Characterization of Wnt Signaling Genes in *Diaphorina citri*

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Abstract

Diaphorina citri is the insect vector of Huanglongbing (HLB), a disease that has devastated global citrus production. In an effort to better understand the insect's biology, the *D. citri* genome has been manually annotated. Here, we report on *D. citri* genes involved in both canonical and noncanonical Wnt signaling. In metazoans, Wnt signaling is important for many biological processes such as patterning, cell polarity, tissue generation, and stem cell maintenance. We have curated a complete repertoire of Wnt signaling genes in *D. citri.* In total, 24 genes associated with canonical and noncanonical Wnt signaling have been annotated including seven Wnt ligands, three *frizzled* homologs, *arrow*, and several receptor tyrosine kinases such as *ROR* and *doughnut.* We were unable to find *Wnt8/D*, *Wnt9*, and *Wnt16* along with the various ligand genes that have been lost to insects such as *Wnt2-4.* The mechanisms of canonical Wnt signaling appear to be mostly conserved and comparable to that found in the model organism, *Drosophila melanogaster.*

Introduction:

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with canonical and noncanonical Wnt signaling have been annotated including seven Wnt ligands, three *frizzled* homologs, *arrow*, and several receptor tyrosine kinases such as *ROR* and *doughnut.* We were unable to find *Wnt8/D*, *Wnt9*, and *Wnt16* along with the various ligand genes that have been lost to insects such as *Wnt2-4.* The mechanisms of canonical Wnt signaling appear to be mostly conserved and comparable to that found in the model organism, *Drosophila melanogaster* (Table 1). A model for canonical Wnt signaling in *D. citri* based on curated genes is shown (Figure 1). This is an important first step for understanding critical biological processes that may be targeted to control the spread of *D. citri* and may provide a broader insight into the mechanisms of Wnt signaling in this important hemipteran vector.

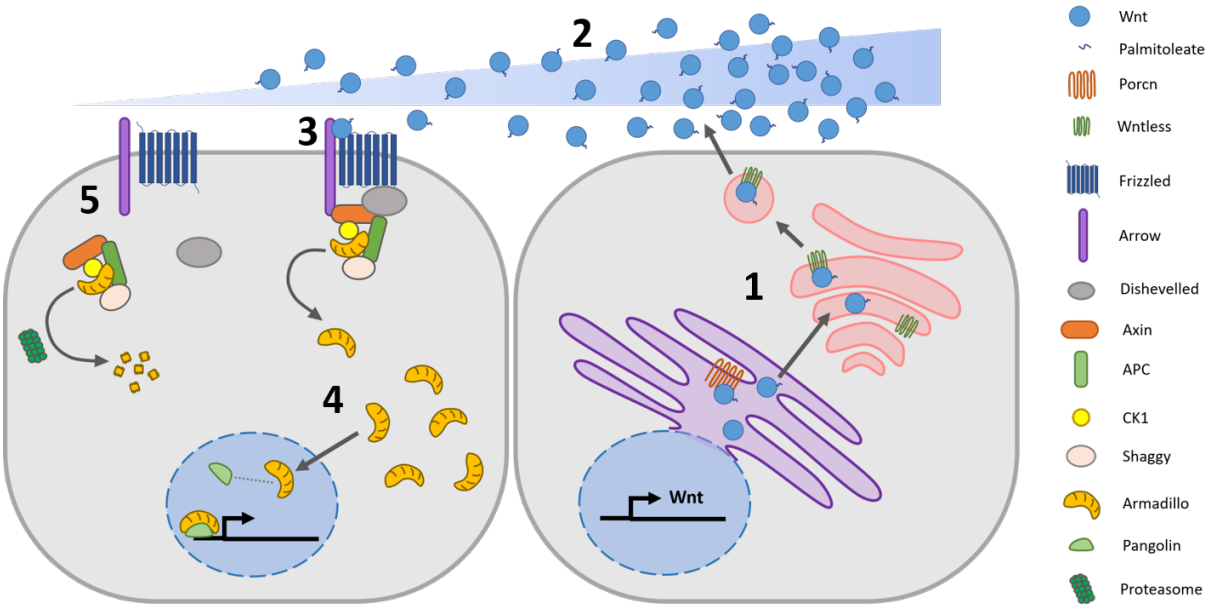

Figure 1: Theoretical model of canonical Wnt signaling cascade in *D. citri* based on curated genes. 1) Wnt is secreted. 2) Wnt concentration gradient forms. 3) Wnt binds to Frizzled and releases Armadillo. 4) Armadillo migrates into the nucleus, associates with Pangolin, and regulates gene expression. 5) Arm is degraded in the absence of Wnt.

