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# Putative orthologues of genetically identified *Drosophila melanogaster* chitin producing and organising genes in *Apis mellifera*

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**Abstract** – The cuticle is a versatile coat allowing insects to survive in a variety of ecological situations. The polysaccharide chitin is a major cuticle component defining cuticle function. The key players of chitin synthesis and organisation, including chitin synthase-1 (CS-1), the chitin deacetylases vermiform (Verm) and serpentine (Serp), the extracellular obstructor-A (Obst-A) protein, the membrane-bound Knickkopf (Knk) and retroactive (Rtv) proteins, the cytoplasmic mummy (Mmy) protein and the transcription factor grainyhead (Grh) have been characterised genetically in the fruit fly *Drosophila melanogaster*. Here, we report on the identification of putative orthologues of these factors in *Apis mellifera* and on their expression at different developmental stages. Overall, differences in gene organisation and expression pattern between these two species may compile different cuticle compositions that reflect their life style and ecology. Our data will serve to elucidate the honeybee-specific mechanisms of cuticle formation.

**cuticle / chitin / chitin synthase / chitin organisation**

## 1. INTRODUCTION

Insects are covered by a cuticle that protects them against dehydration and pathogen entry and allows locomotion. In addition, the cuticle stabilises the body as well as internal organs such as the foregut, the hindgut and the tracheae. It is a stratified extracellular matrix that is produced by the underlying epithelial cells at their apical site (Moussian 2010, 2013). Stratification is the result of ordered sorting and

arrangement of distinct components. The outermost layer is the envelope, which mainly consists of a species-specific mixture of hydrocarbons and wax esters. Underneath the envelope lies the epicuticle that is composed of yet unidentified proteins and probably lipids. Bundles of the polysaccharide chitin associate with chitin-binding proteins and form horizontal sheets (called *laminae*) that are stacked constituting the innermost cuticle layer, the procuticle. While the histology of the cuticle of various arthropods has been meticulously described in the last decades (Locke 2001), the molecular mechanisms of cuticle differentiation are being investigated only recently in detail. Genetic approaches using the fruit fly *Drosophila melanogaster* have advanced our understating especially in the field of chitin production and organisation. Subsequently, several factors iden-

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tified and characterised in *D. melanogaster* have been studied in the red flour beetle *Tribolium castaneum* by RNA interference, largely confirming results obtained with *D. melanogaster* and occasionally adding new notions to their function. For instance, Knickkopf (Knk), a membrane-bound chitin organising factor in *D. melanogaster* (Moussian et al 2006), is additionally needed to protect chitin from chitinase-driven degradation in *T. castaneum* (Chaudhari et al 2011). Hence, despite the equivalence of chitin synthesising and organising factors in *D. melanogaster* and *T. castaneum*, functional differences exist. The significance of these differences remains to be investigated.

In order to deepen our understanding in variability of the molecular mechanisms deployed during insect cuticle formation, we sought to identify those chitin producing and organising factors in the honeybee *Apis mellifera* that had been previously demonstrated to be essential in *D. melanogaster* in genetic experiments. These factors that supposedly constitute the core factors of chitin synthesis and organisation are krotzkopf verkehrt/chitin synthase-1 (Kkv/CS-1), the chitin monomer (GlcNAc) producing enzyme mummy (Mmy, UDP-GlcNAc pyrophosphorylase), the membrane-proteins Knickkopf (Knk) and retroactive (Rtv) and the extracellular proteins obstructor-A (Obst-A), serpentine (Serp) and vermiform (Verm), two chitin deacetylases (Gangishetti et al 2012; Luschnig et al 2006; Moussian et al 2005a, b, 2006; Petkau et al 2012; Tonning et al 2006). Mutations in the respective genes are lethal and cause chitin deficiency (*CS-1/kkv*, *mmy*) or chitin disorganisation (*knk*, *obst-A*, *rtv*, *verm*, *serp*). In addition, we identified the *A. mellifera* grainyhead (Grh) transcription factor that in *D. melanogaster* has been shown to control the transcription of several chitin producing and organising factors such as *knk* (Bray and Kafatos 1991; Gangishetti et al 2012; Pare et al 2012). In order to evaluate especially the presence of newly discovered exons and to study expression dynamics, we also monitored the expression of these genes at different developmental stages.

## 2. MATERIALS AND METHODS

### 2.1. Animal husbandry

Honeybee (*A. mellifera*) larvae and pupae were obtained from the Apicultural State Institute at the University of Hohenheim, Germany.

### 2.2. Identification of putative chitin producing and organising factors in *A. mellifera*

In order to identify putative orthologues of *D. melanogaster* genetically characterised chitin producing and organising factors in *A. mellifera*, we established and followed a five-step protocol. First, we searched the bee genome for respective loci with the coding sequence of *D. melanogaster* factors using the tBLASTx software at BeeBase ([hymenopteragenome.org/beebase](http://hymenopteragenome.org/beebase)) (Munoz-Torres et al 2011). In general, searches yielded fragments coding for protein sequences highly homologous to the *D. melanogaster* sequence. Second, the exons of the annotated sequence encoding the closest homologue were assembled to a continuous coding sequence that was translated to a protein sequence using the SerialCloner 2-6-1 software. Third, the conceptual *A. mellifera* protein sequence was subsequently used to search the *D. melanogaster* protein sequence database at FlyBase for homologous sequences by BLASTp. Without any exception, this search confirmed the closest similarity between the respective initial *D. melanogaster* and *A. mellifera* sequences. Usually, the protein sequences were co-linear. Co-linearity of *D. melanogaster* and *A. mellifera* protein sequences is considered as an indication that we had identified all exons correctly. In parallel, our deduced honeybee protein sequences were aligned with *A. mellifera* annotated protein sequences at the NCBI gene bank by BLASTp. Surprisingly, there were substantial differences in several cases; these differences are dealt within the “Results” section. Ambiguous exons were tested by qualitative PCR whether they are part of respective transcripts or not. The fourth step of our protocol was to use the protein organisation retrieved from the genomes of the honeybee-related species *Bombus impatiens*, *Nasonia vitripennis* and *Camponotus*

*floridanus* to corroborate exon composition of the *A. mellifera* gene. Fifth, expression of genes in *A. mellifera* was recorded at the first (L1) and fourth (L4) instar larval and pink-eyed pupal stages.

