

Section: Biology, Behaviour and Detection

Implications of the *Tribolium* genome project for pest biology

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DOI: 10.5073/jka.2010.425.324

Abstract

The universal availability of the complete *Tribolium castaneum* genome sequence assembly and annotation (Richards et al., 2008) and concomitant development of the versatile *Tribolium* genome browser, BeetleBase (Kim et al., 2010, <http://beetlebase.org/>) open new realms of possibility for stored product pest control by greatly simplifying the task of connecting biology and behavior with underlying molecular mechanisms. This genome has enabled sequence similarity searches that have resulted in a flood of new discovery involving thousands of genes with important functions in digestion, osmoregulation, metamorphosis, olfaction, xenobiotic metabolism, vision, and embryonic and larval growth and development. The value of the *T. castaneum* genome sequence is greatly enhanced by the availability of a sophisticated functional genomic toolkit for laboratory studies of this insect. These tools include high-resolution physical and genetic maps, genomic and cDNA libraries, balancer chromosomes, and effective and reliable techniques for specific knockout of any target gene *via* RNA interference (RNAi). In this paper we briefly discuss just two areas of *Tribolium* biology research that are being revitalized by the availability of the genome sequence, namely olfaction and exoskeleton, or “smell and skin”.

1. Pheromone biology, olfaction and genomics

Many common stored product beetles are long-lived as adults (weeks or months) and use male-produced aggregation pheromones to attract both sexes for mating and for achieving critical population densities for effective conditioning of the microhabitat (Phillips et al., 2000). Examples include species of *Tribolium*, *Tenebrio*, *Sitophilus*, *Cryptolestes*, *Oryzaephilus*, *Rhyzopertha*, and *Prostephanus*. In contrast, adult beetles with female-produced sex pheromones, including stored-product pests in the families Anobiidae, Bruchidae and Dermestidae, tend to be short-lived (days to weeks), and may require only nectar for sustenance. In *Tribolium confusum* (Duval) and *T. castaneum* (Herbst) the predominant male-produced aggregation pheromone is 4,8-dimethyldecanal (DMD; Suzuki, 1980), which has two chiral carbons whose configurations affect biological activity (Levinson and Mori, 1983; Suzuki and Mori, 1983). A 4:1 mixture of the (4R,8R) and (4R,8S) diastereomers elicits a near-optimal attractive response, showing more than ten-fold greater attractive potency than either the corresponding 1:1 blend or the pure (4R,8R) isomer (Suzuki et al., 1984). Since a 1:1 blend of (4R,8R):(4R,8S) is presumably the recipe used in commercially available Tribolure, there is potential for development of a much-improved blend that could be a powerful research tool in studies of *Tribolium* population biology.

As the name implies, this pheromone may function only for aggregation, while other semiochemical cues might be needed to evoke mounting and copulation. The latter idea was first suggested by Keville and Kannowski (1975), who found evidence that 1-pentadecene and other hydrocarbons elicit copulatory behavior in *Tribolium confusum*. It has been observed that, in contrast to the highly effective *Rhyzopertha dominica* (F.) aggregation pheromone, the synthetically-produced *Tribolium* aggregation pheromone (Tribolure) is a relatively weak attractant, to the extent that it is not useful for mass-trapping of large numbers of beetles. Tribolure-baited traps are, however, extremely useful for population monitoring. The possibility that Tribolure contains an unnatural or nonideal blend of diastereomers, reducing its effectiveness, has already been mentioned, but the relatively low attractiveness of Tribolure-baited traps might also be a function of the behavior and ecology of *Tribolium*. In addition, it has been

