Ubiquitin-proteasome pathway annotation in *Diaphorina citri* can reveal potential targets for RNAi-based pest management

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ABSTRACT

Ubiquitination is an ATP-dependent process that targets proteins for degradation by the proteasome. Here, we annotated 15 genes from the ubiquitin-proteasome pathway in the Asian citrus psyllid, *Diaphorina citri*. This psyllid vector has come to prominence in the last decade owing to its role in the transmission of the devastating bacterial pathogen, *Candidatus Liberibacter asiaticus* (CLAs). Infection of citrus crops by this pathogen causes Huanglongbing (HLB), or citrus greening disease, and results in the eventual death of citrus trees. The identification and correct annotation of these genes in *D. citri* will be useful for functional genomic studies to aid the development of RNAi-based management strategies aimed at reducing the spread of HLB. Investigating the effects of CLAs infection on the expression of ubiquitin-proteasome pathway genes may provide new information about the role these genes play in the acquisition and transmission of CLAs by *D. citri*.

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DATA DESCRIPTION

The ubiquitin-proteasome pathway (Figure 1) is responsible for the targeted degradation of most proteins in eukaryotic cells [1]. It regulates the concentration of proteins and degrades misfolded proteins. Ubiquitin modification is an ATP-dependent process. Ubiquitination is initiated by ubiquitin-activating enzymes (E1), which activate and transfer ubiquitin to ubiquitin-conjugating enzymes (E2).

Conjugation of proteins with ubiquitin is mediated by ubiquitin ligases (E3) and tags the target protein for degradation by the proteasome. In its active form, the complete 26S proteasome comprises a 20S catalytic cylindrical core, and one or two 19S regulatory subunits bound to the termini on either or both sides of the core (Figure 2) [3].

The 19S regulatory subunits, which are made up of Rpn and Rpt subunits, bind ubiquitinated proteins and may help to unfold the proteins while translocating them to the 20S catalytic core. The 20S core, often referred to as the 20S proteasome, comprises two β -rings and two α -rings that organize into a cylinder-shaped quaternary structure.

Figure 1. Ubiquitination process and interaction with the proteasome. Created with BioRender [2]. Ub: Ubiquitin.

Figure 2. Proteasome and its subunits. Created with BioRender [2].

Proteolysis occurs at the N-termini of the β -rings where threonine residues form active sites that hydrolyze proteins via threonine nucleophilic hydrolysis [3].

CONTEXT

The ubiquitin-proteasome pathway is highly conserved among eukaryotes and has been the target of many investigations attempting to decode the functions and inner workings of this pathway [1]. Studies involving arthropods [4] have linked the ubiquitin-proteasome pathway to development, immune response, gametogenesis, differentiation, apoptosis and stress response [5]. For example, the main pathways involved in insect resistance to viral and bacterial infections, Toll and JAK/STAT, are both dependent on proteasome activity for degradation of ubiquitinated targets [6, 7].

In this study, we identified and manually annotated 15 genes from the ubiquitin-proteasome pathway in *Diaphorina citri* (NCBI:txid121845), which is the vector for *Candidatus Liberibacter asiaticus* (CLAs), a bacterial pathogen associated with citrus greening disease. Several studies have suggested that the ubiquitin-proteasome pathway is affected by CLAs infection of *D. citri*. Ramsey *et al.* [8] found that several ubiquitin-proteasome pathway proteins are differentially expressed between CLAs(+) and

Table 1. OGS v3.0 genes and the type of evidence used to validate the gene model.