### 2.3. Sequence analyses

Protein domains were initially identified during sequence comparison using the BLASTp software at the National Centre for Biotechnology Information (NCBI). Subsequently, they were studied at the Conserved Domain (NCBI) and Pfam (pfam.sanger.ac.uk/) databases. Putative signal peptides were detected with the PSORT software (psort.hgc.jp). Glycosylphosphatidylinositol (GPI) anchors were predicted using the big-PI Predictor software (mendel.imp.ac.at/gpi/gpi\_server.html). Transmembrane domains were predicted using the TMPred software at embnet.vital-it.ch/software/TMPRED\_form.html. Multiple sequences were aligned using the ClustalO software at [www.uniprot.org/align/](http://www.uniprot.org/align/). For phylogenetic analyses, neighbour-joining trees were constructed using the MEGA 5.2.2. software that allows testing correctness of the relationships by the bootstrap test (1,000 calculations, method: *p*-distance).

To search for possible exons in the 5' region of *GB46725* (coding for the putative grainyhead orthologue), the respective region was scanned manually and by using the software Fgenesh+ ([www.softberry.com](http://www.softberry.com)) and Genscan (genes.mit.edu/GENSCAN.html).

### 2.4. Molecular biology

Total RNA was extracted from 50 first (L1) and two fourth instar larvae (L4) and two pink-eyed pupae using the Qiagen RNEasy kit (Hilden, Germany). For each stage, two independent RNA extractions were carried out. PCR experiments with primers recognising genomic DNA but not complementary DNA (cDNA) proved the absence of genomic DNA (data not shown). Total RNA served as template to produce cDNA by oligodT-primed reverse transcription using the Roche Transcriptor First-Strand cDNA Synthesis kit (Mannheim, Germany). Qualitative PCR was performed on a LightCycler Nano from Roche using the Roche FastStart SYBR Green

Master kit (Mannheim, Germany). In our qualitative PCR experiments, we followed the guidelines proposed by Bustin et al (2009). Amplification of *tubulin* (*GB44134*, most similar sequence to *D. melanogaster tub56*) transcripts was performed to verify that the dynamic range of amplification was linear at three template concentrations (−10-fold, 1-fold, +10-fold). The primers used for transcript amplification were designed with the Primer3 software (biotools.umassmed.edu/bioapps/primer3\_www.cgi) and produced by Sigma-Aldrich; they are listed in Supplementary Table 1. Data based on ddCq values and amplification efficiencies were analysed with the respective Roche LightCycler software and Microsoft Excel; efficiency values for each pair of primers are indicated in Supplementary Table 1. As a control, primers to amplify *tubulin* were used in each experiment.

To analyse the presence of the exon-pairs XIV/XV and XVIII/XIX in chitin-synthase coding transcripts, we first amplified the sequences between exons XIII to XVII and XVI to XX from L4 larval cDNA (generated using the Sigma-Aldrich Enhanced Avian RT First Strand Synthesis Kit) using appropriate primers (Supplementary Table 1). Second, we used the respective amplicons to amplify and determine sequences between exons XIII and XIV or XV and exons XVI and XVIII or XIX using appropriate specific primers (Supplementary Table 1).

## 3. RESULTS

### 3.1. The chitin synthase (CS-1/Kkv)

Chitin is synthesised by the membrane-inserted chitin synthase. Mutations in the *D. melanogaster* chitin synthase coding gene *krotzkopf verkehrt* (*kkv*) are lethal and cause a chitin-deficient collapsed cuticle (Moussian et al 2005a). The *kkv* locus codes for two enzyme isoforms with 1,615 amino acids derived from two alternative transcripts harbouring either exon VIIa or VIIb (Irion 2012). The *kkv*-similar *A. mellifera* *GB49845* locus is annotated to contain 23 exons coding for a conceptual protein with 1,783 amino acids (Figure 1). Sequence comparison suggests that duplicated stretches of sequences encoded by alternative

exons account for the non-linearity and over-length of the *A. mellifera* sequence. For instance, exons XIV and XV (102 aa) both contain the WGTRE motif that has been proposed to be essential for chitin extrusion (Merzendorfer 2006). This motif is at the beginning of a long stretch of a coiled-coil forming sequence (residues 1,121 to 1,224, in *D. melanogaster*: 1,071 to 1,173; Fig. S1). Other insect chitin synthases contain only one WGTRE motif arguing that exons XIV and XV are mutually exclusive. By qualitative PCR (Table II) and sequence determination (data not shown), we confirmed that these exons are not present on the same transcript, i.e. they are most probably alternatively spliced in larvae and pupae.

We next asked whether the duplication of the WGTRE coding exon is specific to *A. mellifera* or Hymenoptera. To answer this question, we characterised the genes coding for the epidermal chitin synthase in the Hymenoptera *B. impatiens* (Apidae, i.e. closely related to *A. mellifera*, (Johnson et al 2013)), *C. floridanus* (Formicidae, i.e. intermediate relative of *A. mellifera*) and *N. vitripennis* (Pteromalidae, i.e. distantly related to *A. mellifera*). *D. melanogaster* served as an out-group (Table I). The duplicated exon is present in *B. impatiens* but not in *C. floridanus*, *N. vitripennis* or *D. melanogaster*. This finding indicates that the exon was duplicated after the separation of the ancestor of *A. mellifera* and *B. impatiens* from the common ancestor of ants, small parasitoid wasps and bees.

