Chitin biosynthesis genes in *Diaphorina citri,* Asian citrus psyllid

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Abstract

The polysaccharide chitin is critical for the formation of many insect structures, including the exoskeleton, and is required for normal development. Here we report the annotation of three genes from the chitin synthesis pathway in the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), the vector of Huanglongbing (citrus greening disease). Most insects have two chitin synthase (CHS) genes but, like other hemipterans, *D. citri* has only one. In contrast, *D. citri* is unusual among insects in having two UDP-N-acetylglucosamine pyrophosphorylase (UAP) genes. One of the *D. citri* UAP genes is broadly expressed, while the other is expressed predominantly in males. Our work helps pave the way for potential utilization of these genes as pest control targets to reduce the spread of Huanglongbing.

Introduction

Chitin is a polysaccharide essential for insect development. It plays a crucial role in the development of the insect cuticle and exoskeleton, the peritrophic membrane of the midgut of some insects, and other structures such as the trachea, wing hinges and eggshell [1]. The biosynthetic pathway for chitin involves several enzymes that act on simple sugars such as glucose, trehalose and glycogen to produce intermediates that are subsequently converted into chitin. In the penultimate step of the chitin biosynthesis pathway, N-acetylglucosamine-1-phosphate is converted into UDP-N-acetylglucosamine. This reaction is catalyzed by the enzyme UDP-N-acetylglucosamine pyrophosphorylase (UAP) [1]. In the final step of the pathway, UDP-N-acetylglucosamine is converted to chitin by enzymes known as chitin synthases (CHS) [1]. Because chitin is essential for insect development, but is not found in mammals, the enzymes involved in its synthesis are considered attractive targets for pest control. Here we report the annotation of one CHS gene and two UAP genes in the Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Liviidae), the vector for the bacterium that causes Huanglongbing (citrus greening disease). Although most insects have two CHS genes [2,3] (Table 1), the presence of a single CHS gene is consistent with reports from other hemipteran genomes [4]. In contrast, *D. citri* seems to be unusual in having two UAP genes. RNA-Seq data indicates that one of the *D. citri* UAP genes is broadly expressed, while the other is expressed predominantly in males. Our manual annotation of these chitin biosynthesis genes provides improved gene targets for future experiments.

Results and Discussion

Chitin Synthases

Chitin synthases are the only enzymes in the chitin biosynthetic pathway that act specifically in the synthesis of chitin, making them an attractive, insect-specific target for RNA interference (RNAi) based insecticides. The two *CHS* genes found in most holometabolous insects have distinct functions. *CHS1*, also referred to as *CHSA*, produces the chitin essential for proper cuticle development [3,5,6]. *CHS2*, also referred to as *CHSB*, is not required for cuticle development, but is instead essential for proper development of the gut peritrophic membrane [3,5,6]. RNAi knockdown of either *CHS* gene is lethal in holometabolous insects [7–10].

Chitin synthase and UAP ortholog number in select insects

	Drosophila melanogaster	***Anopheles gambiae***	***Aedes aegypti***	***Tribolium castaneum***	***Apis mellifera***	***Nasonia vitripennis***	***Acyrthosiphon pisum***	***Bemisia tabaci***	***Diaphorina citri***
CHS1/A	1	1	1	1	1	1	1	1	1
CHS2/B	1	1	1	1	1	1	0	0	0
UAP	1	1	1	2	1	1	1	1	2

Table 1. Gene counts are taken from published reports [2,4,20] or determined from genome data [29-36]. *D. citri* numbers are based on annotation of genome v3.

Previous searches of the *Acyrthosiphon pisum*, *Nilaparvata lugens* and *Rhodnius prolixus* genomes identified *CHS1* but not *CHS2*, suggesting that *CHS2* has probably been lost in the hemipteran lineage [4]. Loss of the chitin synthase gene required for peritrophic membrane development is not particularly surprising, since hemipterans do not have peritrophic membranes [4,11]. Lu et al [12] identified a *D. citri CHS* gene that clustered with other hemipteran CHS genes and was expressed at high levels in most adult body tissues, but at low levels in midgut, as would be expected for a *CHS1* gene. Two groups have shown that RNAi knockdown of *CHS* in *D. citri* causes increased lethality [12,13], supporting the idea that this gene is a good target for pest control.