suggested recently (Verheggen et al., 2007) that minor or trace components of a natural *Tribolium* aggregation pheromone blend might exist and might be critical for maximum activity. Candidate trace constituents include several benzoquinone and hydrocarbon secondary metabolites that are known to be produced in *Tribolium* spp. For example, in *T. confusum* two different 1, 4-benzoquinones and several mono-unsaturated hydrocarbons, previously detected in several *Tribolium* species (Howard, 1987; Markarian et al., 1978), were shown to be attractive in behavioral assays and elicited electroantennogram (EAG) responses in isolated antennae (Verheggen et al., 2007). These include some of the same components previously reported by Keville and Kanno (1975) to be active in eliciting mating behavior. No one has yet reported on attempts to increase the potency of synthetic Tribolure by creating new blends that incorporate these candidate components as minor constituents, but this would seem to be worthy of investigation. It has been observed that Tribolure-baited traps become more attractive after a number of beetles have been captured, perhaps because they are releasing important minor components not included in the commercial pheromone blend (Jim Campbell, unpublished observations).

Chemical, physiological and behavioral studies have revealed much useful information about the olfactory and pheromonal biology of stored-product insects, but, as highlighted above, fundamental questions remain unanswered. The recently completed genome sequence of the red flour beetle, *T. castaneum*, opens a window to a vast, untapped reservoir of opportunity for gaining new knowledge about many aspects of the biology of this pest species, including the biology of olfaction. Establishing or confirming pheromone and secretome biosynthetic pathways (e.g. Kim et al., 2005) will be facilitated by the availability of the genome sequence. In many cases candidate olfaction genes can be readily identified based on sequence conservation, and the powerful technique of RNAi can then be used to knock down candidate genes and enable follow-up functional studies.

Annotation of the *T. castaneum* genome sequence has already revealed unexpectedly large numbers of intact olfactory receptor (OR) and gustatory receptor (GR) genes (259 and 220, respectively, Engsontia et al., 2008), in comparison to other species. For example, *Drosophila melanogaster* has only 62 and 68 OR and GR genes, respectively. In addition to the relatively very large numbers of intact OR/GR genes in the *T. castaneum* genome, there are also 79 OR pseudogenes and 76 GR pseudogenes. RNAi has been done for *TcORI*, which is one of the 259 OR genes in *T. castaneum*, and is a clear ortholog of the *D. melanogaster Or83b* gene, the latter having been demonstrated to be required for function of olfactory reception in *D. melanogaster*. RNAi-mediated knockout of *TcORI* completely eliminated the attractiveness of the synthetic aggregation pheromone DMD (Tribolure) to adult beetles (Engsontia et al., 2008). So far as we are aware, no other olfaction genes have been subjected to functional analysis in *T. castaneum*, but the success of this experiment and the large number of olfaction genes in this species hint at the wealth of functional information that could be revealed by systematic, high-throughput RNAi knockdown studies.

The primary mediators of odor detection in insects are the odorant binding proteins (OBPs) and their smaller, more highly conserved cousins, the chemosensory proteins (CSPs). According to Foret and Maleszka (2006) there are 46 *OBP* genes in the *T. castaneum* genome, which is within the normal range (20~70) as currently defined by the available insect genome sequences. In contrast, the gene count for insect *CSPs* is considerably lower, totaling less than 10 each in *A. mellifera*, *D. melanogaster* and *A. gambiae*. We could find no published data on complete *CSP* annotations in *T. castaneum*, but allusions to individual *CSP* genes can be found in Maleszka et al. (2007), Lu et al. (2007) and Vieira et al. (2007). According to our BLASTP analysis, there are 14 genes encoding highly conserved CSP proteins in *T. castaneum* (Fig. 1), as well as three additional *CSP*-like genes that encode slightly more divergent proteins.

