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Natterin-like and legumain insect gut proteins promote the multiplication of a vector-borne bacterial plant pathogen

Luciana Galetto [a,*], Giulia Lucetti [a], Luca Bucci [a], Francesca Canuto [a], Marika Rossi [a], Simona Abbà [a], Marta Vallino [a], Cecilia Parise [a,b], Sabrina Palmano [a], Marcello Manfredi [c,d], Domenico Bosco [a,b], Cristina Marzachì [a]

[a] Institute for Sustainable Plant Protection, National Research Council (IPSP-CNR), Turin, Italy
[b] Department of Agricultural, Forest and Food Sciences (DISAFA), University of Turin, Grugliasco, TO, Italy
[c] Dipartimento di Medicina Traslazionale, Università del Piemonte Orientale, Palazzo Bellini, Novara, Italy
[d] Center for Translational Research on Autoimmune and Allergic Diseases, University of Piemonte Orientale, Novara, Italy

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ABSTRACT

Phytoplasmas are phloem-limited plant pathogenic bacteria causing diseases in many plant species. They are transmitted by Hemipteran insect species in a persistent-propagative manner. Phytoplasmas are wall-less, and their membrane proteins are involved in pathogen internalization into host cells. We focused on the immunodominant membrane protein (Imp) of Flavescence dorée phytoplasma (FDp), a grapevine quarantine pest and a major threat to European viticulture. *Scaphoideus titanus* is the main natural vector of FDp to grapevine, whereas *Euscelidius variegatus* is commonly used as laboratory vector. Previous works indicated that recombinant Imp of two FDp strains (FD-C and FD-D) selectively interact with gut proteins from vector species rather than those from non-vectors. Here, similar patterns of interacting insect gut proteins were obtained from both vector species, following pull-down with His-tagged FDp Imps. After identification of several targets, four *S. titanus* and five *E. variegatus* proteins interacting with Imp were further characterized by measuring expression in different insect tissues and in healthy vs. infected insects. Specific RNAi silencing of two of these vector genes, namely natterin and legumain, resulted in a significant reduction of phytoplasma multiplication in insects upon pathogen acquisition, compared to control insects. Natterin displays a DM9 domain and legumain possesses a signature of G protein receptor, supporting their involvement as FDp Imp receptors. Outcomes of this work are discussed with particular attention devoted to the gain of knowledge on host/pathogen interaction as well as to the potential impact on improvement phytoplasma disease management.

1. Introduction

Phytoplasmas are phloem-limited and uncultivable plant pathogenic Mollicutes that cause diseases in hundreds of plant species, producing severe economic losses to crops worldwide (Marcone, 2014). These plant pathogens are transmitted by Hemipteran insect species, belonging to the suborder Auchenorrhyncha (Fulgoromorpha and Cicadomorpha) and the family Psyllidae (Trivellone and Dietrich, 2021). Once acquired, phytoplasmas must cross the vector midgut lumen, spread in the hemolymph, colonize the salivary glands, and enter the saliva to be transmitted to another plant (Koinuma et al., 2020). These bacteria possess small genomes due to a progressive gene reduction, and strongly depend on hosts to survive since they lack crucial metabolic pathways, probably as a consequence of their endo-parasitic life style (Debonneville et al., 2022). In the absence of a cell wall, phytoplasma membrane proteins are possibly involved in the internalization process of the host cells. Thus, proteins with domains exposed on the outer layer of the phytoplasma membrane are not only subjected to selective pressure, and therefore highly variable, but also good candidates in mediating interactions with plants and insect vectors. Indeed, different types of phytoplasma membrane proteins have been recognized to play an important role in promoting phytoplasma internalization in insect cells (Rossi et al., 2019b).

Flavescence dorée (FD) is an economically important disease of grapevine caused by a phytoplasma (FDp, 16SrV-C and -D), classified as a Union quarantine plant pest present in the EU, unevenly distributed in

* Corresponding author.
E-mail address: luciana.galetto@ipsp.cnr.it (L. Galetto).

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several grapevine growing areas of Europe (EFSA Panel on Plant Health PLH, 2014). In the absence of resistant *Vitis vinifera* genotypes, the impact of FD is dramatic. The phytoplasma is transmitted to grapevine mainly by the Nearctic leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) (Gonella et al., 2024). Other epidemiological cycles involving different vectors and secondary plant hosts contribute to maintaining infection *foci* of this Palearctic phytoplasma (Malembic-Maher et al., 2020). Flavescence dorée management relies on compulsory control of vector population through insecticide applications, roguing of infected plants to reduce inoculum loads and planting of phytoplasma-free grafted cuttings. The lack of basic knowledge on FDp impairs the development of more specific and innovative management strategies. In the absence of routine axenic cultivation protocols, functional studies of FDp genes are conducted on natural and laboratory pathosystems, namely *V. vinifera* infected by *S. titanus* and *Vicia faba* infected by *Euscelidius variegatus* (Caudwell et al., 1972), respectively. Despite the epidemiological complexity of this obligate pathogen, the integrated use of these two infection systems (natural and artificial) has provided some fundamental basic knowledge, especially on the phytoplasma-vector interaction topic. Indeed, FDp persistent propagative transmission is driven by molecular interactions between vector proteins and phytoplasma membrane proteins (Arricau-Bouvery et al., 2023, 2021, 2018; Canuto et al., 2023; Trivellone et al., 2019), and, on the basis of the primary sequence of few pathogen proteins, it is possible to predict the transmissibility of different FDp strains by *S. titanus* (Malembic-Maher et al., 2020). These studies mainly focused on two FDp membrane proteins, namely the variable membrane protein A (VmpA) and the immunodominant membrane protein (Imp). A combinatorial RNAi-based approach was used as functional validation platform to screen 13 *E. variegatus* genes encoding potential interaction partners of VmpA, and to identify an insect protein with Leucin Rich Repeat domains involved in the adhesion of FDp in vector cell cultures (Canuto et al., 2023). Similarly, silencing of insect clathrin heavy chain, interacting with FDp VmpA, reduces endocytosis-mediated internalization of phytoplasma cells within Drosophila S2 phagocytic lines (Arricau-Bouvery et al., 2023). On the other hand, recombinant FDp Imp displays a complex interaction pattern with insect proteins from six Auchenorrhyncha species with variable FDp acquisition/transmission capabilities, spanning from very efficient to non-competent vectors (Trivellone et al., 2019). The vector status correlates with the ability of insects to *in vitro* bind FDp Imp, and this interaction is especially strong with gut membrane proteins of *S. titanus* and *E. variegatus* (Trivellone et al., 2019). The present work aims at identifying the intestinal proteins of *S. titanus* and *E. variegatus* able to bind FDp Imp and validating their possible involvement as determinants of vector specificity, thus supporting the role of insect gut as the first barrier to be overcome to ensure successful transmission of vector-borne parasites (Shaw and Catteruccia, 2018). Since the epidemiology of FDp is complex and involves different phytoplasma genotypes and insect vector species, the identification of genetic traits regulating transmission specificity paves the way to the possible disruption of the disease spread.

