Two phytoplasmas elicit different responses in the insect vector *Euscelidius variegatus*

Kirschbaum

Running title: Vector-borne plant pathogens modulate insect immunity

Luciana Galetto#, Simona Abbà¹, Marika Rossi¹, Marta Vallino¹, Massimo Pesando¹, Nathalie Arricau-Bouvery², Marie-Pierre Dubrana², Walter Chitarra²,³, Mattia Pegoraro³, Domenico Bosco³, d, Cristina Marzachi³

¹Istituto per la Protezione Sostenibile delle Piante, CNR, National Research Council of Italy, IPSP-CNR, Torino, Italy
²INRA, Univ. Bordeaux, UMR Biologie du Fruit et Pathologie, UMR 1332, Villenave d'Ornon, Cedex, France
³Centro di Ricerca per la Viticoltura e l’Enologia, CREA, Council for Agricultural Research and Economics, CREA-VE, Conegliano (TV), Italy
⁴Dipartimento di Scienze Agrarie, Forestali ed Alimentari DISAFA, Università degli Studi di Torino, Grugliasco (TO), Italy

#Corresponding author: Luciana Galetto, luciana.galetto@ipsp.cnr.it
ABSTRACT

Phytoplasmas are plant pathogenic bacteria transmitted by hemipteran insects. The leafhopper *Euscelidius variegatus* is a natural vector of chrysanthemum yellows phytoplasma (CYp) and a laboratory vector of Flavescence dorée phytoplasma (FDp). The two phytoplasmas induce different effects on this species: CYp slightly improves, while FDp negatively affects insect fitness. To investigate the molecular bases of these different responses, RNA-seq analysis of *E. variegatus* infected with either CYp or FDp was performed. The sequencing provided the first de novo transcriptome assembly for a phytoplasma vector, and a starting point for further analyses on differentially regulated genes, mainly related to immune system and energy metabolism. Insect phenoloxidase activity, immunocompetence, and body pigmentation were measured to investigate the immune response, while respiration and movement rates were quantified to confirm the effects on energy metabolism. The activation of insect immune response upon FDp infection, which is not naturally transmitted by *E. variegatus*, confirmed that this bacterium is mostly perceived as a potential pathogen. Conversely, the acquisition of CYp, which is naturally transmitted by *E. variegatus*, seems to increase the insect fitness by inducing a prompt response to stress. This long-term relationship is likely to improve survival and dispersal of the infected insect, thus enhancing the opportunity of phytoplasma transmission.

INTRODUCTION

Phytoplasmas are wall-less plant pathogenic bacteria of the class Mollicutes, that cause yield losses in many crops worldwide. They colonize plant phloem tissues and are transmitted by phloem-feeding hemipterans (1). In insects, ingested phytoplasmas cross the gut, multiply in the haemocoel and invade salivary glands, before being transmitted during feeding on a new plant (2). Currently, genomes of four phytoplasmas are fully sequenced and annotated and few others are available as drafts (1). Phytoplasmas have small genomes (~750 kb) due to a genome reduction that resulted in the loss of important metabolic pathways: as a consequence, these intracellular bacteria depend on
their hosts for many essential metabolites (3). Due to these strict interactions with hosts and to the difficulty of their axenic cultivation, phytoplasmas need to be studied directly in their hosts. Little is known about pathogenicity mechanism, even if some pathogen-secreted virulence factors have been identified, mainly in strains of ‘Candidatus Phytoplasma asteris’ (2). Studies on this species suggest that phytoplasmas are able to modulate their gene expression during host switching between plant and insect (4, 5).

Flavescence dorée (FD) is an important grapevine disease caused by a 16SrV phytoplasma, mainly transmitted, under field conditions, by the hemipteran cicadellid Scaphoideus titanus. FD phytoplasma (FDp) is a plant quarantine pathogen in the European Union and represents one of the major threats to southern European viticulture. *Vitis vinifera* and *S. titanus* do not represent ideal experimental organisms for laboratory tests: grapevine is a perennial woody plant and the leafhopper is a monovoltine species. Thus, a laboratory model has been established to manage FDp infection cycle with the herbaceous *Vicia faba* as plant and the polivoltine leafhopper *Euscelidius variegatus* as vector (6).

Chrysanthemum yellows phytoplasma (CYp), 16SrI-B ‘Ca. P. asteris’, is associated with a disease of ornamental plants in north-western Italy, where *E. variegatus* is one of the most important natural vectors (7). Like FDp, CYp infections can be obtained under controlled conditions with *Chrysanthemum carinatum* as host plant and *E. variegatus* as vector. The two phytoplasmas have opposite effects on the vector fitness: FDp significantly reduces insect longevity and fecundity (8), meanwhile CYp induces a slight fitness increase (9). CYp shows greater ability than FDp to colonise the salivary glands of the vector and therefore it is more efficiently transmitted by *E. variegatus* (7). So far, physical maps of FDp and CYp genomes, drafts of their genome sequences and an FDp transcriptome analysis (10) are available. By contrast, neither the genome nor the transcriptome of *E. variegatus* is available. Few proteins of the insect have been identified, such as *in vitro* interacting partners of CYp antigenic membrane protein (Amp) (11), which is necessary for CYp acquisition by insect vectors (12). The specificity of FDp transmission is presumably mediated
by variable membrane protein A (VmpA), a phytoplasma protein that is supposed to interact with insect tissues and shows high sequence variability in different strains transmitted by different vector species (13).

In a few studies the plant response to phytoplasma infection was addressed with NGS transcriptomic approaches (1), but to our knowledge this technique has never been used to investigate either the transcriptomic profile of an insect vector infected by these pathogens or the alterations induced by different phytoplasmas in the same host. Here we investigated the effects of two genetically different phytoplasmas on the same insect vector, providing: i) de novo assembly of *E. variegatus* transcriptome, ii) differential expression analysis of *E. variegatus* transcripts under infection with CY or FD phytoplasmas iii) validation of the differential expression profiles and iv) biological experiments to support the transcript profiling results.

**RESULTS**

**RNAseq and differential gene expression.** Diagnostic RT-qPCR assays confirmed the presence of CY and FD phytoplasmas in *E. variegatus* samples (Eva_CY and Eva_FD). The phytoplasma populations, expressed as mean phytoplasma 16S/insect18S ratio, were 4.67E-03 (SEM ± 9.84 E-04) and 2.08 E-04 (SEM ± 6.54 E-05) for insects infected with CYP and FDP, respectively.