Another pair of exons that like their respective exons in other insects (Arakane et al 2004, 2005; Hogenkamp et al 2005; Rezende et al 2008) may be alternatively spliced is exons XVIII (180 bps) and XIX (174 bps). Both exons are predicted to code for transmembrane domains, which are highly similar (66 % identical bases coding for 80 % similar and 69 % identical amino acids, respectively). However, our qualitative PCR data using two independent pairs of primers suggest that in *A. mellifera*, exons XVIII and XIX may be present in the same transcript in larvae and pupae (Table II). If both exons are present in the same transcript, the C-terminus of the resulting protein would be cytoplasmic instead of extracellular as compared to the situation in *D. melanogaster*. To

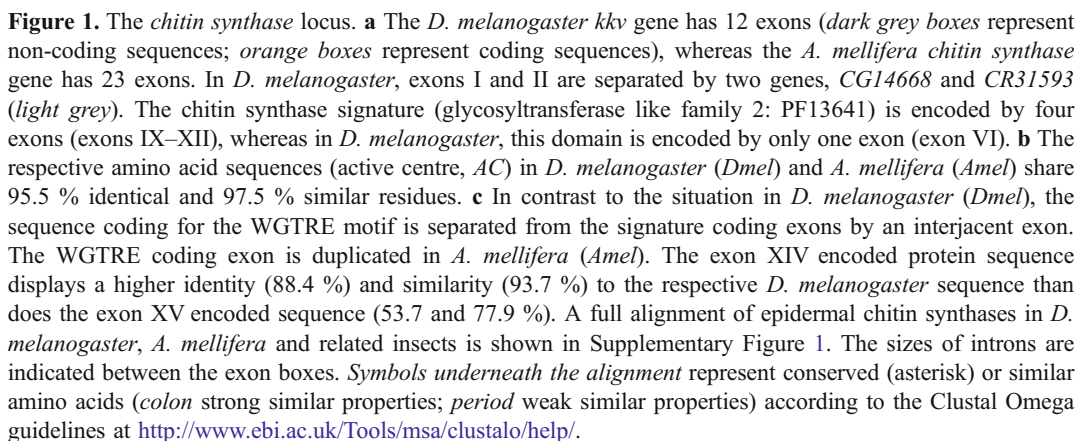
scrutinise whether transcripts with alternative exons XVIII and XIX exist, we performed RT-PCR experiments and determined the sequence of the resulting amplicons. Indeed, the vast majority of amplicons harboured only one of the exons (data not shown).

In summary, the conceptual *A. mellifera* chitin synthase with only one WGTRE motif and excluding the transmembrane domain encoded by exon XIX has 1,623 residues and is largely collinear with the respective *D. melanogaster* protein. Over 1,599 residues, they are 80.2 % similar and 66.4 % identical to each other.

The major non-aligning region is present at the N-terminus, which is variable in chitin synthases in all insects (Hogenkamp et al 2005; Merzendorfer 2006). The predicted NCBI *A. mellifera* chitin synthase protein has 1,632 amino acids that are, except from ten unique N-terminal amino acids, identical to the conceptual BeeBase sequence (Fig. S1). In qualitative PCR experiments, we could not detect the 5' region of the *A. mellifera* chitin synthase transcript deposited at the NCBI site, whereas the BeeBase 5' sequence of the chitin synthase transcript could be amplified (Table II).

### 3.2. GlcNAc pyrophosphorylase

The monomer of chitin, *N*-acetylglucosamine (GlcNAc), which is also a major component of N-glycans, is produced by the Leloir pathway (Moussian 2008). The last enzyme of this pathway catalysing the formation of UDP-GlcNAc is the cytoplasmic GlcNAc pyrophosphorylase, named mummy (Mmy) in *D. melanogaster* (Araujo et al 2005; Schimmelpfeng et al 2006; Tønning et al 2006). *D. melanogaster* larvae homozygous for loss-of-function *mmy* alleles have, among others, a strongly reduced cuticle and die before hatching. The *mmy* gene codes for two isoforms with 520 (A) and 483 (B) amino acids, isoform A having a longer N-terminus (Figure 2). The *A. mellifera* locus *GB44897* codes for a protein with 469 amino acids encompassing the GlcNAc pyrophosphorylase signature from residue 84 to residue 406 (PF01704). It is collinear



Chitin modification by the two secreted chitin deacetylases serpentine (Serp) and vermiform (Verm) have been reported to be essential for chitin organisation (Gangishetti et al 2012;



**Table 1.** Exon composition of *chitin synthase-1* coding genes.

Exons	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
<i>Amel</i>	176	114	245	294	207	345	392	268	263	211	248	193
<i>Bimp</i>	–	113	245	294	210	345	392	268	263	211	248	193
<i>Nvit</i>	–	116	245	294	210	345	392	268	263	459	–	193
<i>Cflo</i>	–	113	251	294	207	342	392	268	263	211	248	193
<i>Dmel</i>	143 <sup>a</sup>	–	244	1,230	–	–	–	271	1,931	–	–	–

Exons	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	Dmel XII
<i>Amel</i>	155	306	306	248	310	180	174	168	232	315	–
<i>Bimp</i>	155	306	306	248	313	177	177	168	229	320	–
<i>Nvit</i>	155	306	–	248	313	177	177	162	232	329	–
<i>Cflo</i>	155	306	–	248	307	177	177	162	229	341	–
<i>Dmel</i>	–	–	–	–	–	177	177	201	366	102	180

In most Hymenopteran species analysed in this work, the chitin synthase-1 coding genes are composed of 19 to 22 coding exons (bp). In *N. vitripennis* (Nvit), exons IX codes for the sequence encoded by exons IX and X in the other Hymenopterans. Exon XIV is duplicated only in *B. impatiens* and *A. mellifera*. Exon XVIII is duplicated in all species present in this table.

