and/or 1 d-old pupae (200 ng per insect). dsRNA

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') ^a
<i>TcY-b</i>	GU111762	05480	8	43.2	Contig6965_Contig294	F:ATGGACAACCTTCGTGTCGC R:GGCAACAACATGACAAGAACC
<i>TcY-c</i>	GU111763	16299	9	40.6	Contig5571_Contig829	F:CAACCATGATCAAATTTATGCTG R:TTGATAAGITTCATCAACATTTGC
<i>TcY-e</i>	GU111765	06227	8	9.4	Contig2833_Contig6624	F:ATGTTACGCCACGCACCTCTTC R:CACTTAACAAATGGTAATCCGCTCG
<i>TcY-e3</i>	GU111764	06229	8	9.4	Contig2833_Contig6624	F:CCAAGATCAATTTGGTGACTC R:CTGCAACAATCTAATCAAAGTTC
<i>TcY-f</i>	GU111766	05565	8	34.8	Contig1938_Contig5361	F:GATCCTCCTCTTTGTGTCC R:TTAATAGTGGCATCCGCCTC
<i>TcY-g</i>	GU111767	06226	8	9.4	Contig2833_Contig6624	F:GAAATGTGGCAAATTCGTGTGC R:CTCAATCGCAGGTTTGATAAGC
<i>TcY-g2</i>	GU111768	05927	8	9.4	Contig2833_Contig6624	F:AAGCATGCTTCGACAAGTCG R:TTACGTATGCACGGGCAATG
<i>TcY-h</i>	GU111769	06230	8	9.4	Contig2833_Contig6624	F:TGGGCAAATTTCCAACCTCC R:TTAGTAGCAATCTTCGCCATC
<i>TcY-y</i>	GU111770	00802	2	8.5	Contig798_Contig4094	F:ATGAACACTCCCTTAACCTA R:TCAAATTACAAACTGTGCAGTTTTC
<i>TcY-1</i>	GU111771	05444	8	44.4	Contig4095_Contig2533	F:CGCAATGAACACTCTCAC R:TTTCACCGTCCCACTAGCAG
<i>TcY-2</i>	GU111772	03539	3	14.8	Contig2778_Contig2095	F:ATGGCCGAATTTGGGTACG R:GCTTTGTCTGTTTGTAGC
<i>TcY-3</i>	GU111773	03898	3	67	Contig4979_Contig4226	F:ATGAACCTCTGTGCGCCTA R:TTAGGATTCTAAATAGCTCTTCG
<i>TcY-4</i>	GU111774	02509	3	67	Contig4979_Contig4226	F:CAATTCTGGATGAACGTAC R:AACCGTAACCCCTTAAGTCG
<i>TcY-5</i>	GU111775	02508	3	67	Contig4979_Contig4226	F:TTCCGCTGCACCACTTGG R:GCGATCCGTAAGAATCCGG

^a F: forward primer, R: reverse primer.

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 allowed for hybridization of 4 independent cRNA samples each for elytra and hindwings. Elytra and hindwings were collected separately from 3–5 d pupae. Twenty-one insects were dissected each day and the samples dissected on three consecutive days were pooled for each biological replicate. A total of four biological replicates was obtained. RNA was prepared from the tissues using the

RNeasy Mini Kit (Qiagen). Two hundred ng of total RNA were used to generate cyanine 3-labeled cRNA with the aid of the Low RNA Input Linear Amplification kit with one-color (Agilent) and purified using the RNeasy Mini Elute kit (Qiagen). Six hundred ng of each labeled cRNA sample were fragmented at 60 °C for 30 min (Agilent Gene Expression Hybridization kit) and then hybridized to the microarray at 65 °C for 17 h. After hybridization, the microarray slide was washed with Agilent Gene Expression wash buffers as follows: buffer 1 at room temperature for 1 min and buffer 2 at 37 °C for 1 min.