2. Materials and methods

2.1. Insects and phytoplasma strains

Oats (*Avena sativa*) and broad beans (*Vicia faba*) from seed as well as potted grapevine cuttings (*Vitis vinifera*) were grown in greenhouse (24 ±2°C) and used to rear healthy colonies of *E. variegatus* (oats) and *S. titanus* (broad beans and grapevines) or as host plants to maintain the FDp isolates (broad beans).

Euscelidius variegatus was originally collected in Piedmont and continuously reared on oats inside plastic and nylon cages in growth chambers at 20–25°C with a L16:D8 photoperiod. *Scaphoideus titanus* has one generation per year and its continuous rearing under controlled conditions is not feasible. To obtain coeval *S. titanus* specimens, two-year-old grapevine canes bearing leafhopper eggs were collected in Veneto (Italy) vineyards during the winter period and kept at 5±1°C. Grapevine branches were caged inside in a glasshouse (at 20–25°C with natural light) with healthy grapevines and broad beans to feed the newly hatched nymphs.

Flavescence dorée phytoplasma strains "FD-C Piedmont" (Galetto et al., 2014) and "FD-D CRA AT" (Galetto et al., 2019) were originally isolated in Piedmont and then routinely maintained on *V. faba* plants with sequential transmission by *E. variegatus* (Galetto et al., 2018). The FD-C and FD-D phytoplasma strains used in this study showed M12 (Map-FD3) and M54 (Map-FD3) profiles, respectively, according to the classification described in (Malembic-Maher et al., 2020).

2.2. Pull-down assays and peptide-mass finger printing

The protocol was optimized from (Rahmeh et al., 2012) with modifications, as detailed in Supplementary Methods. Briefly, histidine-tagged Imp proteins of FD-C (18.2 kDa) and FD-D (18.0 kDa) phytoplasmas (Filippin et al., 2019; Trivellone et al., 2019) or serum albumin (AbCam ab217817, 68.4 kDa) were bound to Ni-NTA agarose beads (Qiagen) and incubated with extracts from homogenized midguts of *E. variegatus* or *S. titanus* (overall 30 insects per species). Insect gut proteins interacting with histidine-tagged baits were eluted from beads and subjected to trypsin digestion and liquid chromatography-mass spectrometry (LC-MS) analysis with a Triple TOF 5600+ (Sciex, Concorde, Canada), according to methods previously described (Albanese et al., 2017). Protein identification was accomplished using the MASCOT database search engine against local *E. variegatus* and *S. titanus* protein databases obtained from TransDecoder-predicted coding regions of TSA transcripts (Transdecoder, 2016). Details of analysis and full results of each replicate are described in Dataset S1. Unbound proteins, washing and elution phases were checked on SDS-PAGE (Fig S1). The experiment was repeated three times for each treatment and each insect species.

2.3. dsRNA production and microinjection

The coding sequences of selected transcripts were predicted by TransDecoder (https://github.com/TransDecoder/TransDecoder) from the TSA sequence databases (BioProject: PRJNA393620 and PRJNA765507) at NCBI under the accession numbers detailed in Dataset S1. Molecules of dsRNAs were obtained as detailed in Supplementary Methods. Briefly, the PCR products driven by primers listed in Table S1 (with T7 promoter sequence at 5') were cloned, sequenced and used as templates for the MEGAscript RNAi Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Newly emerged adults were anesthetized with CO_2 and microinjected between two abdominal segments under a stereomicroscope using a fine glass needle connected to a Cell Tram Oil microinjector (Eppendorf). Adults of *E. variegatus* and *S. titanus* were microinjected with 0.5 μL of dsRNAs at the concentration of 160 ng μL^{-1} (80 ng per insect). Groups of injected insects were then caged on oat plants (*E. variegatus*) or on potted grapevines (*S. titanus*), monitored daily and sampled at defined dpi. Dead insects were removed periodically.

2.4. RNA extraction, cDNA synthesis and gene expression analysis

Total RNAs were extracted from single *E. variegatus* and *S. titanus* adult at different times after dsRNA injection, from 20 samples of healthy or FD-C/FD-D infected *E. variegatus*, and from salivary glands, midguts, ovaries and testes as detailed in Supplementary Methods.

Quantitative RT-PCR (qRT-PCR) was used to quantify the ability of the injected dsRNAs to knockdown target mRNA in insects collected at different sampling dates and to determine expression level of target transcripts in different organs or in healthy vs. infected insects, as detailed in Supplementary Methods.

2.5. Immunofluorescence observation

To confirm reactivity of the commercial antibodies, midguts were excised from *E. variegatus* and *S. titanus* dsGFP-injected adults as detailed above and fixed quickly in 4 % paraformaldehyde, 0.1 M phosphate buffer, pH 7.4, 0.1 % Triton X-100 overnight at 4°C. Organs were washed three times in phosphate-buffered saline, pH 7.4 (PBS), permeabilised at 4°C with PBS and 1 % Triton X-100 for 6 h, washed three times and blocked in PBS containing 1 % BSA for 30 min. Organs were incubated overnight with primary antibodies: *E. variegatus* midguts with anti-legumain (LGMN 101AP rabbit-developed, FabGennix International, diluted 1:10), *S. titanus* midguts with anti-cathepsin L (ab200738 rabbit-developed, Abcam plc, diluted 1:200). Organs were then washed three times with PBS, blocked, incubated for 2 h with a 1:80 dilution of the FITC conjugated antibody (F1262, Sigma-Aldrich), and finally washed three times in PBS. Samples were mounted on microscope glass slides and observed. Control sections were treated in a similar manner but incubated in a rabbit pre-immune serum instead of primary antibody. Labelled midguts were observed under a DM750 (Leica) microscope equipped with a CoolLED pE300white Illumination System, and triple LED cube filter for LED module 365/470/590 nm. Images were acquired with a Leica EC4 camera controlled through the LAS EZ software.

2.6. Western blots

To evaluate the effect of dsRNA treatment on cognate protein expression, Western blots with anti-cathepsin L (ab200738 rabbit-developed, Abcam plc) and anti-legumain (LGMN 101AP rabbit-developed, FabGennix International) were run on total proteins were extracted from single *S. titanus* whole insects (either treated with dsCATH or dsGFP and sampled at 19 dpi) and from midguts dissected from *E. variegatus* (either treated with dsLEG or dsGFP and sampled at 16 dpi), respectively, as detailed in Supplementary Methods.

2.7. Phytoplasma acquisition, diagnosis and quantification

At 14 days after the injection of dsRNAs (targeting either GFP as a control or selected vector genes), insects were allowed to acquire phytoplasma by feeding on infected broad beans for four days, then isolated on healthy plants (oats for *E. variegatus* and grapevine for *S. titanus*) for one week latency and thus collected for molecular analyses at 25 dpi. The load of FDp in infected broad beans were measured just before phytoplasma acquisition by routine DNA extraction and qPCR (Pelletier et al., 2009), and plants showing similar pathogen titer (Dataset S2) were used as source of FDp inoculum.