<sup>a</sup> Coding sequence in an exon that contains also non-coding bases

**Table II.** Expression profiles of chitin producing and organising factors in *A. mellifera*.

Orthologue	Probe	First instar larvae	Pink-eyed pupae
<i>kkv</i>	<i>GB49845 exons VIII–IX</i>	+	+
	<i>5' GB49845 from BeeBase</i>	+	n.d.
	<i>5' GB49845 from NCBI</i>	–	n.d.
	<i>GB49845 exon XIV</i>	+	+
	<i>GB49845 exons XIV–XV</i>	–	–
	<i>GB49845 exon XV</i>	+	+
	<i>GB49845 exon XVIII</i>	+	+
	<i>GB49845 exons XVIII–XIX a</i>	+	+
	<i>GB49845 exon XIX</i>	+	+
<i>serp</i>	<i>GB49845 exons XVIII–XIX b</i>	+	+
	<i>GB45151</i>	+	–
<i>verm</i>	<i>GB45152</i>	–	+
<i>knk</i>	<i>GB50061</i>	+	+
<i>rtv</i>	<i>GB49764</i>	+	+
<i>obst-A</i>	<i>GB50636</i>	+	+
<i>grh</i>	<i>GB46725 exons V–VI</i>	n.d.	+
	<i>GB46725 exons VII–VIII</i>	+	+

The expression of genes (probe) coding for chitin producing and organising factors in *A. mellifera* was recorded in first instar larvae and pink-eyed pupae by qualitative PCR using the primers listed in Supplementary Table 1. The putative *D. melanogaster* orthologues are indicated (orthologue). Expression is marked by a +, non-expression is represented by a –. Expressions of the 5' end of *GB49845* (5' *GB49845* Beebase and 5' *GB49845* NCBI) and of exons V and VI of *GB46725* (*grh*) were not determined in pupae (n.d.)

Luschnig et al 2006). The *A. mellifera* *GB45151* locus codes for a protein with 532 amino acids, which is 89.9 % identical and 95 % similar to the *D. melanogaster* Serp protein (isoform B: 541 aa) over almost the entire protein sequence (516 residues), excluding the N-terminal signal peptide (Figure 3). Over 511 residues, the *GB45151* protein is 61.4 % identical and 77.5 % similar to Verm (549 aa). The neighbouring locus *GB45152* encodes another chitin deacetylase with 549 amino acids that over 519 residues are 84 % identical and 91.3 % similar to Verm and over 545 residues 56.1 % identical and 73.4 % similar to Serp.

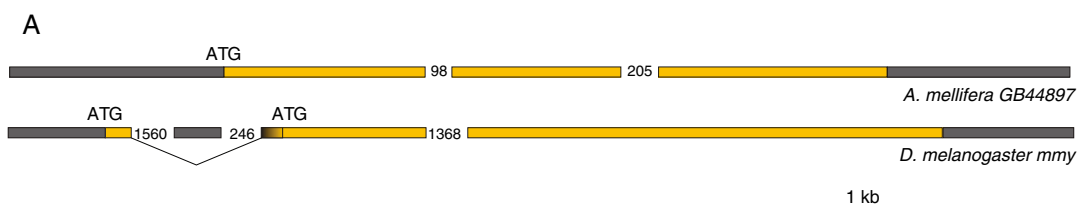
Aside from the sequences in the *A. mellifera* genome, we identified the respective loci in the Hymenoptera *B. impatiens*, *C. floridanus* and *N. vitripennis*. In general, exon composition of the *serp*- and *verm*-like chitin deacetylase coding

genes is conserved among the four Hymenoptera representatives. In phylogenetic analyses, the respective proteins segregate into two distinct clades, each branch harbouring one sequence from each species tested (Fig. S3).

As in *A. mellifera*, the respective chitin deacetylase coding genes *serp* and *verm* in *D. melanogaster* are arranged in tandem in the genome (Luschnig et al 2006). We found that the cognate loci are also arranged in tandem in the genomes of *B. impatiens* and *N. vitripennis* (Figure 4). Incomplete assembly of the *C. floridanus* genome does not allow any conclusion on gene arrangement in this species. Nevertheless, the tandem arrangement suggests an early duplication of a chitin deacetylase-coding gene in the insect lineage.

The putative *A. mellifera* Serp (*GB45151*) and Verm (*GB45152*) orthologues are differen-





**Figure 2.** The *mmy* locus. The *D. melanogaster mmy* locus has two alternative start codons (ATG) and three coding exons (orange boxes; grey boxes represent non-coding sequences). The *A. mellifera* gene GB44897 has only one predicted start codon and three coding exons. RNA sequencing data at BeeBase do not contradict the comparably simple composition of the GB44897 locus. A full alignment of Mmy and Mmy-like proteins in *D. melanogaster*, *A. mellifera* and related insects is shown in Supplementary Figure 2. The sizes of introns are indicated between the exon boxes.

tially expressed (Table II). While the GB45151 transcript is detected at larval stages, the GB45152 transcript is pupal specific.