The slide was scanned using an Agilent microarray scanner (G2565BA) with a setting for one-color using the green channel at 5 μm resolution. Data were extracted with the aid of Feature Extraction software v 9.5.1 (Agilent) and analyzed using GeneSpring GX 10 software. Each array was normalized to its 75th percentile to allow for comparison between arrays. A baseline transformation was performed to normalize each transcript to the median value for all samples. An unpaired t-test and Benjamini-Hochberg correction test were applied to identify all genes with ≥ 2-fold difference in expression at a *p*-value < 0.05. cRNA labeling, chip hybridization and scanning were performed by the Gene Microarray Core Facility at the University of Louisville. Data analysis was performed by the Bioinformatics Center at Kansas State University.

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* and *B. mori* (Albert and Kludiny, 2004; Drapeau, 2001, 2006b; 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.

We searched the *T. castaneum* genome database via BLAST (*tblastn* 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 N-glycosylation sites with the exceptions of TcY-g and -g2. Interestingly, TcY-y does not possess a long C-terminal stretch of more than 100 amino acids present in the Yellow-y orthologs found in Diptera and Lepidoptera (Supplementary Fig. 1). The Yellow-y orthologs from *A. mellifera*, *Nasonia vitripennis* and *Acyrtosiphon pisum* also lack a long C-terminal extension, suggesting that Yellow-y proteins in Diptera and Lepidoptera have gained the additional C-terminal segment rather recently. The TcY genes occur on linkage groups 2 (TcY-y), 3 (TcY-2-5), 8 (TcY-b, -e, -e3, -f, -g, -g2, -h and -1) and 9 (TcY-c).

In *A. mellifera*, 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 apparently are 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-y, -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

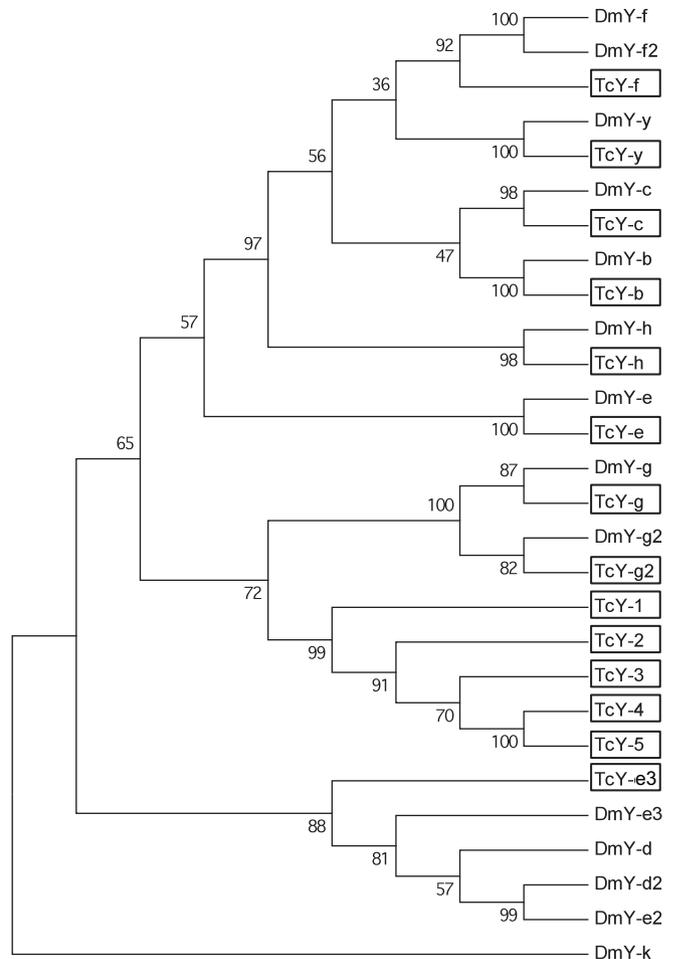


Fig. 1. Phylogenetic analysis of Yellow proteins in *T. castaneum* and *D. melanogaster*. ClustalW software was used to perform the multiple sequence alignment prior to phylogenetic analysis. The phylogenetic tree was constructed by MEGA 4.0 software using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Tamura et al., 2007). Numbers by each branch indicate results of bootstrap analysis from 5000 replications. *T. castaneum* Yellow proteins are boxed.