Results and Discussion:

The loss of Wnt ligand genes is more common in insects than in other metazoans which leads to a highly variable array of *Wnts* from species to species [1]. This may be facilitated by the promiscuous nature of Wnt proteins that allows certain subfamilies to compensate for the loss of others by sharing receptors. Phylogenetic analysis was performed to characterize the *D. citri* Wnt repertoire (Supplementary Figure 1). Seven different *D. citri Wnts* were identified and classified as *Wnt1* (also known as *wingless*), *Wnt5*, *Wnt6*, *Wnt7*, *Wnt10*, *Wnt11*, and *WntA* (Supplementary Figure 1 and 2). In comparison, seven *Wnt* genes have been identified in *D. melanogaster*, nine in *Tribolium castaneum*, and six in *Acyrthosiphon pisum [2,3]*. The collection of *Wnt* genes found in *D. citri* is similar to other insects, and there have been no *Wnt* subfamilies

identified that are unique to *D. citri*. Contrary to what has been previously reported [4], *D. citri* does in fact appear to possess a *Wnt6* gene.

Table 1: Gene copy table.

Gene	*Drosophila melanogaster*	*Apis melifera*	*Tribolium castaneum*	*Acyrthosiphon pisum*	*Diaphorina citri* v3
Wnt1	1	1	1	1	1
Wnt5	1	1	1	1	1
Wnt6	1	1	1	0	1
Wnt7	1	1	1	1	1
Wnt8/D	1	0	1	0	0
Wnt9	1	0	1	0	0
Wnt10	1	1	1	0	1
Wnt11	0	1	1	1	1
Wnt16	0	0	0	1	0
WntA	0	1	1	1	1
pangolin	1	1	1	1	1
armadillo	1	1	2	2	1
wntless	1	1	1	1	1
porcupine	1	1	1	1	1
derailed	2	1	0	1	1
doughnut	1	1	1	1	1
arrow	1	1	1	1	1
frizzled	4	2	3	2	3
ROR	2	2	3	2	2
dishevelled	1	1	1	1	1
shaggy	1	1	1	2	1
Axin	1	1	1	1	1
ck1-gamma	1	1	1	1	1
Apc	2	1	1	1	1

Wnt pathway ortholog numbers in five different insect species. *Drosophila melanogaster, Apis mellifera, Tribolium castaneum,* and *Acyrthosiphon pisum* copy numbers were determined using Flybase, OrthoDB, NCBI Genbank, Uniprot, and several other publications [1,2,9,10]. *Diaphorina citri* numbers represent the number of manually annotated genes in the *D. citri* v3.0 genome.

Wnt1, *Wnt6*, and *Wnt10* typically occur in very close proximity in a highly conserved gene cluster [5,6]. Accordingly, it is believed that this cluster is also conserved in *D. citri* and is supported by the chromosomal length assembly in v3.0 [7]. The close phylogenetic relationship of *Wnt1*, *Wnt6*, and *Wnt10* in *D. citri* (Supplementary Figure 1) supports the hypothesis that this cluster is the result of an ancient duplication event that may predate the divergence of cnidarians and bilaterians [6]. The orientation of these clustered *D. citri Wnt* genes is similar to that found in *D. melanogaster* and differs from what may be a basal organization of *Wnts* found in species

of Coleoptera, Hymenoptera, and Cladocera (Supplementary Figure 3). When present, *Wnt9* is also associated with this gene cluster. However, as with *A. pisum*, *Wnt9* was not found in the *D. citri* genome and appears to have been lost. A second *Wnt* cluster, *Wnt5* and *Wnt7*, also common among non-insect metazoans, is not seen in *D. citri*.