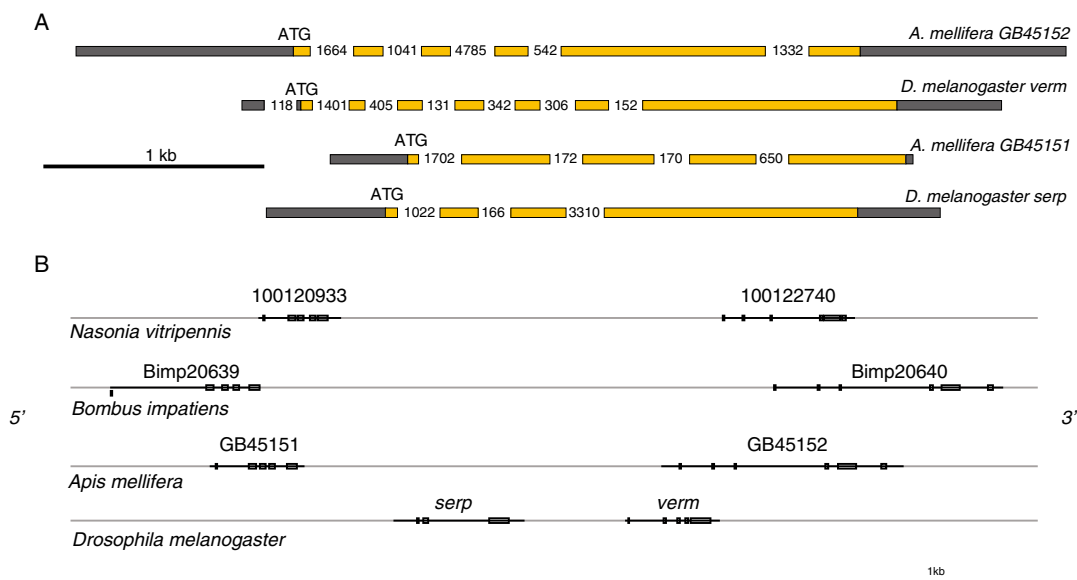
### 3.4. The chitin organising factor Knickkopf

The *D. melanogaster* Knickkopf protein is a membrane-anchored protein that is needed for correct chitin organisation during larval cuticle formation (Moussian et al 2006). In the flour beetle *T. castaneum*, Knk additionally protects chitin against degradation by chitinases before moulting (Chaudhari et al 2011). The *A. mellifera* GB50061 locus consisting of 13 exons codes for a protein with 686 amino acids that displays 57.6 % identity and 72.4 % similarity over its entire length to the *D. melanogaster* Knk protein (689 aa, Figure 5 and S4). The *D. melanogaster* Knk protein has an N-terminal signal peptide, a tandem of DM13 domains, a central DOMON domain and a C-terminal signature allowing the addition of a GPI anchor. GB50061 is predicted to have an N-terminal signal peptide that is cleaved at Gly30. A tandem of DM13 domains and the DOMON domain is present in GB50061, as well. In addition, the C-terminus possesses the signature for GPI anchor attachment. Taken together, the overall architectures of GB50061 and Knk are identical. GB50061 shows significant albeit weaker similarity to two additional *D.*

*melanogaster* proteins, CG34355 (37.9 % identity/56.4 % similarity) and CG43161 (38.7 % identity/56.1 % similarity). The transcript of GB50061 is detected at the larval and pupal stages (Table II).

### 3.5. The chitin organising factor retroactive

The *D. melanogaster* Retroactive (Rtv) protein is a membrane-bound Ly6-type protein that assists chitin organisation during embryogenesis (Moussian et al 2005b, 2006). In *T. castaneum*, the Rtv assists trafficking of Knk to the apical plasma membrane and the extracellular space (Chaudhari et al 2013). The *A. mellifera* GB49764 locus contains three exons coding for a protein with 146 amino acids, which is highly similar to the *D. melanogaster* Rtv protein (Figure 4). Over the length of 136 amino acids, this protein is 52.2 % identical and 66.9 % similar to the *D. melanogaster* Rtv protein (151 aa; Fig. S5). GB49764 has an N-terminal signal peptide and a C-terminal signature for GPI modification (omega-site N122). Structure-based sequence homology searches using the HHpred software (Soding et al 2005) confirm that the sequence between the N-terminal signal peptide and the C-terminal transmembrane domain adopts a Ly6-like folding (data not shown), which in some cases has been



**Figure 3.** The *serp* and *verm* loci. **a** The *D. melanogaster* genes *serp* and *verm* code for chitin deacetylases. The *A. mellifera* GB45151 locus coding for the Serp orthologue has five exons (grey boxes represent non-coding sequences; orange boxes represent coding sequences). The *A. mellifera* GB45152 locus that encodes the Verm orthologue has six exons. According to Flybase, exons III, V and VI of the *D. melanogaster verm* may be alternatively spliced. Exons V and VI correspond to exons II and III in *A. mellifera*, arguing that they may be alternatively spliced, as well. By contrast, *verm* exon III does not show any similarity to any sequence in the *A. mellifera* genome. The sizes of introns are indicated between the exon boxes. **b** In *D. melanogaster*, *A. mellifera*, *B. impatiens* and *N. vitripennis* the genes coding Serp- and Verm-like chitin deacetylase are arranged in tandem. A full alignment of Serp, Verm and Serp/Verm-like proteins in *D. melanogaster*, *A. mellifera* and related insects is shown in Supplementary Figure 3.

shown to mediate partner recognition (Moussian et al 2005b). Taken together, like *D. melanogaster* Rtv, the *A. mellifera* orthologue is probably directed to the plasma membrane where it may interact with a partner to organise chitin microfibrils. The GB49764 transcript is detected at the larval and pupal stages (Table II).