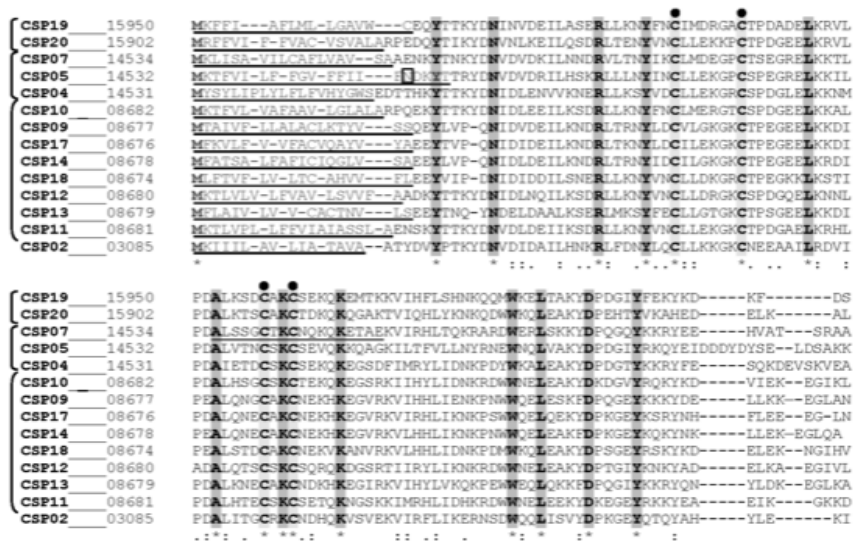


Figure 1 Alignment of Chemosensory Proteins (CSPs) in *Tribolium castaneum*. CSP proteins were detected by BLASTP analysis using a 19-residue query sequence derived from a conserved region of CSP7 (underlined) containing two of the cysteine residues involved in disulfide bridging. Multiple sequence alignment was done using T-COFFEE (Notredame et al., 2000). Fourteen of the 17 known CSP proteins are included. The remaining three (CSP06, 08 and 15) are somewhat divergent from the 14 shown. Secretion signal peptides at the amino termini of each protein, indicated in red font and by underlining, were determined with SignalP 3.0. Invariant residues are indicated by shading and bold font, and the four diagnostic cysteine residues (forming two disulfide bridges) are indicated by dots above the alignment. The curated version of CSP5 lacked a signal peptide and contained a unique, 14-residue insertion, both resulting from an apparent misannotation. We defined a previously unrecognized, 42-nt intron at the Asn (N) residue (boxed) near the terminus of the signal peptide, which reestablished a plausible signal peptide cleavage site and removed the atypical insertion. NCBI protein identities are as follows: CSP18 = NP_001039286.1, CSP17 = NP_001039284.1, CSP09= NP_001039283.1, CSP10= NP_001039278.1, CSP11= NP_001039279.1, CSP12= NP_001039280.1, CSP13= NP_001039281.1, CSP14= NP_001039282.1, CSP19= NP_001039276.1, CSP20= NP_001039274.1, CSP07= NP_001039289.1, CSP05= NP_001039287.1, CSP04= NP_001039285.1, CSP02= NP_001039277.1. The following CSPs are not shown: CSP15= NP_001039291.1, CSP08= NP_001039290.1 and CSP06= NP_001039288.1.

Eight of the 14 proteins shown in Figure 1 are encoded by a closely linked cluster of *CSP* genes located on chromosome 7. Inspection of the tiling array tracks for this chromosomal region in the *Tribolium* genome browser BeetleBase (<http://beetlebase.org/index.shtml>) suggests that the various *CSPs* are differentially expressed and highly regulated (Fig. 2). For example, *CSP10* expression is largely restricted to the larval and early pupal stages, whereas *CSP11* is expressed predominantly in the late pupal and adult stages. *CSP12* appears to have two peaks of expression, one in the early part of the last-instar larval stage and another in the late pupal stage. Still other *CSP* genes (*CSP9*, *CSP13-15* and *CSP17*) have very low or no expression throughout the last larval instar and pupal and adult stages. The latter could have vital but highly localized expression domains (individual sensillae or appendages?) preventing detection of such low-abundance transcripts in whole-animal tiling arrays.