Analyses of the cDNA libraries obtained from Eva_CY and Eva_FD resulted in a combined de novo assembly comprising around 135,000 transcripts, with an average GC content of 40%, a median contig length of 433 bp, and an average contig length of 833 bp. Due to the lack of genomic sequence information of *E. variegatus*, the functional annotation of transcripts was conducted using blastx against the NCBI non-redundant (nr) database. The Blast2GO platform was then used to assign the Gene Ontology (GO) terms to the predicted proteins with known function. The results of the de novo assembly and the following transcript annotations are summarized in Table S1. The species distributions of the best blastx matches for each sequence are shown in Table S2.
Transcriptome profiles of *E. variegatus* infected by the beneficial CY or the pathogenic FD phytoplasmas were compared to elucidate the differential vector responses. Differential expression analysis revealed that 84 transcripts were up-regulated and 13 down-regulated in Eva_CY in comparison with Eva_FD (Tables 1 and 2, S3 and S4). The up-regulated genes could be classified in few main functional categories: immune response (11 transcripts), movement and energy metabolism (34 transcripts), proteases (9 transcripts), extracellular matrix (20 transcripts), nucleic acid binding (6 transcripts), and detoxification (4 transcripts). Down-regulated genes could be ascribed to immune response (10 transcripts), movement and energy metabolism (1 transcript), proteases (1 transcript), and detoxification (1 transcript) functional categories. Some of these putative metabolic functions were further investigated to explore the phenotypes correlated with the altered gene regulation.

**Cellular and humoral immunity in response to phytoplasma infections.** To investigate the effects of the phytoplasma presence on immune response, gene expression analysis of selected transcripts, enzymatic activity and biological assays were performed (Fig. 1A; Tables S5, S6, S7). Healthy controls (Eva_H), not exposed to phytoplasmas and PCR negative, were included in the following experiments to highlight the mechanisms underpinning the differential effects on *E. variegatus* immune response upon infection with the two phytoplasma species. Gender was taken into account, but whenever no sex-related differences were recorded within the same category data were pooled. RT-qPCR validation was run on 42 samples (each made up of five pooled insects), phenoloxidase activity was measured for 36 samples (each made up of haemolymph collected from five insects), pigmentation and immunocompetence assays were tested on 170 and 46 specimens, respectively.

**Gene expression.** Kazal-type 1 serine protease inhibitor and phenoloxidase genes were selected from the RNAseq results and literature search (14), respectively, and analysed by RT-qPCR in CYp-, FDp-infected and healthy insects (Eva_H, _CY, _FD). Similar levels of phenoloxidase transcripts were recorded in the insects, regardless of the sex and the infection status (Fig. 2A),
whereas Kazal-type 1 transcripts were significantly more abundant in Eva_FD males compared to Eva_CY ones (P=0.022) (Fig. 2B). Transcripts of the same gene were significantly more abundant in healthy females compared to healthy (P=0.002) and CYp-infected males (P=0.023) (Table S5).

Up-regulation of Kazal-type 1 serine protease inhibitor in Eva_FD confirmed the differential expression obtained by RNAseq analysis (Table 2).

Enzymatic activity. Phenoloxidase (PO) and Pro-PO activities associated with haemocytes and the plasma fraction of haemolymph were quantified. The specific inhibitor phenylthiourea inhibited PO and ProPO activities in all assays with the exception of PO in haemocytes (data not further analyzed). The PO activities of the plasma fractions were similar irrespective of sex and infection status (Table S6). The plasma ProPO activities were significantly lower in males than in females, irrespective of the infection status (P<0.001, P<0.001 and P=0.018, for H, CY and FD, respectively); this activity was higher in FDp- compared to CYp-infected insects (two-fold increase), although the difference was significant only for males (P=0.046) (Fig. 2C). ProPO activities were similar in haemocytes from females and males of each infection status, whereas the enzymatic activity of Eva_FD was double than that of Eva_CY (P=0.017) (Fig. 2D). Steeper slopes of the linear phases of Eva_FD assays further confirmed faster enzymatic reactions in FDp-infected insects (Table S6).

Pigmentation assay. Since PO activity may be correlated to cuticular colour (15), pigmentation of bodies (dorsal side) and forewings of healthy and phytoplasma-infected E. variegatus was further explored. Pigmentation was expressed as grey intensity: 0 for black to 255 for white (Fig. 2E).

Considering the three experimental conditions (Eva_H, _CY, _FD), males always showed significantly darker bodies and wings than females (P=0.005 for healthy bodies and P<0.001 for all other comparison) (Table S7). Wing pigmentation did not show any significant variation in the presence of phytoplasmas, while bodies of FDp-infected female and male insects were significantly darker than healthy and CYp-infected ones (female: P<0.001 for both comparison; male: P<0.001 and P=0.003 for H vs. FD and CY vs. FD, respectively) (Fig. 2E).
**Immunocompetence assay.** To determine whether the phytoplasma presence could influence insect immune response, nylon threads were implanted in insect abdomens to measure melanization as well as the number of haemocytes adhered to the threads (Fig. 2F, Table S7). There was no influence of sex on melanization index (MI) and number of cells adherent to the nylon threads, irrespective of the infection status, but there was a significant difference of MI s of nylon threads implanted in CYp-infected *E. variegatus* compared to those implanted in healthy insects (Fig. 2F, bars). Up to five times more haemocytes adhered to nylon threads implanted into CYp-infected *E. variegatus* compared to those implanted into H and FDp-infected insects, and this difference was significant in both cases (P<0.001) (Fig. 2F, dots).

**Movement and energy metabolism.** Genes involved in muscle contraction and synthesis of intermediate metabolites for energy production were selected among those differentially regulated according to the RNAseq results and literature search (16) (Fig. 1B; Table S5). To investigate the effects of phytoplasma infection on insect mobility and respiration rate, several parameters were measured (Fig. 3F, G, H), (Table S8). Healthy controls (Eva_H) were included in the following experiments to better describe the metabolic response of *E. variegatus* challenged by the two phytoplasmas, and whenever no sex-related differences were recorded within the same category data were pooled. RT-qPCR validation was run on 42 samples (each made up of five pooled insects), movement assay was tested on 138 specimens and CO$_2$ production was measured in 24 insect groups (each made up of three specimens).