To verify the effective gene silencing in treated insects (first injected with dsRNAs and then exposed to FDp), total RNAs were extracted, retro-transcribed and analysed as detailed above. Moreover, on the same cDNA samples, a multiplex quantitative qPCR was used to detect phytoplasma presence and measure pathogen load, as detailed in Supplementary Methods. Phytoplasma quantity was expressed as ratio between copy numbers of phytoplasma transcripts/copy numbers of insect GAPDH transcripts, as calculated from serial dilutions of plasmid standard curves harbouring either phytoplasma gene or GAPDH insect gene, included in all qPCR plates.

2.8. Statistical analysis

SigmaPlot version 13 (Systat Software, Inc.) was used for statistical analyses, as detailed in Supplementary Methods. When raw data were not normally distributed, they were natural log-transformed before analysis.

3. Results and discussion

3.1. Insect vector proteins interact with phytoplasma immunodominant membrane proteins

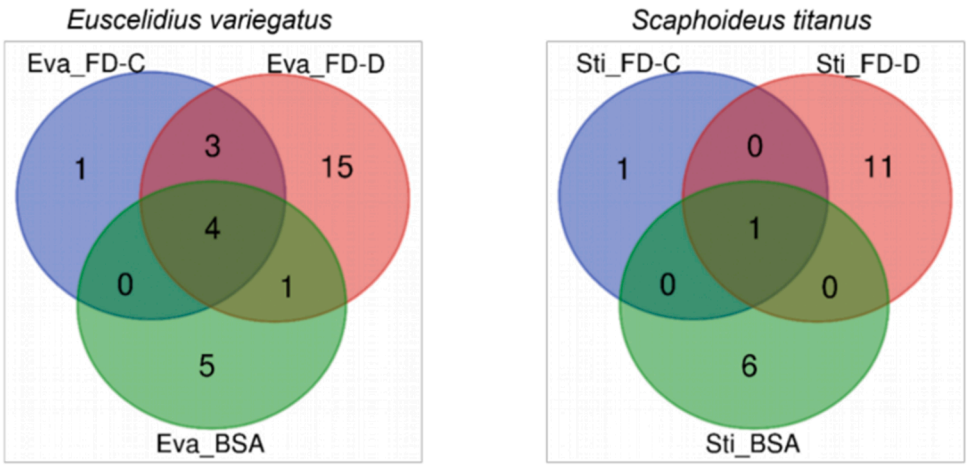
Several proteins extracted from gut of *S. titanus* and *E. variegatus* interacted with FDp recombinant Imp proteins in pull-down assays with Ni^{+}-activated beads (Fig. 1, Dataset S1, Fig S1). Peptide-mass fingerprinting was performed on the elution phases of the three replicates of each treatment with the different histidine-tagged baits (FD-C Imp; FD-D Imp; BSA, negative control) and their interacting gut insect proteins. All detected peptide sequences were matched against the deduced protein database derived from the prediction of candidate coding regions within transcriptomes of both leafhoppers (Dataset S1). For each bait, insect transcripts present in at least two out of three experimental replicates were grouped in Venn's diagrams (Fig. 1). In both insect species, most of the interacting proteins were identified when Imp of strain FD-D was used as bait in pull-down assays, specifically 23 in *E. variegatus* and 12 in *S. titanus*. With a subtractive approach, transcripts present in BSA controls were excluded from further analysis. Entries listed in Fig. 1 were absent in the BSA controls, present in at least two out of three replicates of FD-C and/or FD-D assays and further selected as they showed relevant BlastP hits with significant protein scores in Mascot identification (Dataset S1). Interestingly, Natterin-like proteins were identified in *E. variegatus* (interacting with both FD-D and FD-C Imps) as well as in *S. titanus* (two isoforms, namely 1 and 3, interacting with FD-D Imp only). Cathepsin L and procathepsin L interacted with Imp of FD-D strain in both insect species. Among the three isoforms of legumain-like protein identified in *E. variegatus*, GFTU01008326.1, which showed a transmembrane domain and interacted with both FD-C and FD-D strains, was selected for further analysis. The E3-ubiquitin protein ligase of *E. variegatus* also interacted with phytoplasma Imps of both FD-C and FD-D strains. Few other targets, namely transcription factor kayak, peptidyl-prolyl cis-trans isomerase (*E. variegatus*) and peroxiredoxin-2-like (*S. titanus*), were also selected for further characterization, based on abundance and protein score and/or putative mode of action.

Imp of FD-D phytoplasma strain showed a more complex interactome with gut proteins of both vector species, rather than FD-C Imp. It is noteworthy that FD-C and FD-D phytoplasmas compete during plant and insect colonization (Rossi et al., 2023), and, under field conditions, FD-D is the most prevalent strain (Arnaud et al., 2007; Rossi et al., 2019a). The rich interaction patterns of vector proteins with FD-D Imp may represent one of the molecular explanations of the prevalence of this strain in the field, together with haplotype variability of VmpA and B, other well-known phytoplasma membrane proteins acting as determinants of FDp transmission specificity (Malembic-Maher et al., 2020).

3.2. Selected targets are expressed in insect gut and efficiently silenced by RNAi

To confirm that the identified proteins in pull-down assay were effectively expressed in insect guts and determine whether they were expressed in other organs, the abundance of selected transcripts of *E. variegatus* (five) and *S. titanus* (four) among those interacting in pull-down assays with FD Imp was measured in salivary glands, guts, ovaries, and testes (Fig. 2a). All selected transcripts were expressed in gut, properly supporting the peptide-mass fingerprinting identification that was run indeed on intestinal fresh extracts. Among the analyzed transcripts, natterin 4, legumain and E3-ubiquitin ligase of *E. variegatus* as well as natterin 1, natterin 3 and cathepsin L of *S. titanus* were significantly more expressed in gut than in other organs.

Several dsRNA molecules were designed to silence the selected transcripts (dsNATT4, dsLEG, dsE3UBI, dsKAY and dsISO for *E. variegatus* and dsNATT1, dsCATHL and dsPEROX for *S. titanus*), and they efficiently induced over time gene downregulation after microinjection in both species (Fig. 2b). The transcripts of natterin 4, legumain



E. variegatus transcript ID	Protein identification (BlastP)	Trans-membrane domain (TMHMM 2.0)	Protein presence in 3 replicates (R1 / R2 / R3)					
			FD-C			FD-D		
			R1	R2	R3	R1	R2	R3
GFTU01002968.1	natterin-4-like isoform x1	X	✓	✓	✓	✓	✓	✓
GFTU01008326.1	legumain-like	✓	X	✓	X	✓	✓	✓
GFTU01004003.1	E3 ubiquitin-protein ligase RFWD3-like	X	X	✓	X	X	✓	✓
TR198104_c0_g1_i1	legumain-like	X	X	X	X	✓	✓	✓
TR16114_c1_g1_i1	legumain-like	X	X	X	X	✓	✓	✓
GFTU01009178.1	transcription factor kayak isoform X1	X	X	X	X	✓	✓	✓
GFTU01009823	inactive peptidyl-prolyl cis-trans isomerase	X	X	X	X	✓	X	✓
GFTU01000453.1	cathepsin L	✓	X	X	X	✓	✓	X
GFTU01000454.1	procathepsin L-like	X	X	X	X	X	✓	✓