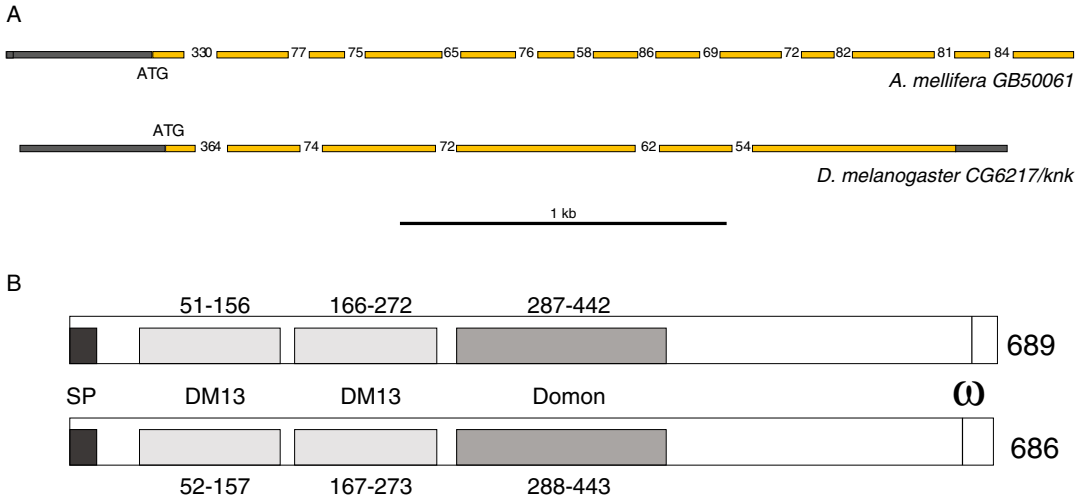
### 3.6. The chitin-binding factor obstructor-A

Obstructor-A (Obst-A) is an extracellular chitin-binding protein that interacts with Knk and Serp to protect chitin from degradation in *D. melanogaster* (Petkau et al 2012). The Obst-A protein has 237 residues constituting an N-terminal signal peptide and three class 2 chitin-binding domains (Fig. S6). The *A. mellifera*

potential orthologous sequence with 233 amino acids that is coded by the locus GB50636 (Figure 6) has the same domain composition. The proteins align over their entire length with 64.6 % identical and 74.7 % similar residues. The GB50636 protein shows significant similarity to a second *D. melanogaster* protein named obstructor-B that is highly similar to GB41227 (data not shown). GB50636 is 48.1 % identical and 63.1 % similar to obstructor-B (gaps 3.4 %). GB50636 is expressed at the larval and pupal stages (Table II).

### 3.7. The transcription factor grainyhead

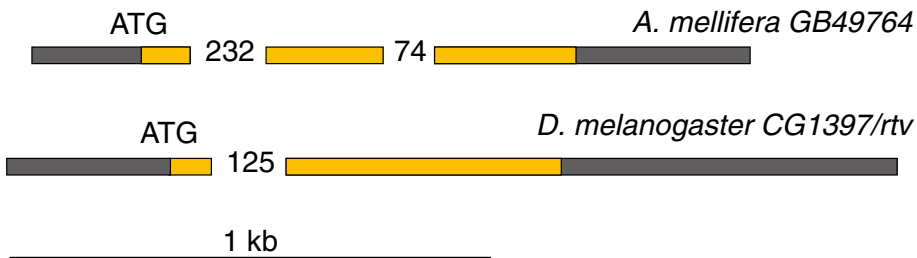
The *D. melanogaster grainyhead* (*grh*) locus is complex. According to FlyBase, the *grh* gene has 19 exons coding for eight isoforms of a



**Figure 4.** The *knk* locus. **a** The *D. melanogaster knk* gene has six exons (grey boxes represent non-coding sequences; orange boxes represent coding sequences) constituting a coding region of 2,067 bps. *GB50061* has 13 exons adding up to 2,058 bps of coding sequence. **b** The resulting proteins align over 658 residues. Both proteins have an N-terminal signal peptide (SP), followed by two DM13 domains (PF10517), a central DOMON domain (PF03351) and a C-terminal signature for GPI modification ( $\omega$ ). The DM13 domains between the proteins are 66 and 56 % identical and 80.2 and 74.8 % similar to each other, respectively. The DOMON domains share 64.1 % identical and 78.8 % similar amino acids. A full alignment of Knk and Knk-like proteins in *D. melanogaster*, *A. mellifera* and related insects is shown in Supplementary Figure 4. The sizes of introns are indicated between the exon boxes.

CP2-type transcription factor ranging from 155 to 1,333 residues (isoforms PH-O) that are needed for terminal epidermal and tracheal differentiation and in a subset of neurons in the *D. melanogaster* embryo (Bray and Kafatos

1991; Gangishetti et al 2012; Uv et al 1997). Experimentally, four isoforms (N, N', O and O') have been described coded by 17 exons (Uv et al 1997). The N isoforms (N 1,063 and N' 1,032 residues) are epidermal, while the O isoforms



**Figure 5.** The *rtv* locus. The 453 bps open reading frame of the *D. melanogaster rtv* gene is split into two exons (grey boxes represent non-coding sequences; orange boxes represent coding sequences). *GB49764* has three exons that together constitute an open reading frame of 438 bps. The position of the first intron is conserved separating the respective sequences coding for the signal peptide from the Ly6 domain (PF00021). A full alignment of Rtv and Rtv-like proteins in *D. melanogaster*, *A. mellifera* and related insects is shown in Supplementary Figure 5. The sizes of introns are indicated between the exon boxes.

(O 1,333 and O' 1,303 residues) are expressed in the nervous system. The *grh* homologous *A. mellifera* locus *GB46725* at BeeBase has 11 exons coding for a protein with 843 amino acids (Figure 7). The *GB46725* transcript is detected at the larval and pupal stages (Table II). Whole transcriptome shotgun sequencing data at BeeBase indicate the additional existence of an alternative isoform with 636 amino acids. Alignment of the large *A. mellifera* protein with the *D. melanogaster* Grh epidermal isoform N reveals an identity of 61.4 % and a similarity of 69.2 % that is confined to the C-terminal half of the *D. melanogaster* protein (after around 600 aa, see Fig. S7). The N-terminal half of the *D. melanogaster* N and O isoforms is characterised by the presence of several stretches of multiple glutamines (12 %). The non-aligning N-terminal region of the *A. mellifera* Grh-homologous protein *GB46725* is serine-rich (15.5 %). Consistently, the N-terminal half of the *B. impatiens* Grh-homologue *Bimp10162* is serine-rich, as well (Fig. S7). Because of the comparably shorter N-terminal region, it is possible that the *GB46725* protein might have additional unmatchable and low-complex N-terminal sequences that have escaped annotation. Scanning of the 5' region as well as the large first and second introns of *GB46725* for repetitive sequences failed to identify undetected exons coding for such sequences.