Our searches of the *D. citri* v3 genome revealed the previously described *CHS* gene, but no additional chitin synthase orthologs (Table 1). Transcriptomic evidence supports the existence of two *CHS* isoforms (Table 2) that differ only in the use of one alternative exon and produce proteins with slightly different C-termini. Similar isoforms of *CHS1/A* have been described in other insects [2,14,15]. Both isoforms of *D. citri* CHS cluster in a monophyletic clade with CHS1 proteins from other insects (Figure 1), so we have named this gene *CHS1*. We retrieved expression data for both isoforms of CHS1 from the Citrusgreening Expression Network (CGEN), which contains RNA-Seq data sets for a variety of life stages and tissues [16]. Data from whole body samples indicate that CHS1 is expressed at all life stages, but is most highly expressed in juvenile stages (Figure 2).

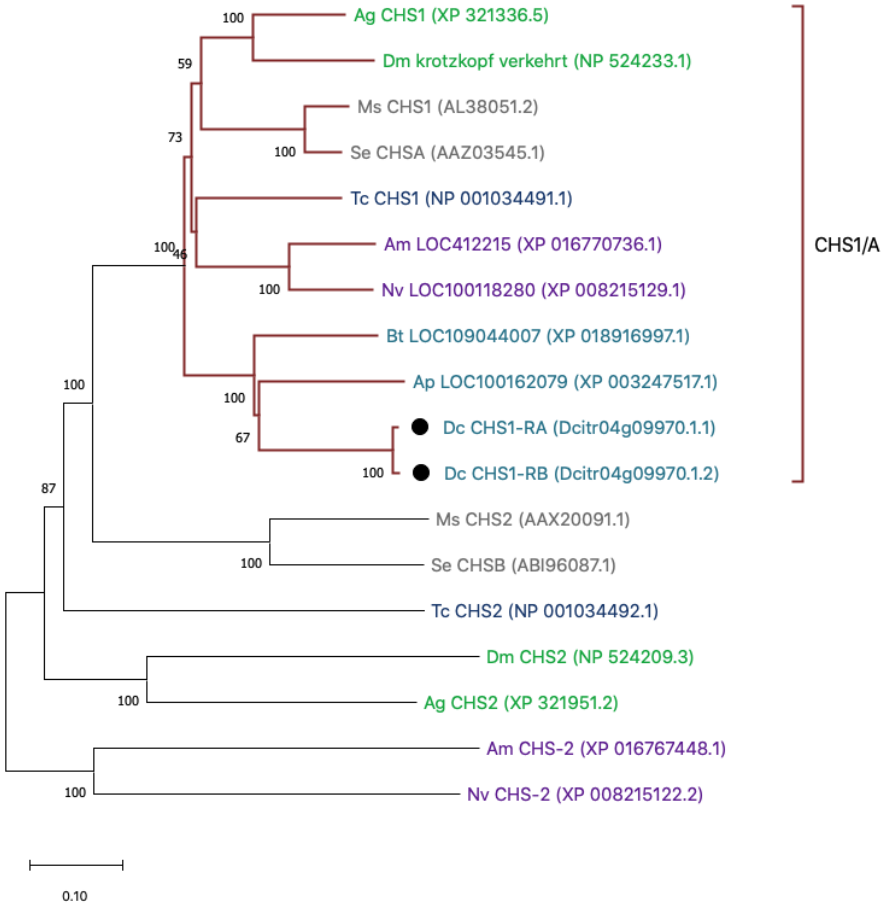

Figure 1: Phylogenetic analysis of insect CHS proteins. Species represented are *Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Tribolium castnaeum* (Tc), *Manduca