**Gene expression.** Myosin light chain, tropomyosin, arginine kinase and maltase were analysed by RT-qPCR in insects of the three experimental conditions (Eva_H, _CY, _FD). The four analysed transcripts were significantly more abundant in males than in females, regardless of the infection status, with the exception of tropomyosin and arginine kinase in healthy insects (Fig 3A-D, Table S5). In general, CYp-infected males showed higher transcript levels compared to healthy and FDp-infected males. These differences were significant only for tropomyosin, with about three times more transcripts in CYp-infected vs. healthy males (P=0.018) and for arginine kinase, with about
twice more transcripts in CYp-infected males vs. healthy and FDp-infected ones (P<0.001 for both comparison). Arginine kinase transcripts were also significantly more abundant in CYp-infected females compared with FDp-infected ones (P=0.024) (Fig. 3C). Up-regulation of arginine kinase and maltase in Eva_CY confirmed the differential expression results of the RNAseq analysis (Table 1).

**Protein expression.** To further characterize the differential expression of proteins involved in movement, Western blot analysis was performed on healthy and phytoplasm-infected insects with anti-tropomyosin commercial antisera (Figs. 3I and S1). Tropomyosin was more expressed in males than in females, regardless of the infection status, therefore confirming the transcriptional analyses. Nevertheless, there were no evident differences in protein expression levels among Eva_H, _CY and _FD categories.

**Movement and respiration functional assays.** Movement parameters (permanence time in a two circle arena and numbers of jumps) showed neither significant variation between males and females, nor among the three experimental conditions, although a trend of a faster movement was observed for CYp-infected males in comparison with healthy and FDp-infected ones (Fig. 3E, bars; Table S8). Consistently, measurements with the gas analyzer showed that Eva_CY produced a significantly higher amount of CO$_2$ than Eva_H and _FD, irrespective of sex (P=0.002 and P=0.027 for H vs. CY and CY vs. FD, respectively) (Fig. 3E, dots; Table S8).

**Protease regulation.** Cathepsin L was selected to investigate the effect of phytoplasma infection on protease regulation. This protein is the major component of the gut digestive enzymes in many invertebrates (17) and, among other functions, is involved in controlling symbiont populations (18). Healthy controls (Eva_H) were included in the following experiments, to dissect the effects of the different phytoplasmas on *E. variegatus* protease regulation.

**Gene expression.** The expression profile of four isoforms (namely 92i3, 92i4, 92i6 and 473) of cathepsin L was analysed by RT-qPCR in insects of the three experimental conditions (Eva_H, _CY, _FD). Isoform 473 was the most strongly up-regulated in Eva_CY within the protease
category (Table 1), whereas isoforms 92i3, 92i4, 92i6 were selected since, among all *E. variegatus* transcripts annotated as “cathepsin L”, they showed the highest identity with the immunogenic peptide recognized by the commercial anti-cathepsin L antibody used for the Western blot.

Significant differences between female and male insects were present for isoform 473, irrespective of the infection status (up-regulated in females, \( P=0.007, P<0.001 \) and \( P<0.001 \), for Eva_H, CY and FD, respectively) and for isoforms 92i3 and 92i6 specifically for FDP-infected *E. variegatus* (up-regulated in males, \( P=0.043 \) and \( P=0.017 \) for 92i3 and 92i6, respectively) (Fig. 4A-D and Table S5).

Transcripts of isoforms 92i3 and 92i4 were significantly up-regulated in CYp-infected females vs. FDP-infected ones (\( P=0.012 \) and \( P=0.023 \) for 92i3 and 92i4, respectively) (Fig. 4A and B). Those of isoform 473 were twice more abundant in CYp-infected females vs. healthy and FDP-infected ones, and these differences were significant (\( P=0.003 \) and \( P=0.012 \), for H vs. CY and CY vs. FD, respectively) (Fig. 4D). Up-regulation of cathepsin L_473 in Eva_CY compared to Eva_FD confirmed the results of the RNAseq analysis (Table 1).

*Putative protein characterization.* The predicted amino acid sequences of the four cathepsin L isoforms were analysed (Fig. 4E and F). All four isoforms showed putative signal peptide from aa 1-16, a pro-region containing the propeptide inhibitor domain and a predicted mature protein including the cysteine protease domain. Putative glycosilation sites were predicted on different isoforms: 2 nitrogen-linked and 5 oxygen-linked. Isoform 92i6 showed a unique putative N-linked site at position 37 and the isoform 473 missed an O-linked site at position 125. Isoforms 92i4 and 92i6 showed highly similar pre-proteins and identical mature forms, whereas isoforms 473 and 92i3 were the most diverse ones (Fig. 4F). The immunogenic peptide, recognized by the commercial antibody used for Western blot, was more similar to isoform 473 than the other ones (Fig. 4F).

*Protein expression.* To further characterize the effect of phytoplasma infection on the expression of cathepsin L protein, Western blot was performed on healthy and phytoplasma-infected insects with anti-cathepsin L commercial antisera (Figs. 4G and S1). The antibody was raised to detect both the pre-protein and the mature form, as it reacts against the immunogenic peptide indicated in Fig. 4E.
Indeed, the Western blots with anti-cathepsin L antibody (Fig. 4G) showed a complex pattern: two faint bands with high MW (around 37-35 kDa) possibly corresponding to pre-proteins, two intense bands with low MW (around 27-25 kDa) possibly corresponding to the mature forms. Similar band patterns were observed from total proteins of *E. variegatus* females, irrespective of the infection status. A similar profile, although less intense, was also evident for healthy males. Surprisingly, almost no signal from the lowest MW bands (25 kDa) was detected from phytoplasma-infected males.

**DISCUSSION**

Relationships between phytoplasmas and their vectors may be pathogenic, neutral or mutualistic (9). CYp and FDp establish different types of relationships with their vector *E. variegatus*: the former slightly improves vector fitness, the latter is pathogenic. The transcriptional landscape of *E. variegatus* infected with the two different phytoplasma species was analysed focusing on long lasting modifications occurring in insects during the response to chronic phytoplasma infection, thus avoiding any possible differences related to CYp and FDp multiplication dynamics during early stages of infection. Sex-specific effects were recorded for several of the tested parameters, as already described for immune response (19, 20) as well as insect movement and dispersal (21).