S. titanus transcript ID	Protein identification (BlastP)	Trans-membrane domain (TMHMM 2.0)	Protein presence in 3 replicates (R1 / R2 / R3)					
			FD-C			FD-D		
			R1	R2	R3	R1	R2	R3
GJQL01127418.1	procathepsin L-like	X	X	X	X	✓	✓	✓
GJQL01144034.1	peroxiredoxin-2-like isoform X1	X	X	X	X	✓	✓	✓
GJQL01050665.1	natterin-1-like	X	X	X	X	✓	✓	✓
GJQL01106150.1	natterin-3-like isoform X1	X	X	X	X	✓	✓	✓
GJQL01088810.1	ATP-dependent RNA helicase pitchoune	X	X	X	X	✓	✓	✓
GJQL01133985.1	glutathione S-transferase-like	X	X	X	X	X	✓	✓
GJQL01068932.1	heat shock 70 kDa protein cognate 4	X	X	X	X	✓	✓	X
GJQL01109497.1	NF-kappa-B essential modulator isoform X3	X	X	X	X	X	✓	✓
GJQL01110349.1	cyclin-K-like isoform X1	✓	X	X	X	✓	✓	X

Fig. 1. Identification of insect proteins interacting with phytoplasma immunodominant membrane proteins. Gut proteins of *Euscelidius variegatus* and *Scaphoideus titanus* interacting in pull-down assays with recombinant immunodominant membrane proteins (Imp) of Flavescence dorée phytoplasma (strains FD-C and FD-D) are presented in Venn diagrams and listed in tables. Venn diagrams include transcripts present in at least two out of three replicates of each treatment. Entries listed in tables are selected as they were absent in BSA controls, showed relevant BlastP hits and produced significant protein scores in Mascot identification (Dataset S1). Proteins selected for further analyses are underlined. Transcript IDs of *E. variegatus* and *S. titanus* can be found in the TSA sequence databases (BioProject: PRJNA393620 and BioProject: PRJNA765507, respectively). Full amino-acid sequences are provided in Dataset S1.

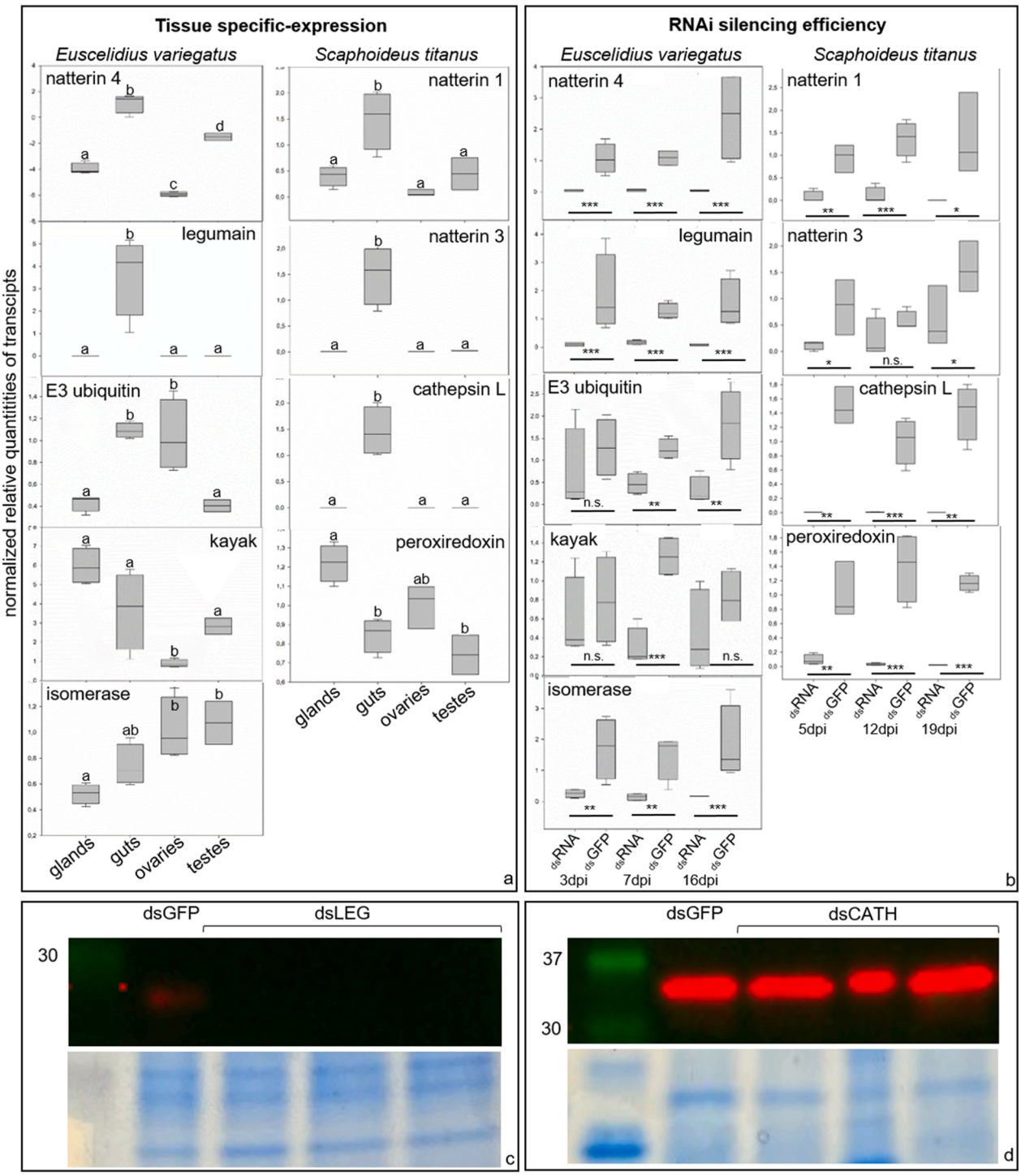


Fig. 2. Characterization of selected insect proteins interacting with phytoplasma immunodominant membrane proteins. Tissue-specific expression of selected transcripts of *Euscelidius variegatus* and *Scaphoideus titanus* among those interacting in pull-down assays with recombinant immunodominant membrane proteins (Imp) of Flavescence dorée phytoplasma measured in salivary glands, guts, ovaries, and testes (panel a). Over time silencing efficiency of microinjected dsRNAs targeting different genes of interest of *E. variegatus* and *S. titanus*, measured as transcript reduction in qPCR (panel b) and as protein abundance in Western blots (panel c and d), in comparison with dsGFP targeting the green fluorescent protein, microinjected as control. Specific dsRNA molecules ($_{ds}$RNA) were designed to silence each target gene, except for *S. titanus* natterin 3 isoform, which was analysed following treatment with dsNATT1. Different letters or asterisks indicate significant differences; n.s.: not significant; dpi: days post injection. Full length images of Western blots are presented in Fig S2. Expected masses of legumain and cathepsin L are 26.7 kDa and 37.7 kDa, respectively.