sexta* (Ms), *Spodoptera exigua* (Se), *Apis mellifera* (Am), *Nasonia vitripennis* (Nv), *Acyrthosiphon pisum* (Ap), *Bemisia tabaci* (Bt) and *Diaphorina citri* (Dc). MUSCLE software was used to perform multiple sequence alignments of full-length protein sequences and the tree was constructed with MEGA X software using the neighbor-joining method with 100 bootstrap replications. The maroon clade shows monophyletic clustering of CHS1/A genes. With the exception of *D. citri* (denoted by black circles), only one isoform per species is depicted. Taxon name color represents insect order: Diptera (green), Coleoptera (navy), Hymenoptera (purple), Lepidoptera (gray), and Hemiptera (teal).

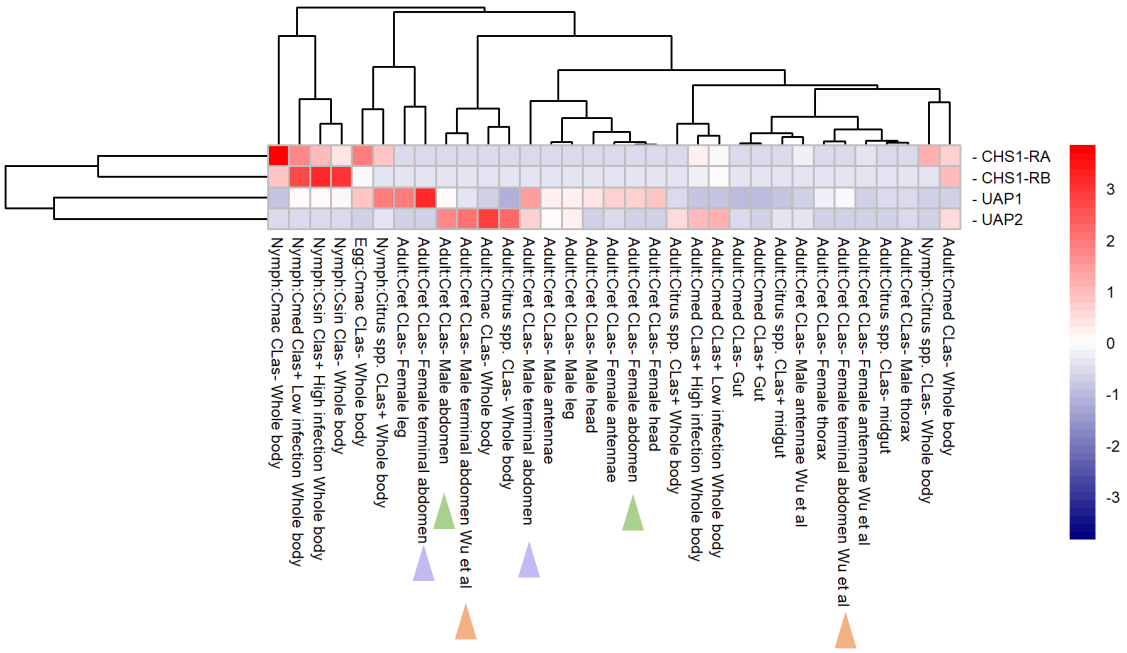

Figure 2. Heatmap representation of chitin biosynthesis gene expression levels in a variety of RNA-Seq datasets. Expression levels were obtained as transcripts per million (TPM) from the Citrusgreening Expression Network [16]. For ease of comparison, colored arrowheads denote pairs of male and female abdominal tissue samples from the same experiments.