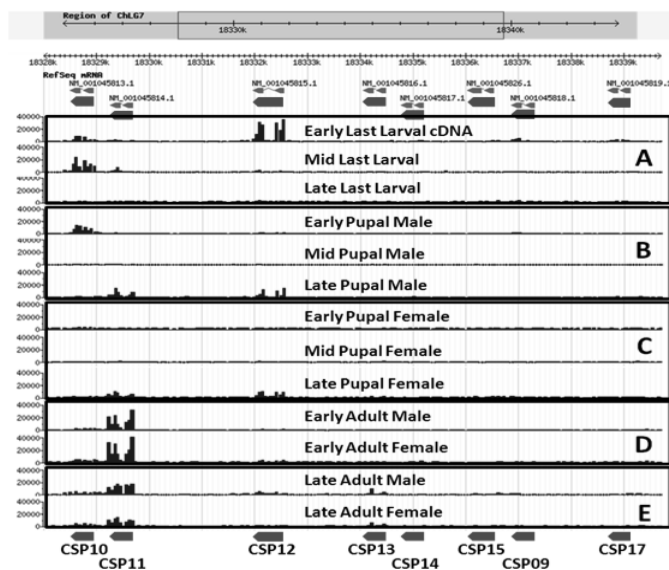


Figure 2 Tiling Array Expression Patterns of a Cluster of Eight Chemosensory Protein (CSP) Genes on Chromosome 7 of *Tribolium castaneum*. Depictions of results from Nimblegen expression tiling array hybridizations were selected in the GBrowse genome viewer in BeetleBase. Vertical axes represent relative expression as determined by hybridization to microarrays containing contiguous 50-mer probes spanning the entire genome (excluding repetitive sequences). cDNAs used for hybridizations had been isolated from whole insects at the stages indicated, ranging from last instar larvae (panel A) through mature adults (panel E). Horizontal red bars (duplicated above and below tiling array panels) indicate positions of genes on the chromosome, and point in the direction of transcription. Computed gene structures and NCBI RefSeq names are shown just above the upper set of bars. Future work in our group will focus on description and RNAi-mediated knockdown of gene products responsible for pheromone biosynthesis, release or perception, with the general goal of understanding the basis for these functions in insect biology, and with a specific goal of knocking down one or more systems in a manner that could facilitate pest management. There is a total of “only” 63 OBP and CSP genes that are the presumed direct mediators of all chemosensory responses in the *Tribolium* repertoire. With specific gene knockdown by RNAi now a routine procedure, the previously impossible task of functionally characterizing all first-response odor detection genes now seems feasible.

2. Chitin biology and genomics

The insect exoskeleton is a noncellular biomaterial that functions both as skin and as waterproof armor that is sufficiently flexible to accommodate growth and enable mobility. This complex and fascinating physiological adaptation, together with other chitinous structures such as the digestive “peritrophic matrix” of the midgut, has contributed to the great evolutionary success of arthropods. We and others have identified more than 200 genes in the *Tribolium* genome that appear to be directly involved in the composition, biosynthesis, deposition and turnover of these structures (Tab. 1), offering a wealth of potential new biotargets for selective pest control. The proteins encoded by these genes can be divided into four major categories, including: (1) structural “cuticle proteins” that, together with the polysaccharide chitin itself, contribute most of the bulk and substance of the finished cuticle; (2) enzymes involved in chitin synthesis, modification or degradation; (3) cuticle assembly proteins involved in deposition and layering of chitin and cuticle structural proteins; and (4) cuticle maturation enzymes that control the processes of tanning, crosslinking and pigmentation to confer the final color, rigidity/elasticity and waterproofing to the finished cuticle. Many of the gene models have been validated by sequence analysis of cDNAs, and many have been functionally characterized by RNAi or other methods (e.g. Arakane et al., 2005a & b, 2008, 2009a & b, 2010; Dixit et al., 2008; Hogenkamp et al., 2008; Jaspurua et al., 2010; Richards et al., 2008; Zhu et al., 2008;).