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

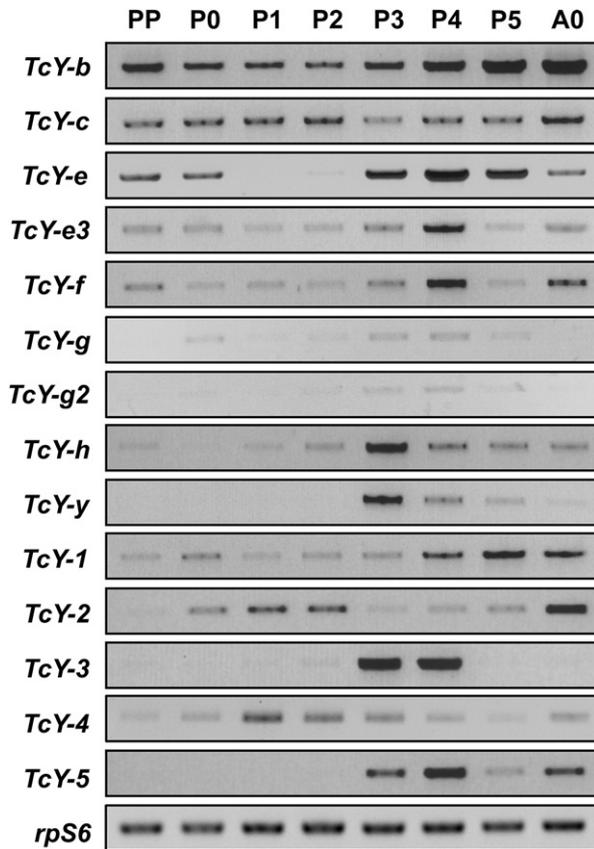


Fig. 2. Expression profiles of yellow genes in *T. castaneum* by RT-PCR. Total RNA was extracted from whole beetles ($n = 5$) at various developmental stages ranging from pharate pupae to young adults. PP, 1–2 d-old pharate pupae; P0, 0 d-old pupae; P1, 1 d-old pupae; P2, 2 d-old pupae; P3, 3 d-old pupae; P4, 4 d-old pupae; P5, 5 d-old pupae and A0, 0 d-old adults.

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-b*, 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
TcYellow genes differentially expressed in elytra and hindwings.

Gene	Fold difference	<i>p</i> -value	Tissue with higher expression
<i>TcY-c</i>	4.9	4.0×10^{-6}	Hindwing
<i>TcY-f</i>	4.4	1.5×10^{-4}	Elytron
<i>TcY-h</i>	3.2	1.6×10^{-4}	Elytron
<i>TcY-y</i>	57	1.7×10^{-3}	Hindwing
<i>TcY-1</i>	8.4	5.5×10^{-5}	Hindwing
<i>TcY-2</i>	12	1.4×10^{-4}	Elytron
<i>TcY-3</i>	22	3.5×10^{-6}	Elytron
<i>TcY-5</i>	168	9.2×10^{-4}	Elytron

Table 3
TcYellow proteins identified by proteomics.

Protein	Peptides matched	Percent coverage	Score ^a	Structure
TcY-b	19	40	195	Elytron
TcY-c	16	36	106	Elytron
	22	57	201	Hindwing
TcY-e	25	69	313	Elytron

^a Scores above 55 are significant ($p < 0.05$).