Annotated *D. citri* orthologs of chitin biosynthesis genes

Gene/Isoform	**OGSv3 ID**	**Evidence supporting annotation**			
		MCOT	**IsoSeq**	**RNASeq**	**Ortholog**
CHS1	Dcitr04g09970.1.1 Dcitr04g09970.1.2	X	X	X	X
UAP1	Dcitr08g04630.1.1		X		X
UAP2	Dcitr05g05060.1.1		X	X	X

Table 2. Each manually annotated gene is full length and has been assigned an OGSv3 gene identifier. Evidence types used for manual annotation are shown for each gene. A description of the various evidence sources and their strengths and weaknesses is included in our online protocol [25].

Our manual annotation of *CHS1* corrects several errors that were present in the previous computationally-predicted annotation for *D. citri* CHS (XP_017303059). Changes to the model include the addition of formerly missing sequence and the removal of artifactually duplicated regions. Domain analysis with TMHMM Server, v. 2.0 indicates that the corrected CHS1-RA and CHS1-RB proteins have 15 transmembrane helices as is typical for insect CHS proteins, rather than the 14 that were reported for the earlier version of the protein [12].

UDP-N-acetylglucosamine pyrophosphorylase (UAP)

In addition to its role in chitin synthesis, UAP is also involved in the modification of other carbohydrates, sphingolipids and proteins. In Drosophila, mutants of *UAP* (also called *mummy*, *cabrio* and *cystic*) have defects in tracheal development, dorsal closure, eye development and nervous system function [17–19]. Some of these developmental defects are due to disruption of the chitin synthesis pathway, while others appear to be caused by effects on other glycoproteins. For example, defects in embryonic dorsal closure have been linked to a role for UAP in regulation of Decapentaplegic signaling [20].

Most insects appear to have a single *UAP* gene [21]. However, a few insects, including *T. castaneum, Locusta migratoria* and *Leptinotarsa decemlineata* have two UAP genes [21-23]. Comparison of the *T. castaneum* and *L. migratoria* gene pairs indicates that they arose through separate, relatively recent lineage-specific gene duplications [22]. RNAi experiments in *T. castaneum* showed that UAP1 is involved in the biosynthesis of chitin both in the cuticle and the peritrophic membrane, while UAP2 is important for the modification of other macromolecules [21]. In *L. migratoria*, *LmUAP1* knockdown caused lethality and defects consistent with disruption of chitin biosynthesis, while *LmUAP2* knockdown did not increase lethality and produced no visible effects [22].