Gene	OGS 3.0 identifier	Evidence supporting annotation			
		MCOT	Iso-seq	RNA-seq	Ortholog
Ubiquitin Thioesterase (OTU1)	Dcitr07g12810.1.1		X		X
Ubiquitin conjugating enzyme 7 (Ubc7)	Dcitr05g04710.1.1		X		X
Regulatory particle non-ATPase (Rpn3)	Dcitr04g11565.1.1		X		X
Small ubiquitin-related modifier (smt3)	Dcitr02g04830.1.1	MCOT07264.0.TT	X		X
Ubiquitin-specific protease 7 (Usp7)	Dcitr07g06000.1.1		X		X
Ubiquitin-like activating enzyme 5 (Uba5)	Dcitr01g11870.1.1	MCOT04043.0.CT MCOT19464.0.CT MCOT01465.4.CT		X	X
E3 ubiquitin-protein ligase (Bre1)	Dcitr08g07680.1.1	MCOT14294.0.CT		X	X
Regulatory particle triple-A ATPase 3 (Rpt3)	Dcitr06g06690.1.1	MCOT00775.0.CT		X	X
Regulatory particle non-ATPase 7 (Rpn7)	Dcitr06g05390.1.1	MCOT18826.2.CT			X
Regulatory particle non-ATPase 11 (Rpn11)	Dcitr01g16470.1.1	MCOT21540.0.CT			X
Regulatory particle non-ATPase 12 (Rpn12)	Dcitr09g09680.1.1	MCOT03904.0.CT	X	X	X
Proteasome α6 subunit (Prosa6)	Dcitr06g06380.1.1				X
Ubiquitin activating enzyme 1 (Uba1)	Dcitr03g10190.1.1	MCOT05061.0.CT			X
Proteasome β6 subunit (Prosβ6)	Dcitr05g14710.1.1	MCOT17488.0.CO			X
Proteasome β5 subunit (Prosβ5)	Dcitr06g03790.1.1		X		X

Manually annotated ubiquitin-proteasome pathway genes in *Diaphorina citri*. Each gene has been assigned an OGS 3.0 identifier and denoted as complete or partial. Evidence types supporting each annotation (MCOT, IsoSeq, RNAseq or ortholog) are indicated in the table. For the exact methods employed, see protocols.io document [15]. MCOT: Maker, Cufflinks, Oases, Trinity pipelines; OGS: Official gene set.

CLas(–) *D. citri*, suggesting that protein degradation may be affected by CLas infection. Two other studies [9] reported differential expression of multiple E3 ubiquitin ligases during CLas infection [10]. With the purpose of finding possible pest control targets, Ulrich *et al.* [11] noted that several ubiquitin-proteasome genes were among the 40 genes associated with the highest lethality percentages in an RNA interference (RNAi) screen of the beetle *Tribolium castaneum* [12], suggesting that this pathway is a possible target for RNAi-based control of insects.

METHODS

Ubiquitin-proteasome genes in *D. citri* genome v3.0 [13] were identified through BLAST (NCBI BLAST, RRID:SCR_004870) searches of *D. citri* sequences with ubiquitin-proteasome orthologs primarily from *Drosophila melanogaster*. Orthology was then verified by reciprocal BLAST of the National Center for Biotechnology Information (NCBI) non-redundant protein database [14]. Genes were manually annotated and validated in Apollo 2.1.0 (RRID:SCR_001936) using available evidence, including RNA-seq reads, Iso-seq and MCOT (MAKER (RRID:SCR_005309), Cufflinks (RRID:SCR_014597), Oases (RRID:SCR_011896), and Trinity (RRID:SCR_013048)) [13] (Table 1). A neighbor-joining phylogenetic tree was constructed in MEGA version 10 (Mega, RRID:SCR_000667) using full-length protein sequences from *D. citri*, *T. castaneum* [12] and *D. melanogaster* [7] (Table 2), employing the CLUSTALW (ClustalW, RRID:SCR_017277) multiple sequence alignment with Poisson correction method and 1000 replicate bootstrap test.

Table 3 shows a list of orthologs evaluated for gene validation. Expression data from the Citrus Greening Expression Network (CGEN) were visualized using the pheatmap [16] (pheatmap, RRID:SCR_016418) package of R (R Project for Statistical Computing, RRID:SCR_001905) or Microsoft Excel (Microsoft Excel, RRID:SCR_016137).

Further details on the annotation process can be found at protocols.io [15] (Figure 3).

Annotating genes in *Diaphorina citri* genome version 3

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D. citri annotation

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Figure 3. Protocols.io protocol for annotating genes in *Diaphorina citri* genome version 3 [15]. <https://www.protocols.io/widgets/doi?uri=dx.doi.org/10.17504/protocols.io.bniimcce>

Table 2. NCBI accession numbers for ortholog proteasome subunit annotations.

Gene	<i>Diaphorina citri</i>	<i>Tribolium castaneum</i>	<i>Drosophila melanogaster</i>
<i>Rpt3</i>	Dcitr06g06690.1.1	XP_967976.1	NP_001285108.1
<i>Rpn3</i>	Dcitr04g11565.1.1	XP_971686.1	NP_001260557.1
<i>Rpn7</i>	Dcitr06g05390.1.1	XP_973643.1	XP_033162910.1
<i>Rpn11</i>	Dcitr01g16470.1.1	XP_008196636.1	NP_608905.1
<i>Rpn12</i>	Dcitr09g09680.1.1	XP_971959.1	NP_648904.1
<i>Prosa6</i>	Dcitr06g06380.1.1	XP_966973.1	NP_001285798.1
<i>Prosβ6</i>	Dcitr05g14710.1.1	XP_968855.1	NP_524115.1
<i>Prosβ5</i>	Dcitr06g03790.1.1	XP_970194.1	NP_652014.1

Ortholog comparison table with NCBI accession numbers of proteins used to construct the phylogenetic tree in Figure 4.