The mechanisms that act to conserve these *Wnt* gene clusters are not well understood. In the basal metazoan, *Nematostella vectensis*, clustered *Wnt* genes do not exhibit similar expression patterns or *Hox*-like collinearity [5] and may not share regulatory elements. Data obtained from Psyllid Expression Network [8] shows varying levels of expression amongst the clustered genes in different life stages of *D. citri* (Supplementary Figure 4). However, it appears that *Wnt1* and *10* are similarly upregulated during embryonic psyllid development and downregulated during the adult stage, and similar transcript levels of *Wnt1* and *6* are seen in the nymphal stage. This suggests there may be shared regulation dependent upon life stage. Furthermore, ordering within the clusters is subject to rearrangement (Supplementary Figure 3)[3,5]. This may indicate that gene directionality is not a factor in conserving this cluster. Yet, our annotation findings support the hypothesis that these *Wnt* clusters are preserved. A better understanding of the regulatory hierarchy that controls *Wnt* expression might shed light on the significance of *Wnt* gene associations in the genome.

The organization of the genomic reference sequence into chromosomal length scaffolds was essential for revealing *D. citri* gene clustering. The previous genome assemblies were often unsupportive in confirming the proximity of genes due to the shorter scaffold lengths. Genome v2.0 assembly errors had likely misrepresented the location of *Wnt10,* making it appear to be separated from *Wnt1* and *Wnt6. A complete Wnt 1-6-10 cluster was found in the improved chromosome length assembly v3.0.* Thus, the quality of the reference genome should be considered when performing phylogenetic studies.

Orthologs for *Wnt2*, *Wnt3*, *Wnt4*, *Wnt8/D*, *Wnt9*, and *Wnt16* were not located in the *D. citri* genome. The close identity of certain *Wnt* subfamilies makes distinguishing between them difficult, however, the loss of *Wnt2–4* is expected as they are absent in all insects [1]. *Apis mellifera* and the hemipteran *A. pisum* have been reported to lack *Wnt8/D*, and perhaps this *Wnt* subfamily has been lost in the divergence from other insect groups [2]. Additionally, *Wnt16* was not found in *D. citri* v3.0. This finding contrasts with the gene predictions of other hemipteran genomes available at NCBI, namely *A. pisum, Sipha flava,* and *Nilaparvata lugens* (Supplementary Figure 1).

Based on whole body RNA extractions, *Wnt6* has the highest average transcript levels of all the *Wnt* genes in both nymph and adult psyllids (Supplementary Figure 5). The relatively high amount of *Wnt6* transcripts suggests that it is important during metamorphosis and adult stage homeostasis and may serve as a good target for molecular therapeutics such as RNA interference. Transcript expression of *Wnt6* in adults is mainly concentrated in the legs and thorax, averaging 102 transcripts per million (TPM) and 272 TPM, respectively. This is considerably higher than all other *Wnt* genes in these tissues which only average between 0.26 and 3 TPM. It is unclear if other *Wnts* can be upregulated to compensate for the loss of *Wnt6*, and perhaps targeting multiple *Wnt* genes or the mechanisms by which Wnt is secreted (i.e. Porcupine and Wntless) would be more disruptive to *D. citri* physiology.

Several receptors and co-receptions that are associated with canonical and non-canonical signaling have been identified (Supplementary Table 1). Three paralogs for the Wnt receptor encoding *frizzled* have been found in *D. citri*. We classified and numerically designated *D. citri's* three *frizzled* genes based on how their encoded protein sequences form clades with *D. melanogaster* orthologs (Supplementary Figure 6). Our analysis showed that *D. citri,* and other hemipterans such as *Halymorpha halys* and *N. lugens,* possess a Frizzled protein similar to *D. melanogaster*'s Frizzled 3. The Hemipteran sequences form a distinct clade separate from the Dipteran sequences (Supplementary Figure 6). The Hemipteran clade suggests that these genes could belong to a different subfamily of Frizzled, maybe one specific to Hemiptera, although this ortholog has not been reported in the *A. pisum* genome [2].

Orthologs for both *ROR1* and *ROR2* have been identified. Interestingly, *ROR1* has two isoforms, the first of which contains an immunoglobulin (IG) domain (Supplementary Figure 7). This first isoform (Dcitr05g14430.1.1) also appears to have higher transcript levels in *D. citri* egg, nymph, and adult stages when compared to the second isoform (Dcitr05g14430.1.2) (Supplementary Figure 8). A large number of transcripts for the first isoform were detected in the psyllid egg which suggests that it plays a very important role in embryo development (Supplementary Figure 8).

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