In the *D. citri* v3 genome, we identified two *UAP* genes located on different chromosome-length scaffolds. The proteins encoded by these apparent paralogs share 50 percent identity distributed throughout the length of the proteins (Figure 3), which is very similar to the level of identity shared with UAP orthologs from closely related insect species. Amino acid residues known to be important for substrate binding in the human UAP ortholog and conserved in the *T. castaneum* UAP proteins [20] are also well conserved in the *D. citri* UAP proteins (Figure 3). Phylogenetic analysis (Figure 4) suggests that the two genes represent a lineage specific duplication. Surprisingly, the *D. citri* UAP proteins do not cluster with the other hemipteran UAP proteins and instead appear as an outgroup to all the other insect UAP proteins, likely suggesting the *D. citri UAP* genes are diverging rather rapidly. We have named the *D. citri* genes *UAP1* and *UAP2*, but this should not be taken to imply direct orthology with duplicated UAP genes in other insects.

```
Dc_UAP1         MVDINEGKDLLTSCNQQ-------------HLLKYWDEINDEEKSILLNE---IKQLNIP
Dc_UAP2         -MDSTKSQDIFTSENSEIDPNKGRTLQKSLELEPNENVSKDKELNILLKDDVSLMNMDIF
                 :* .:.:*::** *.:              *    :  :*:* .***::   : :::*

Dc_UAP1         EACEYFKSANTYSRQAEAQCDSSMKPVPSELYGSAQDTSSDILKSYREIGLQEISEGHVG
Dc_UAP2         KARKYYEEACAVSNAPERFSEVQVFP-PNCLSG-VNTVDASTLGKYRELGLKLISRGDVA
                :* :*::.* : *. .*  .: .: * *. * * .: ..:. * .***:**: ** * *.

Dc_UAP1         VILLAGGQGTRIGVPYPKGMYKVGLPSDKSLFQIQAERIMKLESLAFEQTGKKSIITWFI
Dc_UAP2         VIVLAGGQGTRLGADYPKGMYNIGLPSGKSLFQIQAEKIDKLIEIAKEKFG-SGCLPWFI
                **:********:*. ******::****.*********.* ** .:* *: * .. :.***

Dc_UAP1         MTSESTMEPTKNFFEENKYFGLQKDNVIFFEQGVLPCFTFDGKIIMDSKFKIAKAPDGNG
Dc_UAP2         MTSELTDRPTREYFERNGYFGLDPAHVIFFKQRSMPCFSLSGEILLETRDRVARSPDGHG
                **** *  **.::** * ****:  :****:*  :***::.*:*:::::. .:*.:***:*

Dc_UAP1         GIYIALKKKGILSEMEKRGIEYVHVYSVDNILVKVADPVFMGFCVKSQSDCGVKVVEKKL
Dc_UAP2         GLYHALGATGILDTMHTRGIKHIHVYCVDNILVKVGDPTFLGYCVEQGAHCGVKVVEKIT
                *:* **  .***. * .***:::***.********.**.*:*:**:. : ********

Dc_UAP1         PNEGLGVVCVVDGQYKVVEYSEISSKTA-ELRDADGKLTFRAGNICNHFFSTAFLGQIAN
Dc_UAP2         PGESLGVLCNVDGKHKIVEYSELGNCSVFETQDQTGRLKFNLGSICNHYFSLECLQRMVK
                *.*.***:* ***::*:*****:.. :. * .*  *.*.*. *.****:**   * .:.:

Dc_UAP1         EHESKLKLHIAKKKIPYIDSKGLKVKPEQPNGIKIEKFIFDVFEFCKNLVVWEVAREHDF