(Gompel et al., 2005; Wittkopp and Beldade, 2009; Wittkopp et al., 2002a, 2002b, 2009). In *P. xuthus* and *B. mori* larvae, the expression of *yellow-y* genes, *PxY-y* and *BmY-y*, is also consistent with the appearance and patterning of black pigmentation. Very recently, Ito et al. (2009) reported that *BmY-e* is also required for the normal larval body color pattern in *B. mori*. However, the exact physiological functions of most *yellow* genes are still little understood.

Purified recombinant DmY-f and DmY-f2 exhibited DCE enzymatic activity by catalyzing the conversion of dopachrome to 5,6-dihydroxyindole (Han et al., 2002). In *Manduca sexta*, a Yellow-like protein has been identified and purified from larval hemolymph, and a full-length cDNA corresponding to this protein was cloned. Phylogenetic analysis showed that the *M. sexta* Yellow protein was most closely related to DmY-f/f2, and enzymatic assays revealed that the recombinant *M. sexta* Yellow protein exhibited DCE activity (Sheng and Kanost, unpublished data).

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-f/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

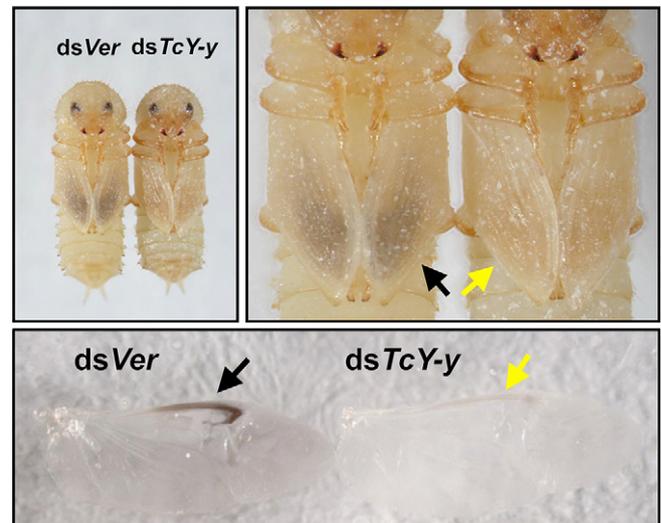


Fig. 3. Effect of dsRNA for *TcYellow-y* on cuticle pigmentation. *dsTcY-y* (200 ng per insect, $n = 40$) was injected into 1 d-old pupae. Injection of *dsTcY-y* had no effect on pupal development or pupal–adult molting. Top panels show 5 d-old pupae (pharate adults) with adult cuticle tanning visible through the old pupal cuticle. Bottom panel shows the hindwings dissected from the resulting adults. *dsTcY-y* injection caused significant defects in the pigmentation of the pterostigma in the hindwings. dsRNA for *T. castaneum* *vermillion* (*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.)

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, *BST/B^{Chr}*, in which *aspartate 1-decarboxylase* mRNA is suppressed, resulting in a deficiency of β -alanine 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 *BST/B^{Chr}* beetles, with the above-noted exception in the hindwing (Fig. 4). Similar results were obtained after co-injection of dsRNAs for *TcY-y* and *Tcebony* (Tomoyasu et al., 2009). These results suggest that *TcY-y* is not critical for the black body pigmentation in the *BST/B^{Chr}* 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