Table 3. Count of orthologous genes in representative insects.

Gene	<i>Drosophila melanogaster</i>	<i>Tribolium castaneum</i>	<i>Acyrtosiphon pisum</i>	<i>Diaphorina citri</i>
OTU1	1	1	1	1
Ubc7	1	1	1	1
Rpn3	1	1	1	1
Smt3	1	1	1	1
Usp7	1	1	1	1
Uba5	1	1	1	1
Bre1	1	1	1	1
Rpt3	1	1	1	1
Rpn7	1	1	1	1
Rpn 11	1	1	1	1
Rpn 12	1	1	1	1
Prosa6	1	1	1	1
Uba1	1	1	1	1
Prosβ6	1	1	1	1
Prosβ5	1	1	1	1

D. melanogaster counts are from FlyBase [17], while *T. castaneum* and *A. pisum* counts were determined by BLAST searches.

DATA VALIDATION AND QUALITY CONTROL

The ubiquitin-proteasome pathway contains hundreds of genes. However, in our study we focused on the genes identified by Ramsey *et al.* [8] and Ulrich *et al.* [11]. We identified a

single ortholog of each of the ubiquitin-proteasome pathway genes described in those studies (Tables 2 and 3), which is consistent with the gene count in *D. melanogaster* [7], *T. castaneum* [12] and *Acyrtosiphon pisum*. Genes were named based on their *Drosophila* orthologs. Since Ramsey *et al.* [8] observed differences in protein levels between CLas(+) and CLas(−) insects for the products of several of these genes, we used CGEN [18] to compare transcriptional expression data from publicly available RNA-seq samples of CLas(+) and CLas(−) *D. citri*.

Two main groups of genes were annotated in this study: ubiquitination-related genes, and proteasome-related genes encoding proteasome-subunits.

UBIQUITINATION-RELATED GENES

Ubiquitin activating enzyme 1 (Uba1)

As the only E1 enzyme in the ubiquitination pathway of insects, Uba1 is critical for initiation of the ubiquitination process [19]. In *D. melanogaster*, clones of cells homozygous for mutations in *Uba1* show changes in the regulation of apoptosis, with weak alleles causing reduced apoptosis and strong alleles causing increased apoptosis [19]. Cells adjacent to clones for strong alleles of Uba1 overproliferate because of increased Notch signaling, suggesting that Uba1, through its role in ubiquitination, may act as a tumor suppressor gene. Flies completely lacking *Uba1* do not survive. However, flies homozygous for weaker alleles sometimes live to adulthood, but have reduced lifespan and motor defects [20]. Knockdown of *Uba1* in *Tribolium* also causes high levels of lethality [11], suggesting that *Uba1* could be a good target for RNAi-based pest control.

Ubiquitin conjugating enzyme 7 (Ubc7)

Ubc7 is one of about 30 E2 ubiquitin-conjugating enzymes known in *D. melanogaster*. It is also known as *courtless*, because male flies lacking Ubc7 display abnormal courtship behavior, as well as defective spermatogenesis [21]. In *D. citri*, Ramsey *et al.* [8] observed almost six-fold higher expression of Ubc7 in CLas(+) organisms (Figure 4). To determine if this difference could be caused by changes in transcription, we analyzed *Ubc7* expression in publicly available RNA-seq data in CGEN. Comparison of *Ubc7* transcript levels in CLas(+) versus CLas(−) samples did show slight upregulation of *Ubc7* in some CLas(+) samples.

E3 ubiquitin-protein ligase (Bre1)

As one of the E3 ligase genes, *Bre1* is a highly conserved gene affecting several other pathways through its role in the ubiquitin pathway. For example, in *D. melanogaster*, the Notch signaling pathway requires Bre1 for histone modification [22]. In CLas(+) psyllids, Bre1 protein levels were greatly reduced (almost 50-fold) compared with CLas(−) psyllids. However, no significant change in transcript levels is seen in the available RNA-seq data (Figure 4).