Among insects of the *Cicadellidae* family, few de novo transcriptome assemblies are available: some were obtained from specific insect tissues, namely salivary glands of *Nephotettix cincticeps* (22) and *Empoasca fabae* (23) and intestinal tract of *Empoasca vitis* (24), others from whole bodies, such as those of *Graminella nigrifrons* (25), *Homalodisca vitripennis* (26) and *Zyginidia pullula* (27). Interestingly, the transcriptomic response of *G. nigrifrons* vector to different plant viruses infections reveals that the expression of cytoskeleton and immunity genes increase in the presence of the persistent propagative rhabdovirus Maize fine streak virus (28).
Phytoplasma infection modulates insect immune response. Molecular and biological analyses indicate that a different modulation of *E. variegatus* immune response occurred following FDp infection compared to CYp infection.

The altered regulation of the immune system was revealed by RNAseq analysis during infection with both phytoplasmas. Among these, transcripts of the Kazal type 1 serine protease inhibitor were more abundant in FDp-infected insects compared to Eva_CY. Similar serine protease inhibitors have antibacterial activity against bacteria (29) as well as antifungal activity against both plant-pathogenic and entomopathogenic fungi, as inhibitors of microbial serine proteases (30). Serpin was the most strongly up-regulated transcript in CYp-infected insects, whereas the snake-like serine protease was among the most strongly down-regulated ones. Clip serine proteases, such as the snake-like ones, are involved in the activation of the ProPO proteolytic cascade in invertebrate immune systems (31, 32), while serpins from different arthropod species inhibit clip domain serine proteases by blocking the activation of ProPO melanization pathway (33, 34). The prophenoloxidase (ProPO) cascade is involved in melanization and encapsulation processes and provides arthropod immunity against bacteria, fungi, protozoan and parasites (34). The opposite regulation of these two transcripts correlates with the lower prophenoloxidase activity and with the less intense cuticular pigmentation observed in CYp-infected compared to FDp-infected insects.

Cuticular color is related to immune response in insects (15), and the darker body pigmentation of FDp-infected *E. variegatus* suggests stimulation of melanization pathway due to a stronger activation of the immune response. The presence of FDp is perceived by the insect as a stress status and therefore it elicits an intense production of melanin. Indeed, prophenoloxidase activities of both plasma and haemocyte lysates were more intense in Eva_FD compared to _CY and _H. On the other hand, the infection with the two phytoplasmas had neither effect on naturally activated phenoloxidase (PO) activity, a good estimate of invertebrate immunocompetence (35), nor on the abundance of the corresponding transcripts. This could be due to the fact that the analyses were performed at late, chronic stages of phytoplasma infection, when colonization of the insect body...
was complete (12). A burst of activated phenoloxidase is, in fact, expected at the onset of the infectious event, as a defence reaction to the immunological challenge (35), as reported for *Micrococcus luteus* infection of the leafhopper *Circulifer haematoceps* (36). Surprisingly, when insects are challenged by an additional stress (wounding through nylon thread), the scenario changes. In the immunocompetence assay, insertion of a nylon thread in the insect body mimics a parasite invader and induces encapsulation. The response of FDp-infected insects is similar to that of the healthy ones. On the contrary, CYP-infected insects showed higher MI and higher number of haemocytes, indicating a better capacity of these insects to react to and isolate an invader. *E. variegatus* is a natural vector of CYP and they share the same ecological niches: these factors could have shaped the insect immune system to fight more promptly against incoming pathogenic organisms.

The Kruppel-like factor, a zinc finger DNA-binding protein, is crucial to mediate white spot syndrome virus (WSSV) infection in two different shrimp species (37), and two transcripts of this gene were oppositely regulated in *E. variegatus* (more abundant in FDp-infected category), suggesting a role for this protein in response to phytoplasma infection. On the other hand, hexamerin transcripts were up-regulated upon CYP infection. Members of this protein family are inducible effector proteins in insect immunity upon bacteria ingestion and have a putative role in gut repair (38). Moreover, in the closely related mollicute-leafhopper association (*C. haematoceps/Spiroplasma citri*) hexamerin is up-regulated following infection and is required for vector survival after spiroplasma inoculation (36). Besides their role in energy metabolism (see below), arthropod arginine kinases (AK) are also involved in stress response and innate immunity: *Apis cerana* AK is induced by abiotic and biotic stresses (39), pacific oyster AK modulates bactericidal immune response in haemolymph (40), and AK from shrimp *Fenneropenaeus chinensis* has been hypothesized as putative receptor of the WSSV virus envelope protein (41). AK transcript was up-regulated upon CYP infection, suggesting different potential roles for this protein during infection with the two phytoplasmas.
Disulfide bonds are redox-controlled switches for pathogens invasion, and are involved in regulating pathogen entry into the endocytic pathway of vertebrate (42) and some invertebrates (43, 44). Recently, a vesicle-mediated colonization of salivary glands has been suggested for CYp infection of *E. variegatus* (12). Consistently, the protein disulfide-isomerase (five transcripts) and the gamma-interferon inducible lysosomal thiol genes were up-regulated upon CYp infection, supporting the involvement of the endocytic pathway in phytoplasma colonization of the host, as described for *Leishmania*, *Listeria* and *Chlamydia* spp (42). Other bacterial pathogens have developed strategies to interfere with host lipidation mechanisms (45). For example, *Salmonella enterica* and *Legionella pneumophyla* exploit host prenylation to direct effector proteins to the pathogen containing vacuole of the host cell (46). Interestingly, transcripts of the farnesyl – geranylgeranyl transferases, the key enzyme of the prenylation pathway, were oppositely regulated in *E. variegatus* (down-regulated in CYp-infected insects) suggesting different alteration of vesicular trafficking upon infection with CYp and FDp. Phytoplasma may modulate the host metabolism through active secretion of effector molecules and the diversity of the effector arsenals among phytoplasmas (2, 47) may explain the opposite transcription profiles of this gene. **Chrysanthemum yellows phytoplasma infection increases energy metabolism.** Molecular and biological analyses indicate an activation of *E. variegatus* energy production metabolism and an increased locomotion activity upon CYp infection. According with RNAseq results, movement and energy production metabolism was the functional category with the highest number of gene transcripts altered upon phytoplasma infection.