and peptidyl-prolyl cis-trans isomerase of *E. variegatus* as well as of natterin 1, cathepsin L and peroxiredoxin of *S. titanus* were significantly down-regulated at all the three tested dates after injection of specific dsRNAs, in comparison with corresponding transcripts measured in dsGFP-treated control insects. Overall, dsRNA molecules showed variable silencing efficiencies: most of them were extremely efficient, whereas few others were not. Lack of gene silencing may be due to the sequence and/or structure of dsRNAs, as it has been demonstrated that

morphological features of the molecules may have dramatic and unanticipated effects on RNAi efficiency (Silver et al., 2021). Cleavage of dsRNAs can be affected by their sequence, although the mechanisms that cause the variability in the sensitivity of different portions of a target gene to RNAi are not entirely clear (Silver et al., 2021).

Among the two natterin isoforms of *S. titanus* identified in pull-down assays, we initially focused on natterin 1, as the most similar to that identified among gut proteins of *E. variegatus* (82.3 % identity at amino-acid level). We therefore produced dsRNAs targeting natterin 1, but, in the same dsNATT1-treated *S. titanus* insects, we also monitored the expression of isoform 3, that was indeed reduced, although with lower efficiency (Fig. 2b). The hypothesis of cross-amplification between *S. titanus* natterin isoforms in qPCR should be excluded, as the primers specific for isoform 3 exhibit numerous mismatches on isoform 1. On the other hand, the sequence of dsNATT1 showed a 55.8 % identity with natterin 3, with several stretches of identical consecutive nucleotides. Consistently, a perfect match of at least 16 nucleotides in the central part of the siRNA is required to efficient drive mRNA recognition and cleavage (Du, 2005), and identical stretches of 19 nt could lead to non-target effects in Drosophila (Kulkarni et al., 2006). Thus, it is reasonable that dsNATT1 can silence both *S. titanus* isoforms.

Immunolabelling of guts dissected from dsGFP-treated insects confirmed the specific reactivity of commercial antibodies raised against legumain and cathepsin L (Fig S2). The former antibody used in Western blots confirmed the effective gene silencing of legumain at gut protein level at 16 dpi (Fig. 2c). On the other hand, the abundance of cathepsin L

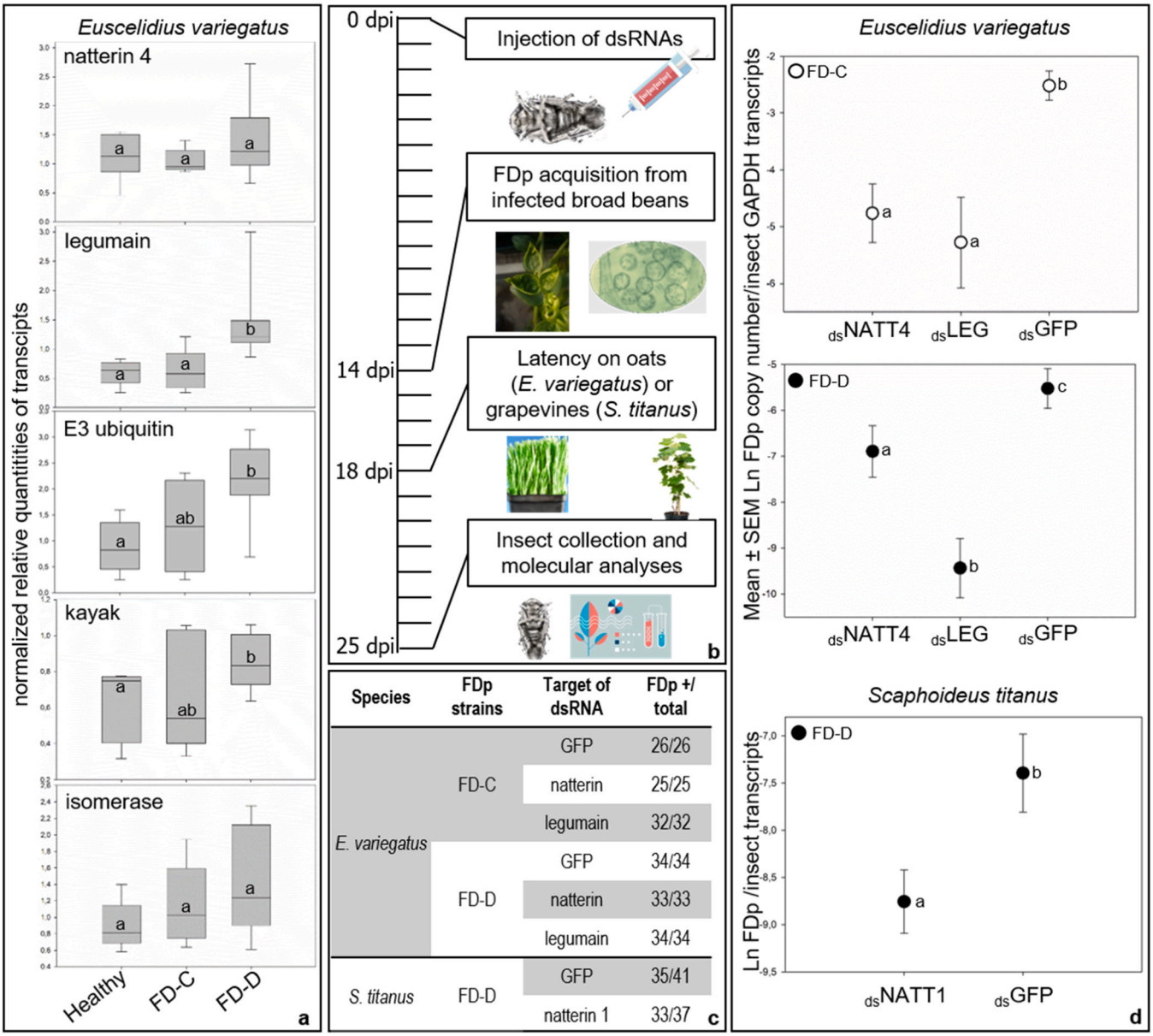


Fig. 3. ***In vivo*** **validation of interactions between Flavescence dorée (FD) phytoplasma and the selected insect proteins.** Normalized expression of selected transcripts of *Euscelidius variegatus* measured in insects healthy or infected by FD-C or FD-D phytoplasma strains (panel a). Diagram of the experimental procedure used to assess the silencing effect of selected targets on the ability of dsRNA-treated insects to acquire phytoplasmas (panel b). Frequency of phytoplasma acquisition (FD-C or FD-D strains) by *E. variegatus* and *Scaphoideus titanus* insects after silencing of natterin and legumain (panel c). Mean transcript amount of FD-C (white dots) or FD-D (black dots) phytoplasma strains ± standard error of the mean (SEM), measured in insects sampled at 25 days post injection of dsRNAs targeting natterin (dsNATT4 or dsNATT1), legumain (dsLEG) or green fluorescent protein (dsGFP) (panel d). The graphs in panel d include data from *E. variegatus* females only and from *S. titanus* males/females pooled together. Phytoplasma quantity was expressed as natural logarithm of ratio between copy numbers of phytoplasma transcripts/ copy numbers of insect GAPDH transcripts. Different letters indicate significant differences; dpi: days post injection.