## 4. DISCUSSION

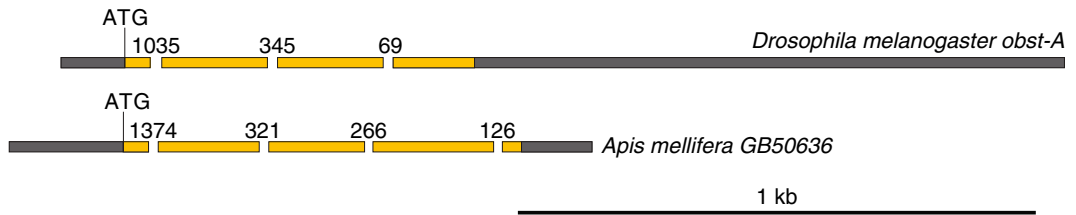
### 4.1. Chitin synthesis and organisation in *D. melanogaster* and *A. mellifera* are generally conserved

Our data reveal that genes coding for factors highly similar to those that are essential for chitin synthesis and organisation in *D. melanogaster* are present in *A. mellifera*. In other words, for each *D. melanogaster* gene, there is a counterpart in the *A. mellifera* genome. A central question is whether these factors are true orthologues or not. There are at least three criteria for true orthology. First, two factors in two species should have a common origin, i.e. they should be homologous. Second, the spatial and temporal expression patterns of these genes coding for homologous factors in two

different species should be similar. Third, two homologous factors in two different species have to exert the same molecular and cellular function. Thus, the homologous factor from one species should be able to rescue the phenotype caused by a mutation in the respective gene in another species in transgenic experiments. Based on accurate sequence analyses, we present evidence that the chitin synthesis and organisation factors identified in *A. mellifera* are most probably orthologues of the respective factors, which have been genetically characterised in *D. melanogaster*. A strong argument that we have been comparing orthologous sequences is the determination of bidirectional best hits (BBH), a simple method to infer orthology (Altenhoff and Dessimoz 2012): the most similar sequence retrieved by tBLASTx in *A. mellifera*, recognises the initial sequence of *D. melanogaster* as the most similar. In the case of Knk, unambiguous reciprocal identification of similar sequences among three homologues in each species supports the assumption that *GB650061* is the orthologue of Knk. Besides BBH, we have additional arguments supporting the assumption that we have identified orthologues in the other cases.

**Chitin synthase** Insects have two genes coding for chitin synthases (Merzendorfer 2006). The defining trait of the epidermal chitin synthase versus the midgut version is an extended coiled-coil region including the WGTRE motif. Hence, the presence of this region indicates that *GB49845* is the true orthologue of Kkv.

**Serp and Verm** Serp and Verm belong to the group I of insect chitin deacetylases that harbours two isoforms per insect (Dixit et al 2008). They are characterised by an N-terminal chitin-binding domain, a middle LDL-like domain and a chitin deacetylase signature. In *D. melanogaster*, *A. mellifera*, *B. impatiens* and *N. vitripennis*, the respective genes are located in tandem in the genome. These features unambiguously support the assumption that the respective enzymes have a common origin and are true orthologues.



**Figure 6.** The *obst-A* locus. **a** The *A. mellifera* GB50636 locus that codes for an Obst-A homologous protein has five exons, whereas the *D. melanogaster* reference locus has four exons (grey boxes represent non-coding sequences; orange boxes represent coding sequences). A full alignment of Obst-A and Obst-A-like proteins in *D. melanogaster*, *A. mellifera* and related insects is shown in Supplementary Figure 6. The sizes of introns are indicated between the exon boxes.

**Rtv and Mmy** These two proteins are unique sequences in each insect. It is hence conceivable that GB44897 and GB49764 are orthologues of Rtv and Mmy, respectively.

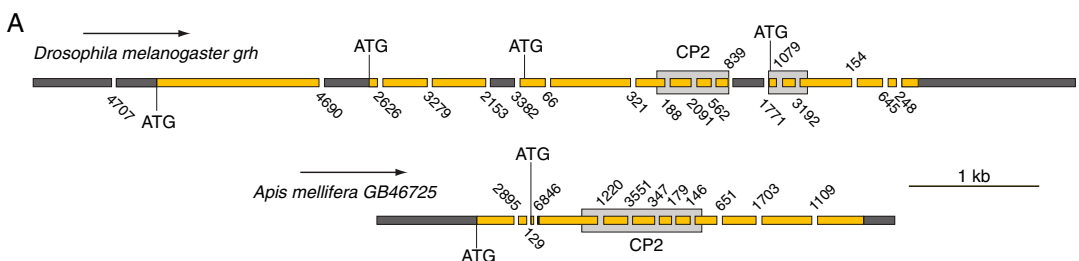
**Obst-A** Similarly to Obst-B and Obst-B-like proteins, Obst-A and GB50636 have three class 2 chitin-binding domains. However, Obst-A and Obst-A-like proteins have a C-terminal ER retention signal suggesting that the cell biology between Obst-A-proteins and Obst-B-proteins differs. In turn, this feature together with the BBH argument argues that Obst-A and GB50636 are orthologues.