Table 1 *Tribolium castaneum* genes involved in assembly and metabolism of cuticle and peritrophic matrix

Category	Gene	Genbank/GLEAN accession #	dsRNA knock-down phenotypes	proposed function
Chitin synthases (CHS)	TcCHS-A-8a	AY291475	Prevents L-L, L-P and P-A molting	chitin synthesis
	TcCHS-A-8b	AY291476	Prevents adult eclosion	chitin synthesis
	TcCHS-B	AY291477	loss of PM chitin, starvation	chitin synthesis
UDP-NAG pyrophosphorylases	TcUAP1	GU228846	not tested	chitin synthesis
	TcUAP2	GU228847	not tested	chitin synthesis
	TcCHT2	GLEAN_09872	not tested	chitin degradation
	TcCHT4	EF125543	No visible phenotype	chitin degradation in digestive peritrophic matrix
	TcCHT5	AY675073	Affects P-A molting	chitin degradation
	TcCHT6	EFA00965	No visible phenotype	chitin degradation
	TcCHT7	DQ659247	arrested pupal wing expansion and pumping motion	chitin degradation
	TcCHT8	DQ659248	No visible phenotype	chitin degradation in digestive peritrophic matrix
	TcCHT9	DQ659249	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT10	DQ659250	Prevents L-L, L-P and P-A molting	chitin degradation
	TcCHT11	GLEAN_15665	No visible phenotype	chitin degradation
	TcCHT12	GLEAN_09178	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT13	DQ659252	not tested	chitin degradation in digestive peritrophic matrix
Chitinases	TcCHT14	GLEAN_09628	No visible phenotype	chitin degradation in digestive peritrophic matrix
	TcCHT15	GLEAN_09629	No visible phenotype	chitin degradation in digestive peritrophic matrix
	TcCHT16	AY873915	No visible phenotype	chitin degradation in digestive peritrophic matrix
	TcCHT17	GLEAN_09625	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT18	GLEAN_09630	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT19	GLEAN_09175	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT20	AY873913	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT21	AY873916	not tested	chitin degradation in digestive peritrophic matrix
	TcCHT22	DQ659251	not tested	chitin degradation in digestive peritrophic matrix
	TcIDGF2	DQ659253	No visible phenotype	cell proliferation/remodeling
TcIDGF4	DQ659254	Prevents P-A molting	cell proliferation/remodeling	
N-Acetyl glucosaminidases (NAG)	TcNAG1	EF592536	Prevents L-L, L-P and P-A molting	chitin degradation
	TcNAG2	EF592537	Prevents L-L, L-P and P-A molting	chitin degradation
	TcNAG3	EF592538	compromises L-L, L-P and P-A molting	chitin degradation
	TcFDL	EF592539	Prevents L-L, L-P and P-A molting	N-glycan processing/chitin catabolism
	TcCDA1	EU019711	Prevents L-L, L-P and P-A molting	chitin modification
	TcCDA2a	EU019712	Nonarticulation of femoral-tibial joints	chitin modification
	TcCDA2b	EU019713	Affects epidermal cuticle morphology	chitin modification
Chitin deacetylases (CDA)	TcCDA3	EU190485	No visible phenotype	chitin modification
	TcCDA4	EU190486	No visible phenotype	chitin modification
	TcCDA5A	EU190487	No visible phenotype	chitin modification
	TcCDA5B	EU190488	No visible phenotype	chitin modification
	TcCDA6	EU190489	No visible phenotype	chitin modification
	TcCDA7	EU190490	No visible phenotype	chitin modification
	TcCDA8	EU190491	No visible phenotype	chitin modification
	TcCDA9	EU190492	No visible phenotype	chitin modification