protein was not affected by the injection of cognate dsRNA, at least at the tested date (19 dpi) (Fig. 2d). Although the dsRNA-mediated gene silencing of *S. titanus* cathepsin L is indisputable at transcript level, the reduction of the corresponding protein was not detectable. Such discrepancy between transcript and protein levels could be explained by the several isoforms of cathepsin L expressed in leafhoppers (Galetto et al., 2018, p. 2) presumably recognized by the same antisera. Moreover, this protein is essential for cell viability, as it is involved in several biological processes (*i.e.* development, apoptosis, and immunity of arthropods (Saikhedkar et al., 2015; Waniek et al., 2012)), and, as such, known to be subjected to fine post-translational regulation mechanisms that modify its dynamics (Katunuma, 2010).

3.3. Phytoplasma infection upregulates three selected E. variegatus genes

To explore the role of selected insect genes in response to phytoplasma infection, their expression was measured in FD-C and FD-D infectious *E. variegatus* insects (sex-balanced pooled samples), collected at the peak of infectivity (28 days post acquisition), and compared with level of corresponding transcripts measured in coeval healthy insects (Fig. 3a). Interestingly, a general trend of overexpression upon phytoplasma infection was observed for most of the analysed genes, and particularly legumain, E3-ubiquitin ligase and transcription factor kayak resulted significantly more expressed in FD-D infected than in healthy insects, confirming their putative role during phytoplasma infection of insect vectors.

3.4. Silencing of natterin and legumain reduces multiplication of acquired phytoplasmas

To evaluate the effects of silencing of selected genes on phytoplasma acquisition, we focused on natterin and legumain of *E. variegatus* (with FD-C and FD-D phytoplasma strains), and on natterin of *S. titanus* (with FD-D strain only, due to the very low level of interaction between FD-C Imp and *S. titanus* proteins observed in the pull-down assay). The selection of these targets and the choice of the FDp strains derived from the combinatorial analysis of the results obtained in the multiple experiments performed to determine i) vector/pathogen interactions by pull-down assays, ii) silencing efficiency, iii) survival upon dsRNA administration (Fig S3), and iv) tissue-specific expression. In the absence of effective and consistent gene silencing (Fig. 2b), transcription factor kayak and E3-ubiquitin ligase of *E. variegatus* were excluded from experiments on the efficiency of FD phytoplasma acquisition. Cathepsin L of *S. titanus* was also excluded, as no protein reduction was observed after dsRNA injection (Fig. 2d), despite the strong reduction observed at transcript level (Fig. 2b). Finally, peptidyl-prolyl cis-trans isomerase of *E. variegatus* and peroxiredoxin of *S. titanus* were also excluded, as their silencing induced a significant mortality rate (Fig S3), a factor incompatible with the long timeframe required by phytoplasmas acquisition assays.

The methodological design, depicted in Fig. 3b, consisted in i) dsRNA injection to silence the identified and selected insect genes, ii) subsequent phytoplasma acquisition (FD-C or FD-D) by feeding on infected broad bean plants with comparable phytoplasma loads measured by qPCR (Dataset S2), iii) latent phase on healthy oat (*E. variegatus*) or grapevine (*S. titanus*) plants and iv) final insect sampling to verify the effective RNAi silencing and quantitatively detect the acquired phytoplasmas.

As expected, both natterin 4 and legumain genes were significantly silenced in insects treated with the corresponding dsRNA molecules in comparison with dsGFP-treated controls (Fig S4). Insects injected with either dsRNAs or dsGFP successfully acquired the respective FD-C or FD-D phytoplasmas during feeding on infected plants. Indeed, all *E. variegatus* and over 80 % of *S. titanus* analyzed insects tested positive in RT-qPCR for the presence of the expected phytoplasma strain (Fig. 3c). Irrespective of the treatment and the FDp strain, *E. variegatus* males showed a significant higher phytoplasma amount than corresponding females subjected to the same treatment (Fig S5, Dataset S2). Interestingly, *E. variegatus* treated females showed a significant lower amount of both FD-C and FD-D phytoplasma strains in comparison with dsGFP control females (Fig. 3d, Dataset S2). On the other hand, in the case of *S. titanus* no gender effect in phytoplasma quantity was observed and therefore male/female samples were pooled and analysed together (Fig. 3d). Also here, the effective silencing of natterin 1 and 3 (Fig S4) determined a significant lower amount of FD-D pathogen strain than in the dsGFP control insects (Fig. 3d, Dataset S2).

A gender effect has been described in several associations of phytoplasma/leafhopper vector species, but not always (Beanland et al., 1999). In line with *E. variegatus* males harbouring higher pathogen titre, in the case of *Macrosteles quadrilineatus* (vector of aster yellows phytoplasma) male leafhoppers acquire phytoplasma at a greater rate than females, despite females are then able to transmit the pathogen with a higher efficiency (Beanland et al., 1999). Moreover, *M. quadrilineatus* males or females differently behave in the attraction phenotype mediated by the phytoplasma effector SAP54 expressed in transgenic *Arabidopsis thaliana* leaves (Orlovskis et al., 2024). There may be behavioural, physiological and molecular differences between male and female leafhoppers that would contribute to the unequal phytoplasma multiplication by gender, that should however be assessed in a case-by-case manner on a given pathogen/vector association.