Dc_UAP2         E-DAALKFHMARKKIPCLDEQGISQRPNKPNGIKLEKFLFDAFPLCENLVAWEVTRD-EF
                * :: **:*:*.**** :*.:*:. .*::*****:***:**.* :*:***.***:*: :*

Dc_UAP1         SALKNSNAEK-TENPTTCCLALYDLHKSYIEAAGGTVKPDAVGNVVCEISPSVSYDGEGL
Dc_UAP2         SPLKNSPLDSASDNPVTCCQAVHALHARWIETAGGVVVADETGNTVCEIAPRVSYEGEGL
                *.****  :. ::**.*** *:: **  :**:***.* .* .**.****:* ***:****

Dc_UAP1         KPIVNGNTFESPILLK
Dc_UAP2         EERVKGKVLQTPLLLE
                :  *:*:.:::*:**:
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Figure 3. Alignment of D. citri UAP1 and UAP2. Alignment was performed using MUSCLE [25]. Individual amino acid alignments are denoted as identical (*), highly similar (:) or similar (.). Residues important for substrate binding by human UAP1 and conserved in *T. castaneum* are shaded according to their level of conservation. Identical residues are shaded blue and non-identical (but similar) residues are shaded red. The green shaded residue denotes the position of an alanine important for substrate binding in human UAP1 that is a cysteine in *T. castaneum* and other insects.

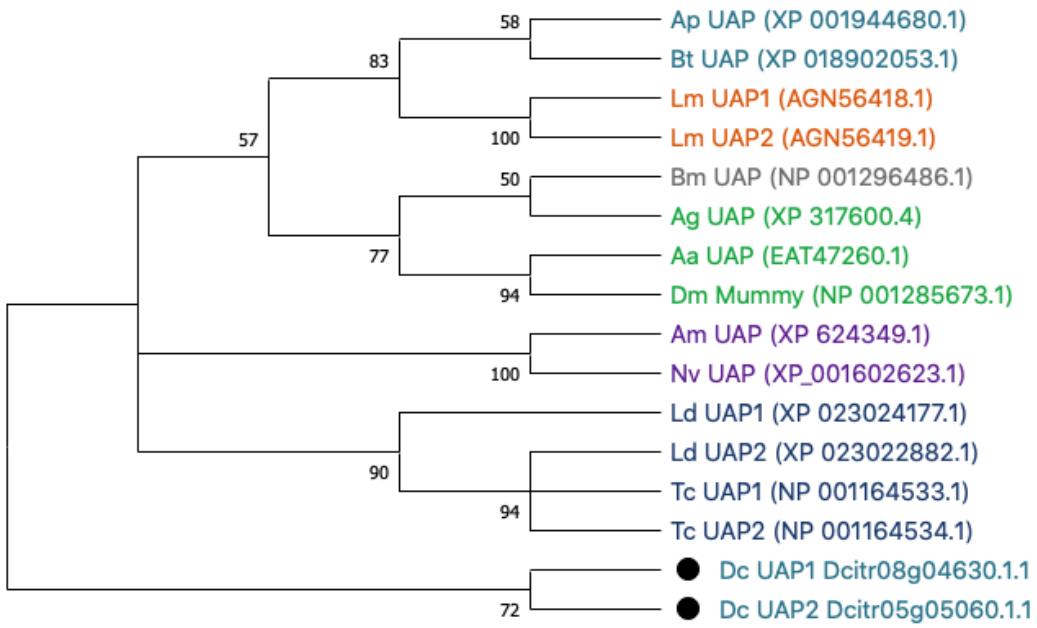

Figure 4. Phylogenetic analysis of representative insect UAP orthologs. Species shown are *Drosophila melanogaster* (Dm), *Anopheles gambiae* (Ag), *Aedes aegypti* (Aa), *Bombxy mori* (Bm), *Tribolium castaneum* (Tc), *Leptinotarsa decemlineata* (Ld), *Apis mellifera* (Am), *Nasonia vitripennis* (Nv), *Locusta migratoria* (Lm), *Acyrthosiphon pisum* (Ap), *Bemisia tabaci* (Bt) and *Diaphorina citri* (Dc and black circles). ClustalW software was used to perform the multiple sequence alignment of full-length protein sequences and a bootstrap consensus tree was constructed with MEGA X software using the neighbor-joining method with 100 bootstrap replications. Colors denote insect orders: Hemiptera (teal), Orthoptera (orange), Lepidoptera (gray), Diptera (green), Hymenoptera (purple) and Coleoptera (navy).

We compared available expression data from the two *D. citri UAP* genes using CGEN [16]. *D. citri UAP1* is expressed in all tissues and stages examined, although expression levels vary (Figure 2). A few samples (e.g. female terminal abdomen and female leg) show high expression of *UAP1*, but these are single replicate samples that would need further verification. In the case of female terminal abdomen, single replicate data from a separate experiment shows only a moderate level of expression. Interestingly, *D. citri UAP2* appears to show a sexually dimorphic expression pattern. It is expressed at a low to moderate level in most male tissues,