Category	Gene	Genbank/GLEAN accession #	dsRNA knock-down phenotypes	proposed function
Cuticle assembly genes	TcRtv	GLEAN_07384	not tested	cuticle assembly
	TcKNK1	GLEAN_10653	not tested	cuticle assembly
	TcKNK2	GLEAN_12301	not tested	cuticle assembly
	TcKNK3	GLEAN_02304	not tested	cuticle assembly
Peritrophic matrix-associated genes	TcPMP1-A	GU128096	not tested	peritrophic matrix structural protein
	TcPMP1-B	GU128097	not tested	peritrophic matrix structural protein
	TcPMP1-C	GU128098	not tested	peritrophic matrix structural protein
	TcPMP2-A	GU128099	not tested	peritrophic matrix structural protein
	TcPMP2-B	GU128100	not tested	peritrophic matrix structural protein
	TcPMP2-C	GU128101	not tested	peritrophic matrix structural protein
	TcPMP3	GU128102	not tested	peritrophic matrix structural protein
	TcPMP5-A	GU128103	not tested	peritrophic matrix structural protein
	TcPMP5-B	GU128104	not tested	peritrophic matrix structural protein
	TcPMP9	GU128105	not tested	peritrophic matrix structural protein
	TcPMP14	GU128106	not tested	peritrophic matrix structural protein
	TcLac1	AY884065	No visible phenotype	ferroxidase
	TcLac2A	AY884061	Required for larval, pupal and adult cuticle tanning	tanning
	Phenoloxi-dases	TcLac2B	AY884062	Required for larval, pupal and adult cuticle tanning
TcLLP		GLEAN_15880	Prevent pupal development	unknown vital function
TcTyr1		AY884063	No visible phenotype	Immune-related melanization?
TcTyr2		AY884064	No visible phenotype	Immune-related melanization?
TcTH		EF592178	Required for larval, pupal and adult cuticle tanning	cuticle tanning-related
TcDDC		EU019710	Delays adult cuticle tanning, darkens body color	cuticle tanning-related
TcADC		ABU25221	Black body color phenotype	cuticle tanning-related
TcNAT1		FJ647798	Dark pigment around elytral sensillae	cuticle tanning-related
Tcebony		FJ647797	Black body color phenotype	cuticle tanning-related
Tctan		GLEAN_03448	not tested	cuticle tanning-related
TcYellow-b		GU111762	not tested	pigmentation/sclerotization
TcYellow-c		GU111763	not tested	pigmentation/sclerotization
TcYellow-e		GU111765	not tested	pigmentation/sclerotization
TcYellow-e3		GU111764	not tested	pigmentation/sclerotization
Cuticle tanning - related genes	TcYellow-f	GU111766	Prevents P-A molting	pigmentation/sclerotization
	TcYellow-g1	GU111767	not tested	egg cuticle stabilization
	TcYellow-g2	GU111768	not tested	egg cuticle stabilization
	TcYellow-h	GU111769	pigmentation/sclerotization	pigmentation/sclerotization
	TcYellow-y	GU111770	prevents hindwing melanin production	pigmentation/sclerotization
	TcYellow-1	GU111771	not tested	pigmentation/sclerotization
	TcYellow-2	GU111772	not tested	pigmentation/sclerotization
	TcYellow-3	GU111773	not tested	pigmentation/sclerotization
	TcYellow-4	GU111774	not tested	pigmentation/sclerotization
	TcYellow-5	GU111775	not tested	pigmentation/sclerotization