3.5. Sequences of natterin and legumain possess features of protein/protein interaction and pathogen recognition

Peptide-mass fingerprinting analyses identified five peptides along the deduced amino-acid sequences of natterin 4 from *E. variegatus* and both isoforms from *S. titanus* (identified peptides are underlined in Fig. 4a). Natterins were firstly described as major toxins in the spine venom of the fish *Thalassophryne nattereri*, consisting of an N-terminal lectin domain for mannobiose (DM9 domain) and a C-terminal aerolysin-like domain (Hatakeyama and Unno, 2023). The three natterin sequences of FDp vectors all showed the presence of a DM9 domain overlapping with a DUF3421 domain, which is functionally uncharacterized (Fig. 4a). Interestingly, proteins displaying DM9 domains act as pattern recognition receptors (Li et al., 2024) to recognize pathogen-associated molecular patterns and participate in the immune responses in arthropods (Liu et al., 2023). Moreover, a protein present in *Anopheles* spp. with two 'natterin-like' domains acts as a receptor in the recognition of the transmitted parasite *Plasmodium falciparum*. The Pfs47 protein of *P. falciparum* is recognized by the P47Rec (natterin-like) receptor protein present in the gut of vector dipterans and, when P47Rec is silenced, parasite infection in mosquitoes is reduced (Molina-Cruz et al., 2020). *P. falciparum* has been shown to evade insect immunity by disrupting apoptosis of colonized gut cells (Ramphul et al., 2015). This mechanism is mediated by the anti-caspase activity of the mosquito heat shock protein 70 cognate 3, which interacts with the natterin-like P47Rec (Alves E Silva et al., 2023). Thus, the recognition Pfs47 by its natterin receptor favors plasmodium evasion of early immune responses and accelerates the pathogen dynamics, through the activation of the Hsc70–3 anti-apoptotic pathway. It is worth mentioning that the heat shock protein 70 cognate 4 was identified in the FDp Imp interaction complex,of *S. titanus* (Fig. 1). Remarkably, Pfs47 is a polymorphic protein with multiple haplotypes clustered in a striking geographic population structure and the specific haplotype present in a parasite is a major determinant of its compatibility with a given anopheline vector (Molina-Cruz et al., 2015). Analogously in our system, the immunodominant membrane protein Imp has high genetic variability among phytoplasmas, being under strong positive selection pressure (Kakizawa et al., 2009; Siampour et al., 2013), and is a possible determinant of transmission specificity in different combination of insect vectors/phytoplasma strains (Ding et al., 2022; Siampour et al., 2011; Trivellone et al., 2019). Evidence gathered in the present work therefore

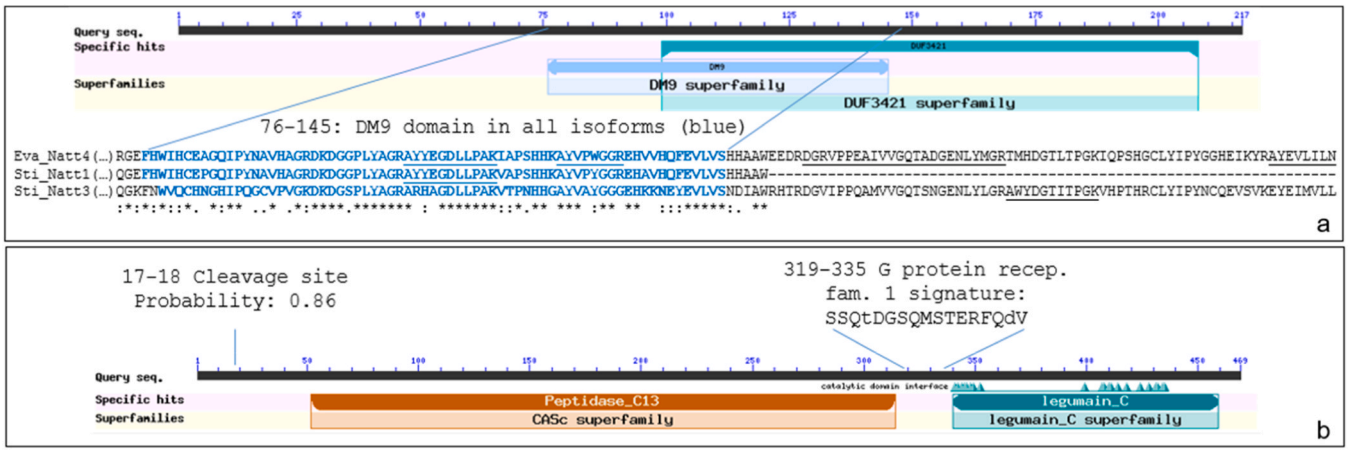


Fig. 4. Descriptive analysis of vector natterin and legumain sequences. Prediction of putative conserved domains along the deduced amino-acid sequences of *Euscelidius variegatus* natterin 4 (**a**) and legumain (**b**). In panel **a**, DM9 domains are indicated in blue in the alignments of partial natterin sequences of *E. variegatus* (Eva_Natt4) and *Scaphoideus titanus* (Sti_Natt1 and Sti_Natt3), whereas peptides identified in mass spectrometry are underlined. Full-length alignment of these sequences is shown in Fig S6.

suggests that natterin localized in gut tissues of *E. variegatus* and *S. titanus* vector species could play a receptor role in the interaction with FDp Imp.

The deduced amino-acid sequence of legumain from *E. variegatus* showed the predicted presence of two putative conserved domains, namely Peptidase C13 (CASc superfamily) and the C-terminal prodomain of legumain (Fig. 4b). Moreover, a cleavage site between position 17 and 18 and a signature of G-protein coupled receptors family 1 at the C-terminal were predicted with Signal P and Prosite, respectively. Consistently with the predicted presence of two peptidase domains and a signal peptide in *E. variegatus* sequence, legumain is an asparaginyl endopeptidase, usually found in endolysosomal system, but also retrieved extracellularly as a secreted protein. It was first identified in plants and more recently characterized in a large variety of metazoans, where it is associated with food digestion under acidic conditions (Fuzita et al., 2015). In addition to its proteolytic activity that leads to the degradation or activation of different substrates, legumain has also been shown to have a nonproteolytic ligase function Rho GTPase (Pan et al., 2022), in line with the presence of the signature of G-protein coupled receptors predicted in *E. variegatus* legumain. In mammals, this protein contributes to antigen processing for class II MHC presentation and plays important roles in the immune system, but its functions at "non-canonical" compartments are poorly defined and often related to pathological situations, such as cancer or Alzheimer's disease (Dall and Brandstetter, 2016; Solberg et al., 2022). More specifically in arthropods, it seems that this gut enzyme is important to animals which have liquid/liquefied diets (Fuzita et al., 2015), such as ticks (Fernando and Fischer, 2020; Hartmann et al., 2018) and some mites (Santamaría et al., 2012; Schicht et al., 2013), and it also plays an important role in yolk accumulation and ovarian maturation of oriental river prawn *Macrobrachium nipponense* females (Jiang et al., 2022). In the plant-sap feeding mite *Tetranychus urticae*, the anomalous high number of retrieved legumain genes (19 instead of the common 1–3 copies) has been possibly correlated with the extremely polyphagous behaviour of this pest: legumains, together with other abundant peptidases, may act as a first barrier against ingested plant defensive proteins (Santamaría et al., 2015). Intriguingly, in most insect orders (including Diptera, Coleoptera, Hymenoptera and Lepidoptera) legumain gene is missing, with the exception of hemipterans (Fuzita et al., 2015). Several lines of evidence, namely i) the *in vitro* interaction between intestinal *E. variegatus* legumain and FD-Imp, ii) the overexpression of this gene in infected insects and iii) the reduced phytoplasma multiplication in silenced insects, indicate that this protein plays a crucial role in FDp transmission mechanism. Beside the direct interaction with FD-Imp, it cannot be ruled out that the reduced phytoplasma level could be also due to a reduced feeding ability in insects in which this peptidase was silenced. A reduced digestive capacity in insects feeding on infected plants could impair their feeding capacity and thus the amount of phytoplasma acquired.