with highest expression in abdominal samples, but shows little or no expression in the same tissues from females (Figures 2,5). While these observations are intriguing, the technical difficulty of creating RNA-Seq libraries from miniscule amounts of dissected tissue while maintaining integrity of the RNA in addition to the lack of statistical power provided by single replicate samples mean that the expression data currently available should be interpreted with caution. More detailed analysis of *UAP1* and *UAP2* expression and function in individual males and females will be necessary to resolve these questions.

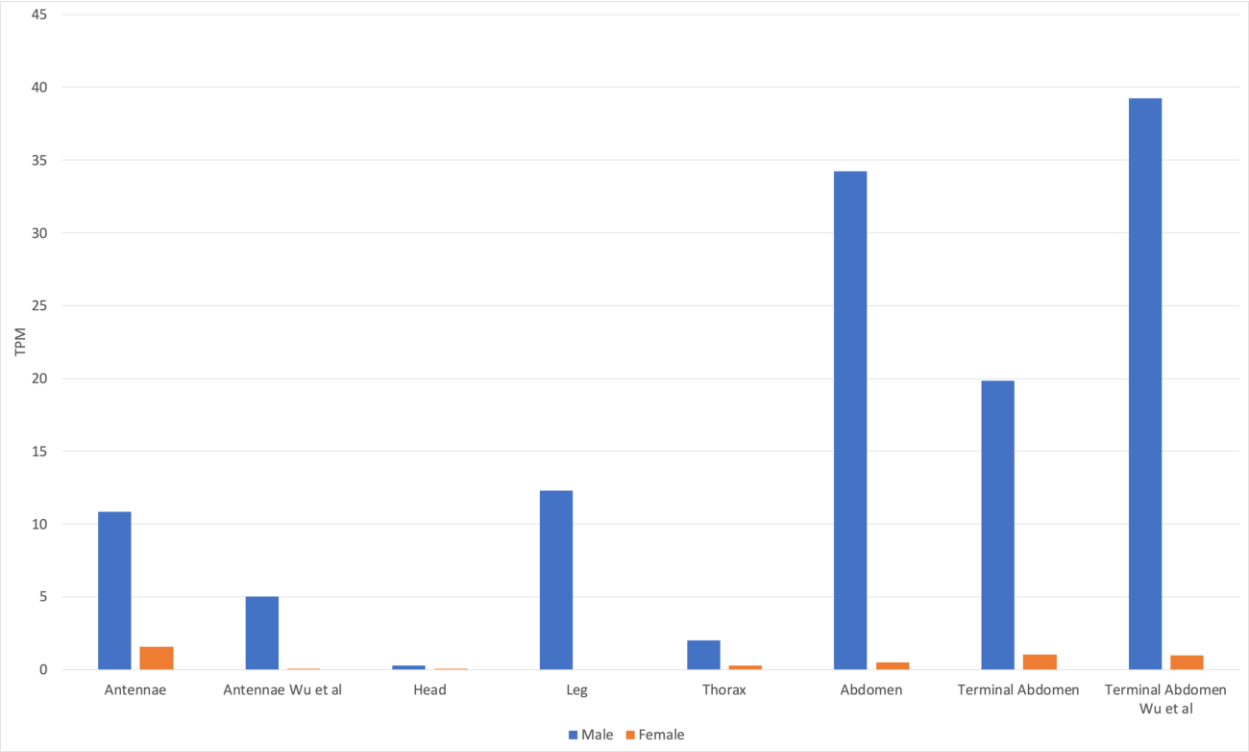

Figure 5. Expression levels of *UAP2* in male and female tissues. Expression levels were obtained from the Citrusgreening Expression Network [16]. Tissue types are shown on the X axis and expression levels (TPM) on they Y-axis. Blue bars denote expression levels in males and orange bars denote expression levels in females (all single replicate data). RNA-Seq data from tissues labeled Wu et al were sequenced in [37]. Data for the remaining tissues are from NCBI BioProject PRJNA448935.

Materials and Methods

D. citri genes in genome v3 [24] were identified by BLAST analysis of *D. citri* sequences with insect CHS and UAP orthologs. Reciprocal BLAST of the NCBI non-redundant protein database was used to confirm orthology. Manual annotation of genes was performed in Apollo 2.1.0 using RNA-Seq reads, IsoSeq transcripts and *de novo*-assembled transcripts as evidence. A more detailed description of the annotation workflow is available via protocols.io [25]. Multiple alignments of the predicted *D. citri* proteins and their insect homologs were performed using MUSCLE [26]. Phylogenetic trees were constructed using full-length protein sequences in MEGA7 or MEGAX. Orthologs used in tree construction are listed in Table 3. Gene expression levels were obtained from the Citrusgreening Expression Network [16] and visualized using Excel and the pheatmap package in R [27-28].

<Insert Table 3 - See Appendix>

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Funding Acquisition	S.J.B., T.D., W.B.H., L.M.
Writing - Original Draft Preparation	S.M., T.D.S
Writing - Review and Editing	S.J.B., S.S.

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Appendix