Category	Gene	Genbank/GLEAN accession #	dsRNA knock-down phenotypes	proposed function
Cuticle protein (CP) genes	RR1 CP	~50 genes	not tested	cuticle structural proteins
	RR2 CP	~50 genes	not tested	cuticle structural proteins
	RR3 CP	~10 genes	not tested	cuticle structural proteins
	CPLC	~15 genes	not tested	cuticle structural proteins
	other CPs*	~15 genes	not tested	cuticle structural proteins
	TcCPAP3-A1	EF125544	not tested	cuticle structural protein
	TcCPAP3-A2	EF125545	not tested	cuticle structural protein
	TcCPAP3-B	EF125544	not tested	cuticle structural protein
	TcCPAP3-C5a	EF125545	not tested	cuticle structural protein
	TcCPAP3-C5b	EF125546	not tested	cuticle structural protein
	TcCPAP3-D1	EF125544	not tested	cuticle structural protein
	TcCPAP3-D2	EF125545	not tested	cuticle structural protein
	TcCPAP3-E	EF125546	not tested	cuticle structural protein
	TcCPAP1-A	EF125546	not tested	cuticle structural protein
	TcCPAP1-B	EF125546	not tested	cuticle structural protein
	TcCPAP1-C	EF125546	not tested	cuticle structural protein
TcCPAP1-D	EF125546	not tested	cuticle structural protein	
TcCPAP1-E	EF125546	not tested	cuticle structural protein	
TcCPAP1-F	EF125546	not tested	cuticle structural protein	
TcCPAP1-G	EF125546	not tested	cuticle structural protein	
TcCPAP1-H	EF125546	not tested	cuticle structural protein	
TcCPAP1-I	EF125546	not tested	cuticle structural protein	
TcCPAP1-J	EF125546	not tested	cuticle structural protein	

*includes tweedle, CPF and CPFL genes

With respect to category (1) above, insects employ an amazing variety of what appear to be structural protein genes during manufacture of the chitinous matrices. In *T. castaneum* there are approximately 160 cuticle protein genes, encoding approximately 110 RR motif proteins, ~15 “cuticle proteins of low complexity” (CPLCs), 18 “cuticle proteins analogous to peritrophins” CPAPs) and several other minor categories of cuticle proteins, each represented by only one or a few genes (Table 1 and unpublished observations). Why insects should require such a large array of protein structural components for cuticle and peritrophic matrix is still uncertain, but it is becoming clear that the various cuticle proteins are not uniformly expressed, and that different regions of cuticle and peritrophic matrix have different protein composition, probably reflecting the different physical properties and functions needed in different regions of the exoskeleton and digestive sac (Willis, 2010).

A great number and variety of genes are also utilized for chitin-modification and degradation (24 *chitinases*, 4 *N-acetyl glucosaminidases* and 9 *chitin deacetylases*) and for cuticle tanning and pigmentation (26 genes). Of particular interest in the latter category are the 14 *yellow* genes, each of which may be specialized for a unique function. For example, we found that one of the *yellow* genes is required specifically for wing pigmentation (but not that of elytra or body wall) while another *yellow* gene has a specific, vital role in tanning of the cuticle of the adult body wall (Arakane et al., 2010).

3. Summary

In summary, the *T. castaneum* genome project has opened a fast-track to gene discovery in this stored product pest insect for all areas of *Tribolium* biology, two of which are briefly discussed here. These examples reveal the complexity and sophistication of genetic regulation of insect adaptations, but also illustrate the rapid progress towards understanding biological mechanisms made possible by the availability of this genome sequence. Any gene can be quickly categorized as either essential or dispensable by the powerful technique of RNAi. For essential genes, the timing and mode of RNAi-induced death give clues about specific gene functions, supplementing insights gained from protein sequence homology and conserved domain analysis. More subtle and detailed functional inferences can be gleaned by more careful scrutiny of beetles after gene knockdown or by more sophisticated bioassays. Examples include monitoring for changes in responsiveness to pheromone after knockdown of candidate olfaction genes, monitoring for abnormalities in stereotyped premolting behavior after knockdown of

candidate effector genes involved in the endocrine regulation of molting, or monitoring cuticle composition after knockdown of genes with suspected roles in the metabolism or recycling of the exoskeleton. Assessment of gene/protein expression patterns and biochemical studies of purified or recombinant proteins can complement and supplement gene RNAi studies. The resulting improvements in our knowledge of the basic biology of pest insects will fuel the next generation of pest control technologies.

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