Titin, twitchin and protein unc-89 are members of the giant cytoskeletal kinase family that mediate sensing and transduction of mechanical signals in the myofibril. These big proteins display elastic conformational deformation and regulate muscle tissue in adaptation to external stimuli (48).

Several isoforms of these gene transcripts were up-regulated in CYp-infected *E. variegatus*. These kinases participate in regulating protein turnover in muscle, and, in particular, unc-89 regulates...
ubiquitin-mediated protein degradation, through recruitment of E3 ubiquitin ligases (48), which indeed was also up-regulated upon CYp infection. Two isoforms of PDZ and LIM domain protein 3 were up-regulated in CYp-infected insects. PDZ/LIM genes encode a large group of proteins that play important and diverse biological roles, but that functionally can all influence or be associated with the actin cytoskeleton (49). Ryanodine receptor (RYR) is the main calcium release complex of the sarcoplasmic reticulum involved in the excitation-contraction coupling of muscle cells (50), and the dihydropyridine receptor (DHPR) is the plasma membrane L-type calcium channel involved in opening of the RYR by a calcium-induced calcium release mechanism (51). Transcripts of these genes were inversely regulated (RYR up-regulated and DHPR down-regulated) upon CYp infection, suggesting an altered Ca\textsuperscript{2+} regulation in the cytosol of muscle cells in response to phytoplasmas. Indeed, transcripts of the calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum (SERCA) and of the sarcalumenin were up-regulated in CYp-infected insects. The former is a pump involved in translocation of cytosolic calcium into the sarcoplasmic reticulum to allow relaxation of muscle fibers (16), the latter is a calcium-binding protein involved in fine regulation of cellular calcium storage (52). Moreover, transcripts of the main proteins involved in contraction and cytoskeletal motion, tropomyosin, troponin, myosin, actin and dynein (16) were all up-regulated upon CYp infection. The analysis of tropomyosin confirmed the stronger expression in males than in females, as revealed by RT-qPCR and Western blot, but no evident differences were detected among different infection categories (H, CY, FD), possibly due to several isoforms derived from alternative splicing, a well-known phenomenon for this gene (53). Increased insect movement has been observed in some pathogen-vector associations, such as *Diaphorina citri* infected with ‘*Candidatus* Liberibacter asiaticus’ (54) and *Bombyx mori* with BmNPV (55). We tested the intriguing hypothesis that CY phytoplasma, which is naturally transmitted by the insect host, can manipulate the vector movement to increase its transmission, but the parameters recorded during the movement assays did not clearly support this hypothesis. Despite that, muscle contraction is also involved in active insect respiration, so the
higher expression of the above-mentioned genes in CYp-infected insects could be related with an increase of the respiration rate. This was indeed the case, as higher CO\textsubscript{2} levels were produced by CYp-infected *E. variegatus* in the respiration assay compared to FDp-infected and healthy insects. Additionally, the up-regulation of maltase, hydroxybutyrate dehydrogenase and arginine kinase (AK) transcripts in CYp-infected *E. variegatus* indicates a stronger activation of the energy production metabolism. Altogether these data point to an augmented movement in CYp-infected insect, which may positively influence phytoplasma transmission.

**Phytoplasma infection alters protease regulation.** Cathepsins are proteases generally stored in lysosomes, involved in several processes like development, apoptosis and immunity of arthropods (17, 56). Upon CYp infection, transcripts of cathepsin L were up-regulated and those of cathepsin D down-regulated, suggesting different roles of these enzymes in response to phytoplasma infection.

To confirm RNAseq analysis, four isoforms were chosen among all the *E. variegatus* “cathepsin L” transcripts: 473, derived from differential expression analysis, and 92i3, 92i4, 92i6, showing the highest identity with the immunogenic peptide recognized by the anti-cathepsin L antibody used in Western blot. The up-regulation of cathepsin L was confirmed by RT-qPCR for three of the four isoforms upon CYp infection in comparison with FDp. The same up-regulation was not present at protein level. Molecular weights of cathepsin L mature proteins differed from the theoretical ones and this may be explained by different glycosylation, one of the post translational-processes occurring during cathepsin maturation (57). Indeed the anti-cathepsin L antibody detected proteins of different sizes, and intriguingly the lowest ones were poorly present in healthy males and nearly absent in phytoplasma-infected males. As transcripts of isoform 473 were significant up-regulated in females, these lowest protein bands are presumably its mature forms. Indeed isoform 473 showed one glycosilation site less than other isoforms and could migrate faster. The absence of mature form of this isoform in infected males might indicate that phytoplasma presence could prevent cathepsin L maturation. Expression of this gene is altered upon microbial infection, either in pathogenic combinations, such as *Serratia marcescens* in the pea aphid *Acyrthosiphon pisum* (18), and in
symbiotic associations, such as *Burkholderia* symbionts ingested by the bean bug *Riportus pedestris* (58). Indeed, both in pathogenic and mutualistic associations, bacteria need to avoid lysosomal degradation to establish an intracellular association and this could be true also for CY and FD phytoplasmas.

In conclusion, transcriptomic and phenotypic results shed some light on the molecular mechanisms underlying the different effects of the two phytoplasmas on the insect vector *E. variegatus*. Our data show that *E. variegatus* perceives FD as a pathogen, since it activates an immune response. Lack of natural interactions between FD phytoplasma, mainly restricted to *Vitis* spp., and the laboratory vector *E. variegatus*, which does not feed on grapevine, may explain perception of this phytoplasma as non-self. On the other hand, the long-lasting interactions between CY phytoplasma and *E. variegatus* (that are sympatric) might have driven towards a mutualistic relationship.

The prompt and aggressive response to the menace of an external pathogen, mimicked by the nylon thread, may be due to an immune priming activated by CYp and together with the increased energy metabolism are likely to provide an ecological advantage to both the vector and the phytoplasma.

**MATERIALS AND METHODS**

*Insect and phytoplasmas.* *E. variegatus* isolate to-1 was collected in Piedmont (Italy) and reared on oat, *Avena sativa* (L.) (7). Chrysanthemum yellows phytoplasma (CYp) was isolated in Italy, and maintained by insect transmission on daisy, *Chrysanthemum carinatum* Schousboe (7). Flavescence dorée phytoplasma (FDp) was isolated in Italy, and maintained by insect transmission on broad bean, *Vicia faba* L. Daisies, broad beans and oats were all grown from seed in greenhouses (59).