Ubiquitin Thioesterase (OTU1)

In *D. melanogaster*, Ubiquitin Thioesterase 1 (OTU1) is a hydrolase responsible for removal of conjugated ubiquitin and regulates protein turnover by halting the ubiquitination process and retaining proteins [23]. In *D. citri*, Ramsey *et al.* [8] observed differential expression of OTU1 during CLas infection, with CLas(+) psyllids showing 13-fold higher expression than CLas(−). In nymphs fed on CLas(+) *Citrus* spp., we saw an approximately

Figure 4. Phylogenetic comparison of proteasome subunit orthologs. The numbers are bootstrap values and the *D. citri* proteins are marked with a triangle.

six-fold increase in OTU1 transcripts compared with nymphs fed on uninfected plants of the same species. However, this was a single replicate study and none of the other CLas(+) versus CLas(−) comparisons showed a significant difference in OTU1 expression (Figure 4).

Ubiquitin-specific protease 7 (Usp7)

Usp7 is another deubiquitinase/protease, and has been demonstrated in *D. melanogaster* to regulate aging and autophagy [24]. In *D. melanogaster*, specimens with knocked down Usp7 have difficulty coping with oxidative stress and starvation [24]. Furthermore, Usp7 has been shown to interact with the p53 tumor suppressor gene [25] and inhibition of Usp7 through small molecule targeted approaches have shown promise in increasing cytotoxicity and inducing tumor cell death [25]. In contrast to OTU1, which also negatively regulates ubiquitin-mediated degradation, Usp7 protein showed three-fold downregulation in CLas(+) organisms [8]. We saw no evidence of differential expression at the transcript level (Figure 4).

Ubiquitin-like activating enzyme 5 (Uba5)

Uba5 is an E1 enzyme for the ufmylation pathway, which modifies proteins in a manner similar to the ubiquitination pathway [26]. In ufmylation, the ubiquitin-like protein Ubiquitin fold modifier 1 (UFM1) is conjugated to target proteins. The functions of ufmylation are not well understood, but it seems to be important for tumor suppression, response to DNA damage, and regulation of protein homeostasis in the endoplasmic reticulum [27]. Absence of Uba5 in *D. melanogaster* causes cerebellar ataxia, resulting in severe locomotive defects and a shortened lifespan [28]. Uba5 shows an almost three-fold reduction in protein levels in CLas(+) than CLas(−) psyllids [8]. However, there seems to be no consistent effect of CLas infection on *Uba5* transcript levels (Figure 4).

Small ubiquitin-related modifier (smt3)

The *smt3* gene encodes the Small Ubiquitin-Like Modifier (SUMO) protein [28]. Conjugation of SUMO to target proteins, known as sumoylation, occurs by a process analogous to ubiquitination, but uses a distinct set of E1, E2 and E3 enzymes. Sumoylation has the opposite effect of ubiquitination in that it inhibits protein degradation [29, 30]. Studies in *D. melanogaster* have shown that sumoylation may potentiate the immune response against infections [29]. Ramsey *et al.* [8] observed a four-fold reduction in *D. citri* Smt3 protein in CLas(+) versus CLas(−) psyllids. We do see a reduction in *smt3* transcript levels in adult psyllids fed on *Citrus* spp. infected with CLas, but this difference is not seen in nymphs or in adults fed on *Citrus medica* (Figure 4).

PROTEASOME GENES

Regulatory particle non-ATPase (Rpn3), Regulatory particle non-ATPase 7 (Rpn7), Regulatory particle non-ATPase 11 (Rpn11), Regulatory particle non-ATPase 12 (Rpn12)

The Regulatory particle non-ATPase (Rpn) subunits of the proteasome are responsible for the ‘lid’ complex of the proteasome. They are directly responsible for capturing polyubiquitinated proteins and moving them into the cylindrical complex [3]. Furthermore, Rpn11 has been recognized as being responsible for deubiquitinating these proteins. RNAi knockdown of Rpn subunits in *Nilaparvata lugens*, a member of the same insect order as *D. citri*, caused multiple defects in female reproduction, suggesting that the proteolytic activity of the proteasome is important for these processes [31]. Moreover, Rpn7, Rpn11 and Rpn12 were shown by both Ulrich *et al.* [11] and Knorr *et al.* [32] to be potential high lethality RNAi targets in *T. castaneum*.