**Grh** The *A. mellifera* and *D. melanogaster* genomes have been shown to code for two CP2-type transcription factors (Huang et al 2012). Our

BLAST analyses revealed that GB46725 corresponds to Grh, whereas GB48238 is the cognate of Gemini. Grh and Grh-like proteins in the species examined have several yet uncharacterised regions C-terminal to the CP2-domain, which are completely missing in respective Gem proteins. Thus, despite N-terminal sequence divergences discussed below, the CP2-type transcription factors described in this work are most probably Grh orthologues.

#### 4.2. Sequence divergences and expression differences

Despite the identical set of chitin synthesis and organising factors in the fruit fly and the



**Figure 7.** The *grh* locus. **a** According to the Flybase database, the *D. melanogaster* *grh* gene has 19 exons (dark grey boxes represent non-coding sequences; orange boxes represent coding sequences). The *A. mellifera* locus GB46725 is predicted to have 11 exons. To date, the *D. melanogaster* *grh* locus is annotated with four start codons (ATG). The respective *A. mellifera* locus has only one start codon. **b** The CP2 domains (PF004516) of *D. melanogaster* (*Dmel*) and *A. mellifera* (*Amel*) are 85.8 % identical and 90.7 % similar to each other. A full alignment of Grh and Grh-like proteins in *D. melanogaster*, *A. mellifera* and related insects is shown in Supplementary Figure 7. The sizes of introns are indicated between the exon boxes.

honeybee, differences exist in their sequence composition (*kkv* and *grh*) and expression pattern (*serp* and *verm*). Intriguingly, for instance, in *A. mellifera* and *B. impatiens* but not in other Hymenoptera tested, the sequence coding for the WGTRE motif of the epidermal chitin synthase is duplicated yielding two alternatively spliced exons. Of note, the WGTRE-coding sequence is also unique in other insect species belonging to orthopterans (*Locusta*), dipterans (e.g. *Drosophila* and *Anopheles*), coleopterans (e.g. *Tribolium*) and lepidopterans (e.g. *Ostrinia*) (Arakane et al 2004, 2005; Moussian et al 2005a; Qu and Yang 2011, 2012; Zhang et al 2010, 2012). Underlining the importance of this motif, a missense mutation changing the Gly to an Asp completely abrogates enzyme activity in *D. melanogaster* (Moussian et al 2005a). Moreover, the etoxazole sensitive isoleucine<sup>1056</sup> (Van Leeuwen et al 2012) preceding the WGTRE motif is conserved in both alternative sequences. In summary, the alternative use of an important albeit molecularly uncharacterised motif in the *A. mellifera* and *B. impatiens* epidermal chitin synthases adds a level of complexity in chitin production that is absent in insects outside the Apoide clade.

Another major sequence difference in chitin synthesis and organising factors between *D. melanogaster* and *A. mellifera* concerns the N-termini of the respective Grh proteins. The N-terminal half of epidermal Grh versions in *D. melanogaster* is glutamine-rich, a feature, which has been shown to enhance the activity of transcription factors (Atanesyan et al 2012). By contrast, the N-terminus of GB46725 in *A. mellifera* is serine-rich, a feature needed for transcription activation in Sp1-related transcription factors (Suske 1999). Considering the non-perfect alignment of N-termini between Grh-like proteins in *A. mellifera*-related species *B. impatiens*, *C. floridanus* and *N. vitripennis*, we conclude that in general, the N-terminal region of insect Grh-like proteins is frequently modified during evolution.

Along with sequence divergences also discrepancies in gene expression profiles account for differences in chitin synthesis and organisation between *D. melanogaster* and *A. mellifera* beyond

the basic scheme. Notably, the genes *GB45151* and *GB45152* coding for chitin deacetylases are expressed at different developmental stages in *A. mellifera*, whereas their orthologues *serp* and *verm* in *D. melanogaster* are expressed concomitantly at embryonic stages and act redundantly (Luschnig et al 2006). These genes have probably a common enhancer in *D. melanogaster*, whereas in *A. mellifera*, different enhancers control their expression at different developmental stages. This observation suggests a level of expression control in chitin organisation that is missing in *D. melanogaster*.

### 4.3. Conclusion and outlook

Recent publications on honeybee and fruit fly cuticle proteins underline considerable variances in cuticle composition in these two species. Whereas, for example, the *D. melanogaster* genome harbours 27 tweedle proteins, only two have been shown to be present in the *A. mellifera* genome (Guan et al 2006; Soares et al 2011). Moreover, sequences homologous to the three *A. mellifera* low-complex cuticle proteins called apidermins are missing in the *D. melanogaster* genome (Kucharski et al 2007). By contrast, in this study, we have shown that—despite some differences—the core of chitin synthesis and organisation machinery is most probably conserved between *D. melanogaster* and *A. mellifera*. Altogether, these findings underline that data from the fruit fly are only partially translatable to the honeybee and generally to other insects. Dissection of the molecular mechanisms of honeybee cuticle formation combined with a thorough histological study is hence crucial to contribute to the understanding of honeybee biology and ecology.

Our sequence analyses will serve to characterise essential chitin producing and organising factors in RNA interference experiments. Efforts focussing at pupal and pharate stages that have been histologically excellently described (Elias-Neto et al 2009) would be particularly rewarding. Ultimately, we expect to learn more about the interaction between honeybee



and the pathogen vector *Varroa destructor* that transmits viruses through the cuticle (Rosenkranz et al 2010).

**Orthologues putatifs des gènes identifiés chez *Drosophila melanogaster* impliqués dans la synthèse et l'organisation de la chitine chez *Apis mellifera***

cuticule / chitine / synthèse de la chitine

**Mutmaßliche Orthologe der bei *Drosophila melanogaster* identifizierten, zur Produktion und Organisation von Chitin benötigten gene bei *Apis mellifera*. Kutikula / Chitin / Chitin Synthase / Chitin-Organisation**

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