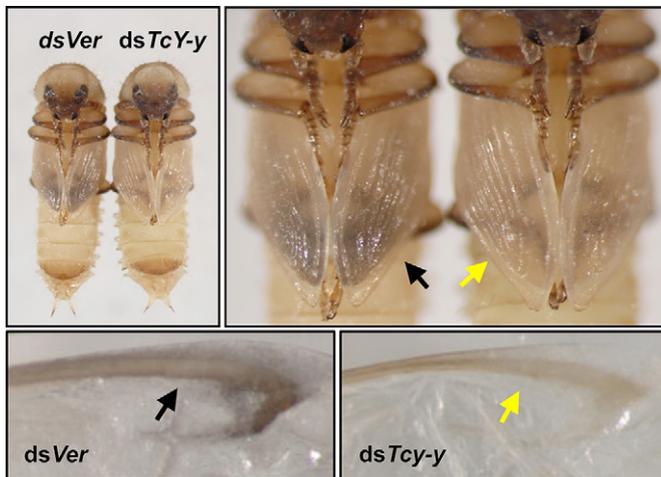


Fig. 4. Effect of dsRNA for *TcYellow-y* on cuticle pigmentation of the *T. castaneum* black body color mutant strain. RNAi for *TcY-y* was carried out using the black body color mutant, B^{ST}/B^{chr} , in which *aspartate 1-decarboxylase* (*TcADC*) mRNA is undetectable, and content of dopamine (for production of dopamine-melanin) is greatly increased (Arakane et al., 2009). *dsTcY-y* (200 ng per insect, $n = 40$) was injected into 1 d-old pupae. Top panels show 5 d-old pupae (pharate adults) when adult cuticle tanning is visible through the old pupal cuticle. Bottom panels show the pterostigma of the hindwings dissected out from resulting adults. As in the case of *TcY-y* RNAi using the wild-type GA-1 strain, there was no effect on adult cuticle pigmentation in the B^{ST}/B^{chr} strain, except for loss of black color in the pterostigma of the hindwing. dsRNA for *T. castaneum vermillion* (*dsVer*) was injected as the negative control for cuticle effects and as a positive control for effectiveness of injected dsRNA. 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.)

pupal cuticle including the setae, gin traps and urogomphi showed normal tanning. Adult cuticle could also be viewed through the translucent pupal cuticle. Adult cuticle tanning including the head, mandibles and legs was initiated on schedule (pupal day 4–5). In addition, unlike the results of the *dsTcY-y* injection, there was no defect in the black pigmentation of the hindwing including the pterostigma, indicating that *TcY-f* is not required for pupal and adult cuticle pigmentation in *T. castaneum* (left panel in Fig. 5). The subsequent

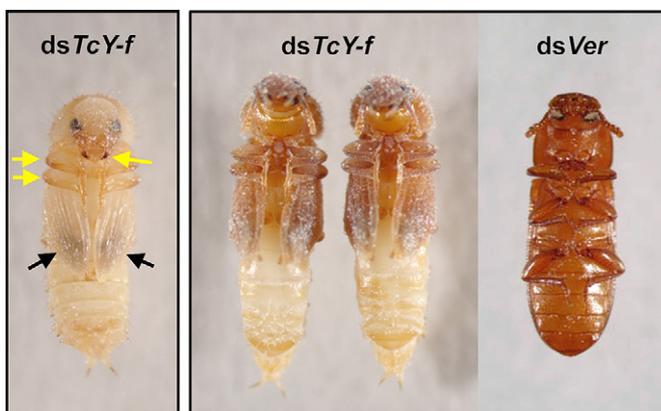


Fig. 5. Effect of dsRNA for *TcYellow-f* on pupal–adult molting. *dsTcY-f* (200 ng per insect, $n = 20$) was injected into late-stage larvae. *dsTcY-f* injection had no effect on larval–pupal molting or pupal development. Left panel shows absence of effect of *dsTcY-f* on adult cuticle tanning including that of the adult body wall, head, mandibles and legs (yellow arrows) as well as the hindwing including the pterostigma (black arrows), as observed through the pupal cuticle at the pharate adult stage (5 d-old pupa). However, these animals could not shed the old pupal cuticle and died without undergoing eclosion (right panel). Right panel shows the result of injection of dsRNA for *T. castaneum vermillion* (*dsVer*) as the negative control. (For interpretation of the references to colour in figure legends, the reader is referred to the web version of this article.)

pupal–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 its 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 *dsTcY*-family-treated insects using dynamic mechanical analysis to help determine their functions in *T. castaneum* development (Arakane et al., 2009).

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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.

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