For each acquisition access period (AAP), the sanitary status of source plants was confirmed by symptom observation and PCR as detailed in (4).

Three experimental conditions were set up. Fifth instar healthy nymphs were separately fed on i) healthy daisies and broad beans (Eva_H), ii) CYp-infected daisies (Eva_CY) or iii) FDp-infected...
broad beans (Eva_FD) for an AAP of 7 days and then transferred on oat for a 28-day latency period (LP). At 35 days post acquisition survived insects were sexed, analyzed for phytoplasma infection and used.

**RNA extraction.** Total RNA was extracted from 64 samples (each made of 5 insects), nearly 20 for each experimental condition (10 female and 10 male) using Direct-zol RNA Mini Prep Kit (Zymo Research). RNA was analyzed in a Nanodrop spectrophotometer and in a Bioanalyzer 2100 Expert Agilent Technologies, to evaluate concentration, purity and quality of the samples.

**Phytoplasma detection and quantification.** Total RNA was treated with Turbo RNase-free DNase I (Applied Biosystems). For CYp and FDp diagnosis, cDNA was synthesized from total RNA (800 ng) using High Capacity cDNA reverse transcription kit (Applied Biosystems). Two µl of cDNA were used as template in qPCR with iTaq Universal Probes Supermix (Bio-Rad) and primers CYS2Fw/Rv and TaqMan CYS2Probe (4). The same primers and probe, targeting phytoplasma 16SrRNA, and primers MqFw/Rv with TaqMan MqProbe, targeting insect 18SrRNA (4) were used to quantify phytoplasma load. Four serial 100-fold dilutions of p-GemTEasy (Promega) plasmids, harboring portions of ribosomal genes from phytoplasma and insect, were included to calculate phytoplasma16S/insect18S ratio.

**RNA-seq, differential gene expression and sequence analysis.** Six micrograms of RNA extracted from insects fed on phytoplasma-infected plants (Eva_CY; Eva_FD) and showing similar phytoplasma amount were sent to Macrogen (South Korea) for cDNA libraries construction and sequencing, as detailed in (59). Each library was obtained from a pooled sample of 20 males and 20 females. To generate a comprehensive landscape of the *E. variegatus* transcriptome, the datasets generated by the cDNA libraries (two biological replicates for each condition) were pooled, trimmed by Trimmomatic v0.32 (60), quality checked by FastQC v0.9(61), *de novo* assembled using Trinity v2.0.6 (62) and clustered by cd-hit-est (63) with a sequence identity cut-off = 0.98. Each transcript was analyzed by blastx against the NCBI nr database with a cut-off Expected value of 1e-04. Only transcripts with arthropods as best top hits species were retained for further analysis.
Functional annotation for each of the selected transcripts was obtained by loading the corresponding XML output files in Blast2GO and running the mapping and the annotation options with default parameters to retrieve GO terms and assign reliable functions, respectively. In addition, sequences were analyzed for orthology predictions with eggnog (64) with DIAMOND mapping mode. Open reading frames (ORF) were predicted by TransDecoder (65) using “--single_best_orf” and “--retain_pfam_hits” options, which allow to retain only the single best ORF for each transcript according to the presence of a significant Pfam hit.

Reads were loaded to NCBI's Sequence Read Archive (SRA) database with the following accession numbers: SRR5816888, SRR5816889, SRR5816890, SRR5816891.

For differentially expressed gene (DEG) identification, DESeq2 package (66) v. 1.14.1 was run on a 60 core and 256 GB RAM local machine, running Ubuntu server 12.04 LTS. DEG selection was based on an adjusted p-value ≤ 0.01 and a log2FC (Fold Change) ≥ 0.5 for up-regulated genes and ≤ -0.5 for down-regulated genes.

SignalP 4.1 (67), Prosite (68) and GlycoEP (69) were used to predict putative signal peptide, active and glycosylation sites on cathepsin L isoforms, respectively. KEGG pathway database was used for Fig. 1B preparation (70).

**qPCR validation.**

Some genes were selected from the RNAseq results and literature search and analyzed by RT-qPCR in CYP-, FDp-infected and healthy insects (Eva_H, _CY, _FD). Reverse transcriptase reactions were performed on the RNA extracted from 42 samples (each made up of five pooled insects): seven samples of males and seven of females for each of the three conditions. These samples included those used for library construction as well as new ones. Complementary DNA was used as template for qPCR with primers in Table S9 and iTaq Universal SYBR Green Supermix (Bio-Rad) with an annealing/extension temperature of 60°C. Primers were checked to target unique isoform in the whole *E. variegatus* transcriptome. Among the six putative reference genes tested, the insect elongation factor-1α, glutathione S-transferase and heat shock protein 70-1 were selected as the
most stable under the three conditions (Eva_H, Eva_FD and Eva_CY) (Table S10) and used for qPCR gene expression analysis, according to (71). Normalized relative quantities for each condition were compared.

**Phenoloxidase activity.** The enzymatic activity of naturally activated phenoloxidase (PO) and proenzyme prophenoloxidase (ProPO) were measured in plasma and haemocyte lysate supernatant (HLS) as described (35, 36). About 5 haemolymph samples were tested for each sex and condition. The optical density (OD) at 490 nm was determined immediately, after 30 min and then every hour for 15 h using a Bio-Rad Microplate Spectrophotometer. One unit of activity was defined as a change of 0.001 OD490nm per minute in the linear phase of reaction. Specificity was tested using phenylthiourea (Sigma, 4 mg/ml) to inhibit enzyme activity.

**Pigmentation assay.** The pigmentation of forewing and body (dorsal side) devoid of appendices was calculated through image analysis for about 50 insects for each condition. Images were taken under a stereomicroscope with a D5000 Nikon controlled by Camera Control Pro 2 software and analysed with Fiji software (72). The outline of the object to be measured was marked by the freehand selection tool. Light conditions, camera and software setting were not changed throughout image acquisition of the whole set of samples. Nevertheless, measure of each object was normalized against a white area used as internal standard. Mean degree of grey intensity was expressed as a numerical reading ranging from 0 for black to 255 for white.