Orthologs used in phylogenetic analysis

Species	Accession	Name in NCBI	Name in Tree
Tribolium castaneum	NP_001034491.1	chitin synthase 1	Tc CHS1
Anopheles gambiae	XP_321336.5	AGAP001748-PA	Ag CHS1
Apis mellifera	XP_016770736.1	PREDICTED: uncharacterized protein LOC412215 isoform X1	Am LOC412215
Nasonia vitripennis	XP_008215129.1	PREDICTED: uncharacterized protein LOC100118280 isoform X1	Nv LOC100118280
Acyrthosiphon pisum	XP_003247517.1	PREDICTED: uncharacterized protein LOC100162079	Ap LOC100162079
Bemisia tabaci	XP_018916997.1	PREDICTED: uncharacterized protein LOC109044007 isoform X1	Bt LOC109044007
Drosophila melanogaster	NP_524233.1	krotzkopf verkehrt, isoform A	Dm krotzkopf verkehrt
Manduca sexta	AAL38051.2	chitin synthase	Ms CHS1
Spodoptera exigua	AAZ03545.1	chitin synthase A	Se CHSA
Tribolium castaneum	NP_001034492.1	chitin synthase 2	Tc CHS2
Manduca sexta	AAX20091.1	chitin synthase 2	Ms CHS2
Spodoptera exigua	ABI96087.1	chitin synthase B	Se CHSB
Drosophila melanogaster	NP_524209.3	chitin synthase 2	Dm CHS2
Anopheles gambiae	XP_321951.2	AGAP001205-PA	Ag CHS2
Apis mellifera	XP_016767448.1	chitin synthase chs-2	Am CHS-2
Nasonia vitripennis	XP_008215122.2	chitin synthase chs-2	Nv CHS-2
Drosophila melanogaster	NP_001285673.1	mummy, isoform D	Dm Mummy
Anopheles gambiae	XP_317600.4	AGAP007889-PA	Ag UAP
Aedes aegypti	EAT47260.1	AAEL001627-PA	Aa UAP
Bombyx mori	NP_001296486.1	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	Bm UAP
Tribolium castaneum	NP_001164533.1	UDP-N-acetylglucosamine pyrophosphorylase 1	Tc UAP1
Tribolium castaneum	NP_001164534.1	UDP-N-acetylglucosamine pyrophosphorylase 2	Tc UAP2
Apis mellifera	XP_624349.1	UDP-N-acetylhexosamine pyrophosphorylase	Am UAP
Nasonia vitripennis	XP_001602623.1	UDP-N-acetylhexosamine pyrophosphorylase	Nv UAP
Acyrthosiphon pisum	XP_001944680.1	UDP-N-acetylhexosamine pyrophosphorylase	Ap UAP
Bemisia tabaci	XP_018902053.1	PREDICTED: UDP-N-acetylhexosamine pyrophosphorylase	Bt UAP
Locusta migratoria	AGN56418.1	UDP N-acetylglucosamine	Lm UAP1

		pyrophosphorylases 1	
Locusta migratoria	AGN56419.1	UDP N-acetylglucosamine pyrophosphorylases 2	Lm UAP2
Leptinotarsa decemlineata	XP_023024177.1	UDP-N-acetylhexosamine pyrophosphorylase-like	Ld UAP1
Leptinotarsa decemlineata	XP_023022882.1	UDP-N-acetylhexosamine pyrophosphorylase-like protein 1	Ld UAP2

Table 3. Species, NCBI Accession numbers, full names and abbreviated names used in phylogenetic trees are listed for all orthologs included in phylogenetic analyses (Figure 1,4).