Regulatory particle triple-A ATPase 3 (Rpt3)

The six Regulatory particle triple-A ATPase (Rpt) subunits are organized into a hexameric ring, responsible for unfolding the proteins and opening the α gates within the proteasome, where the protein will be ultimately degraded [3]. Rpt3 is one of the three subunits that contains the conserved C-terminal hydrophobic-tyrosine-X (HbYX) [3], which has been shown to be directly responsible for facilitating gate opening. In fission yeast, Rpt3 has been associated with CENP-A, a variant of histone H3 responsible for kinetochore establishment [33, 34]. In RNAi knockdown experiments in *Tribolium*, *Rpt3* was one of the top 11 genes with respect to lethality percentage.

Proteasome $\alpha 6$ subunit (Prosa6), Proteasome $\beta 6$ subunit (Pros $\beta 6$), Proteasome $\beta 5$ subunit (Pros $\beta 5$)

The Proteasome (Pros) alpha proteins are the ‘gating’ subunits, which receive the unfolded proteins provided by the Rpt subunits [3]. The Pros beta subunits provide the peptidases and catalytic enzymatic activity that degrade the proteins within the proteasome. In *D. melanogaster*, mutations in the Pros units cause accentuated muscle degradation and a 25% decrease in lifespan [35]. The three Pros subunit genes annotated here were identified as good targets for RNAi-based pest control because of high lethality percentages [11].

The protein sequences of the various proteasome subunits are often quite similar, making it challenging to establish orthology. We performed phylogenetic analysis to ensure

that we had correctly identified all the annotated *D. citri* proteasome subunit genes (Figure 4). All *D. citri* subunit proteins clustered with the expected *D. melanogaster* and *T. castaneum* orthologs, indicating that the annotated genes are named correctly.

In Ramsey *et al.* [8], a very distinct difference can be observed between upregulated and downregulated proteins, with as much as 50-fold differences in expression. When these data are displayed as a clustered heatmap scaled by gene (Figure 4) we can compare the relative expression levels between samples. There is very little differential expression of genes between CLas(+) and CLas(−) samples. The apparent differences between CLas effect on protein and gene expression could indicate that there is post-transcriptional regulation of these genes/proteins. Post-transcriptional regulation of proteasome genes in yeast [25] has been observed before, and could be a possible explanation for these discrepancies.

Conclusion and future work

The ubiquitin-proteasome pathway is highly conserved in most eukaryotes [1] and, therefore, a potential candidate for comprehensive studies. Disruption of this pathway has shown promise as a pest control mechanism in various insects, as explored by Ramsey *et al.* [8] and Ulrich *et al.* [11], among many others. This has led us to annotate 15 ubiquitin-proteasome pathway genes from *D. citri*. Previous reports also suggested that the pathway might be affected by CLas infection [8, 9]. Our analyses found little effect of CLas infection on transcription of the subset of ubiquitin-proteasome genes we annotated. More comprehensive studies will be needed to understand how CLas influences this pathway in *D. citri*.

RE-USE POTENTIAL

The models annotated in this work will be helpful to researchers studying this pathway in many organisms, as this pathway is present in most eukaryotic species. Future studies are required to confirm the validity of the ubiquitin-proteasome pathway as an RNAi target for control of *D. citri* populations.

DATA AVAILABILITY

The *D. citri* genome assembly, official gene sets, and transcriptome data can be downloaded from the Citrus Greening website [36]. Additional datasets supporting this article are available in the GigaScience GigaDB repository [37].

EDITOR'S NOTE

This article is one of a series of Data Releases crediting the outputs of a student-focused and community-driven manual annotation project curating gene models and if required, correcting assembly anomalies, for the *D. citri* genome project [38].

DECLARATIONS

LIST OF ABBREVIATIONS

CLas: *Candidatus Liberibacter asiaticus*; NCBI: National Center for Biotechnology Information, CGEN: Citrus Greening Expression Network; RNAi: RNA interference; RNA-seq: RNA sequencing; Iso-seq: Isoform sequencing; MCOT: Maker, Cufflinks, Oases, Trinity.

ETHICAL APPROVAL

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHOR CONTRIBUTIONS

WBH, SJB, TD and LAM conceptualized the study; TD, SS, TDS and SJB supervised the study; SJB, TD, SS and LAM contributed to project administration; WT, AT and CM conducted the investigation; PH, MF-G and SS contributed to software development; PH, MF-G, SS, TDS and JB developed methodology; SJB, TD, WBH and LAM acquired funding; WT prepared and wrote the original draft; TD, SJB, SS, TDS, CM, WBH and JB reviewed and edited the draft.

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