**Immunocompetence assay.** A nylon thread (length 2-4 mm, Ø 80 μm) was implanted in abdomen of CO2 anaesthetized insects under a stereoscope. About 50 insects were treated for each condition. Insects were transferred to oat for 72 h, collected and dissected to recover the nylon thread in 900 μl 10% PBS. Following overnight fixation at 4°C (4% paraformaldehyde, 0.1% Triton X100 in 10% PBS), the threads were washed, DAPI stained and photographed under light and UV microscope. Three images were taken at different z axes, to ensure the best count of nuclei of cell adherent to the thread. Image analyses was performed with Fiji software (72), and Melanization Index (MI) was calculated as the ratio between the integrated density per surface unit of nylon portions inside and
outside the body of each insect. About 15 insects were analysed for each condition. Number of adherent cells were calculated by summing the DAPI stained nuclei in the three pictures of each thread.

**Western blots.** For each category, proteins were extracted from four samples (each made up of five pooled insects), quantified by Bradford reagent (Bio-Rad), and load on 12% polyacrylamide gels (12 µg/lane), together with pre-stained and unstained broad range standards (Bio-Rad) (11). Gels were either stained with colloidal Coomassie or blotted on PVDF membrane. Membranes were blocked for 1 h (3% BSA in TBS 0.1% Tween, BSA-TBST), incubated overnight at 4°C with primary antibodies (ab50567 rat-developed anti-tropomyosin, and ab200738 rabbit-developed anti-cathepsin L, Abcam plc) both diluted 1:1000 in BSA-TBST, washed, incubated 2 h with corresponding horseradish peroxidase conjugated secondary antibodies (A4416 GAM-HRP, and A0545 GAR-HRP, Sigma, respectively) both diluted 1:10000 in BSA-TBST, washed and developed with West Pico SuperSignal chemiluminescent substrate (Pierce) in a VersaDoc 4000 MP (Bio-Rad). Each experiment was repeated three times.

**Movement and respiration assays.** To evaluate insect movement among the three conditions, insects were anaesthetized for 30 sec and put one at a time in the middle of two concentric circles (Ø 2 and 6 cm) drawn on a paper, covered with a glass cylinder (height 20 cm) and continuously observed for 5 min. Time required to leave the two circles and numbers of jumps were recorded. About 20 insects were tested for each sex and condition.

To evaluate insect respiration, CO₂ production was monitored within the standard “broad leaf chamber” of a LCpro+ (ADC BioScientific) gas analyzer, as described for *Drosophila* (73). To measure gas exchange, groups of three adults (same sex and same category) were put in a mini-cage (1.5 ml tube, deprived of bottom and sealed with net). To allow better survival, 200 µl of feeding solution (12) were put in the mini-cage cap and covered by a parafilm layer. For each sex and category, 4 groups were analysed. Each mini-cage was left 30 min in the chamber before measure
(5 reading replicates). The CO$_2$ production, expressed by the analyzer in $\mu$mol/sec m$^2$, was transformed in $\mu$l/h per insect according with (73).

Statistical analyses. Depending on normal or not normal distribution of data, t-test or Mann-Whitney test were used for sex comparison, ANOVA or Kruskal-Wallis for category comparison (H vs. CY vs. FD) (Table S11). Tukey or Dunn post-hoc tests were used following ANOVA or Kruskal-Wallis, respectively. Whenever no sex-related differences were recorded within the same category, female and male data were pooled. SIGMAPLOT 11 (Systat Software) was used.
Contributions of authors

Design of experiments: LG SA CM MR MV DB; RNAseq, bioinformatic analysis and differential gene expression profiling: SA; RT-qPCR validation, statistical analysis and protein expression: LG;
phenoloxidase enzymatic activity: LG CM MPes; pigmentation assay: MR MV SA;
immunocompetence assay: CM NAB MPD; movement assay: SA MR MV MPes LG; respiration assay: LG WC; insect rearing and plant production: MPeg. LG SA MR MV CM wrote the paper and all authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

ACKNOWLEDGMENTS

The Authors thank Brigitte Batailler for helping with immunocompetence assay, Francesco Pennacchio and Gennaro Di Prisco, University of Naples Federico II, for helpful discussion and suggestions.

This work was part of the ‘FitoDigIt’ Project funded by Fondazione Cassa di Risparmio di Torino, Torino (Italy), within the ‘Richieste Ordinarie 2014’ and ‘Richieste Ordinarie 2015’ calls. MR and MPeg were supported by a fellowship funded by the following grant-making foundations:

Fondazione Cassa di Risparmio di Cuneo, Fondazione Cassa di Risparmio di Torino, and Fondazione Cassa di Risparmio di Asti in the framework of the INTEFLAVI project. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES


<table>
<thead>
<tr>
<th>Contig accession</th>
<th>TPM (Average TPM)</th>
<th>Euc_CV</th>
<th>Euc_FD</th>
<th>Log-2 FC</th>
<th>P value</th>
<th>Sequence description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFTU000000001</td>
<td>99.0±0.8</td>
<td>36.7±0.5</td>
<td>-1.802</td>
<td>2E-14</td>
<td></td>
<td>PREDICTED: copia BS-like</td>
</tr>
<tr>
<td>GFTU000000002</td>
<td>61.8±0.3</td>
<td>36.7±0.5</td>
<td>-2E-23</td>
<td></td>
<td></td>
<td>hypothetical protein A57531 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000003</td>
<td>54.8±0.8</td>
<td>22.0±0.8</td>
<td>-1E-21</td>
<td>2E-22</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000004</td>
<td>35.8±0.8</td>
<td>22.0±0.8</td>
<td>-2E-22</td>
<td>9E-15</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000005</td>
<td>35.8±0.8</td>
<td>22.0±0.8</td>
<td>-2E-22</td>
<td>9E-15</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000006</td>
<td>35.8±0.8</td>
<td>22.0±0.8</td>
<td>-2E-22</td>
<td>9E-15</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000007</td>
<td>35.8±0.8</td>
<td>22.0±0.8</td>
<td>-2E-22</td>
<td>9E-15</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000008</td>
<td>35.8±0.8</td>
<td>22.0±0.8</td>
<td>-2E-22</td>
<td>9E-15</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
<tr>
<td>GFTU000000009</td>
<td>35.8±0.8</td>
<td>22.0±0.8</td>
<td>-2E-22</td>
<td>9E-15</td>
<td></td>
<td>PREDICTED: extracellular protein A55421 (Protein Disulfide Isomerase (PDI) family, redox active TRX domain)</td>
</tr>
</tbody>
</table>

**Table 1. Overview of Euscelidius variegatus transcripts up-regulated during chrysanthemum yellows (CYp) infection, compared with insects infected by Flavescence dorée (FDp) phytoplasmas.** Transcripts were classified into functional categories according to the putative identification assigned by a blastx search.
32
<table>
<thead>
<tr>
<th>Contig accession</th>
<th>FPKM (Average±SD)</th>
<th>Log2 Fold change</th>
<th>P value</th>
<th>Sequence description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detoxification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFTU01009741.1</td>
<td>23.5±0.6</td>
<td>13.8±0.3</td>
<td>2E-14</td>
<td>PREDICTED: venom carboxylesterase-6</td>
</tr>
<tr>
<td>GFTU01002689.1</td>
<td>13.1±4.4</td>
<td>4.9±2.1</td>
<td>3E-03</td>
<td>PREDICTED: cytochrome P450-4C1-like</td>
</tr>
<tr>
<td>GFTU01002688.1</td>
<td>17.8±5.7</td>
<td>6.4±1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFTU01000057.1</td>
<td>7.8±0.4</td>
<td>5.1±0.4</td>
<td>1E-05</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Overview of *Euscelidius variegatus* transcripts down-regulated during chrysanthemum yellows (CYp) infection, compared with insects infected by Flavescence dorée (FDp) phytoplasmas. Transcripts were classified into functional categories according to the putative identification assigned by a blastx search.

* Contig accessions correspond to BioProject PRJNA393620.
FIGURE LEGENDS

FIG 1 Regulation of prophenoloxidase cascade and muscle contraction pathway. During *Euscelidius variegatus* infection with Flavescence dorée (FDp) or Chrysanthemum yellows (CYp) phytoplasmas, prophenoloxidase cascade, which produces melanine as innate immunity response, is oppositely regulated, being activated in FDp and inhibited in CYp-infected insects (A). The expression of most of genes involved in muscle contraction was altered upon CYp infection (indicated in colored boxes) (B). Scheme was modified from KEGG map 04260: DHPR, dihydropyridine receptor; RyR2, ryanodine receptor; TnC, troponin C; SERCA2a, calcium-transporiting ATPase sarcoplasmic/endoplasmic reticulum.

Heatmap of expression is indicated: red and green correspond to up- and down-regulation level, respectively, during CYp infection compared with infection by FDp.

FIG 2 Phytoplasma infection modulates insect immune response. Gene expression profile indicated as mean normalized relative quantities ± standard error of phenoloxidase (A) and Kazal-type 1 serine protease inhibitor (B) in healthy (H) *Euscelidius variegatus* insects or infected by chrysanthemum yellows (CYp) or Flavescence dorée (FDp) phytoplasmas. Mean enzymatic activities (U) ± standard error of prophenoloxidase (ProPO) measured in plasma (C) and haemocyte lysate (D) fractions of H, CY, FD insects. (E): Box plot of grey intensity calculated for body (dorsal side) of H, CY, FD insects. Grey intensity ranges from 0 (black) to 255 (white). Within the same category (H, CY, FD), asterisks indicate significant differences between female and male of each category. Within the same gender, different letters indicate significant differences among the categories (capitalized for females and small for males). Whenever no sex-related differences were recorded within the same category, female and male data were pooled. (F): Mean melanization index (MI, bars) and mean number of adherent cells (dots) (± standard error) measured on nylon threads implanted into H, CY, FD insects. MI ranges from 0 (black) to 1 (white). Different letters
indicate significant differences among the categories (small for MI and capitalized for adherent cells).

**FIG 3** Chrysanthemum yellows phytoplasma infection increases energy metabolism. Gene expression profile indicated as mean normalized relative quantities ± standard error of myosin light chain (A), tropomyosin (B), arginine kinase (C) and maltase (D) in healthy (H) *Euscelidius variegatus* insects or infected by chrysanthemum yellows (CYp) or by Flavescence dorée (FDp) phytoplasmas. Mean time (seconds) required to leave the 1st circle (bars, Time spent in 1st circle) and mean CO₂ production (dots, Respiration) ± standard error measured in H, CY, FD insects (E). Concentric circles used for movement assay (F) and leaf mini chamber used for respiration assay (G and H). Western blot with anti-tropomyosin antisera (I) and SDS-PAGE for internal loading control (L) of total proteins extracted from H, CY, FD insects. Within the same category (H, CY, FD), asterisks indicate significant differences between female and male of each category. Within the same gender, different letters indicate significant differences among the categories (capitalized for females and small for males). Whenever no sex-related differences were recorded within the same category, female and male data were pooled.

**FIG 4** Phytoplasma infection alters protease regulation. Gene expression profile indicated as mean normalized relative quantities ± standard error of cathepsin L isoforms 92i3 (A), 92i4 (B), 92i6 (C) and 473 (D) in healthy (H) *Euscelidius variegatus* insects or infected by chrysanthemum yellows (CYp) or by Flavescence dorée (FDp) phytoplasmas. Within the same category (H, CY, FD), asterisks indicate significant differences between female and male of each category. Within the same gender, different letters indicate significant differences among the categories (capitalized for females and small for males). Whenever no sex-related differences were recorded within the same category, female and male data were pooled. Alignment of predicted amino acid sequences for cathepsin L isoforms (E): position of signal peptide (grey highlighting), cathepsin propeptide...
inhibitor domain (I29) and mature protease domains are indicated above the alignment; predicted glycosylation sites are indicated as single boxed amino acid (white: N-glycosilation; grey: O-glycosilation); highly conserved regions containing the three cathepsin L-typical consensus sequences are boxed, bold and required conserved amino acids within each consensus sequence are located above the sequences; immunogenic peptide sequence is boxed and aligned below the sequences; three predicted active sites are indicated by black full arrowheads; predicted isoelectric point and molecular weight of pre-proteins and mature forms are indicated at the end of each sequence. Percent identity matrix of the four cathepsin L isoforms and immunogenic peptide (F): values for pre-proteins are indicated bottom left of the matrix, mature forms in italics up right of the matrix. Western blot with anti-cathepsin L antisera (G) and SDS-PAGE for internal loading control (H) of total proteins extracted from H, CY, FD insects.