4. Conclusions

Several lines of evidence support the crucial role of the immunodominant membrane protein Imp of FD phytoplasma as one of the molecular determinants of transmission specificity. Here, the gut insect proteins natterin and legumain were identified as putative receptors/interactors of FD Imp. The prominent role of insect natterin was particularly confirmed by the fact that its identification and the functional biologic validation by RNAi occurred in the two different vector species. Moreover, the specific silencing of *E. variegatus* legumain impaired the multiplication of both pathogen strains, suggesting a possible role for this protein common to a broad range of pathosystems. Although hypothesized on the basis of conserved domains, the subcellular localization of natterin and legumain as well as their role in mediating the infection process by the pathogen will need to be further investigated. On the other hand, this work provides a comprehensive list of FD Imp-interacting partners (Dataset S1), which have been partially or not at all investigated here, but which represent an important wealth of knowledge and a starting point for future lines of research.

As control of phytoplasma diseases is based on insecticides against vectors, this work elucidates key stages of FDp transmission specificity and vector infection mechanisms, which might be targeted to disrupt the epidemiological cycle, providing tools to integrate chemical treatments for more sustainable pest control. Moreover, basic knowledge gained here could be valuable for broader research community, as the identified targets are conserved among arthropods and have prominent and crucial roles in host/pathogen interaction.

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CRediT authorship contribution statement

Luca Bucci: Validation, Methodology, Investigation. **Francesca Canuto:** Writing – review & editing, Investigation. **Marika Rossi:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Simona Abbà:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Marta Vallino:** Writing – review & editing, Methodology, Investigation. **Cecilia Parise:** Visualization, Investigation. **Sabrina Palmano:** Writing – review & editing, Methodology, Conceptualization. **Marcello Manfredi:** Investigation, Formal analysis, Data curation. **Domenico Bosco:** Writing – original draft, Resources, Project administration, Funding acquisition. **Cristina Marzachì:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Luciana Galetto:** Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giulia Lucetti:** Validation, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127984.

Data availability

Data will be made available on request.

References

Albanese, P., Melero, R., Engel, B.D., Grinzato, A., Berto, P., Manfredi, M., Chiodoni, A., Vargas, J., Sorzano, C.Ó.S., Marengo, E., Saracco, G., Zanotti, G., Carazo, J.-M., Pagliano, C., 2017. Pea PSII-LHCII supercomplexes form pairs by making connections across the stromal gap. Sci. Rep. 7, 10067. https://doi.org/10.1038/s41598-017-10700-8.

Alves E Silva, T.L., Canepa, G.E., Sweeney, B., Hessab Alvarenga, P., Zhao, M., Vega-Rodríguez, J., Molina-Cruz, A., Barillas-Mury, C., 2023. The heat shock protein Hsc70-3 mediates an anti-apoptotic response critical for *Plasmodium* evasion of *Anopheles gambiae* immunity. Microbiol Spectr. 11, e00940-23. https://doi.org/10.1128/spectrum.00940-23.

Arnaud, G., Malembic-Maher, S., Salar, P., Bonnet, P., Maixner, M., Marcone, C., Boudon-Padieu, E., Foissac, X., 2007. Multilocus sequence typing confirms the close genetic interrelatedness of three distinct Flavescence dorée phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. Appl. Environ. Microbiol 73, 4001–4010. https://doi.org/10.1128/AEM.02323-06.

Arricau-Bouvery, N., Dubrana, M.-P., Canuto, F., Duret, S., Brocard, L., Claverol, S., Malembic-Maher, S., Foissac, X., 2023. Flavescence dorée phytoplasma enters insect cells by a clathrin-mediated endocytosis allowing infection of its insect vector. Sci. Rep. 13, 2211. https://doi.org/10.1038/s41598-023-29341-1.

Arricau-Bouvery, N., Duret, S., Dubrana, M.-P., Batailler, B., Desqué, D., Béven, L., Danet, J.-L., Monticone, M., Bosco, D., Malembic-Maher, S., Foissac, X., 2018. Variable membrane protein A of flavescence dorée phytoplasma binds the midgut perimicrovillar membrane of *Euscelidius variegatus* and promotes adhesion to its epithelial cells. Appl. Environ. Microbiol. 84, 8–17. https://doi.org/10.1128/AEM.02487-17.

Arricau-Bouvery, N., Duret, S., Dubrana, M.-P., Desqué, D., Eveillard, S., Brocard, L., Malembic-Maher, S., Foissac, X., 2021. Interactions between the flavescence dorée phytoplasma and its insect vector indicate lectin-type adhesion mediated by the adhesin VmpA. Sci. Rep. 11, 11222. https://doi.org/10.1038/s41598-021-90809-z.

Beanland, L., Hoy, C.W., Miller, S.A., Nault, L.R., 1999. Leafhopper (Homoptera: Cicadellidae) Transmission of Aster Yellows Phytoplasma: Does Gender Matter?: Fig. 1. Environ. Entomol. 28, 1101–1106. https://doi.org/10.1093/ee/28.6.1101.

Canuto, F., Duret, S., Dubrana, M.-P., Claverol, S., Malembic-Maher, S., Foissac, X., Arricau-Bouvery, N., 2023. A knockdown gene approach identifies an insect vector membrane protein with Leucin Rich Repeats as one of the receptors for the VmpA adhesin of flavescence dorée phytoplasma. Front. Cell. Infect. Microbiol. 13, 1289100. https://doi.org/10.3389/fcimb.2023.1289100.

Caudwell, A., Kuszala, C., Larrue, J., Bachelier, J., 1972. Transmission de la Flavescence dorée de la fève à la fève par des cicadelles des genres *Euscelis* et *Euscelidius*. Ann. Phytopathol. No. hors S. érie 181–189.

Dall, E., Brandstetter, H., 2016. Structure and function of legumain in health and disease. Biochimie 122, 126–150. https://doi.org/10.1016/j.biochi.2015.09.022.

Debonneville, C., Mandelli, L., Brodard, J., Groux, R., Roquis, D., Schumpp, O., 2022. The complete genome of the "Flavescence dorée" phytoplasma reveals characteristics of low genome plasticity. Biology 11, 953. https://doi.org/10.3390/biology11070953.

Ding, L., Lu, W., Yang, Y., Zhong, Q., Zhou, T., Wang, G., Song, C., Ma, W., Chen, W., Wu, Y., 2022. Immunodominant membrane protein (Imp) promotes the transmission of wheat blue dwarf (WBD) phytoplasma by directly interacting with α-tubulin in leafhoppers. Eur. J. Plant Pathol. 162, 357–367. https://doi.org/10.1007/s10658-021-02408-3.

Du, Q., 2005. A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. Nucleic Acids Res. 33, 1671–1677. https://doi.org/10.1093/nar/gki312.

EFSA Panel on Plant Health PLH, 2014. Scientific opinion on pest categorisation of grapevine Flavescence dorée. EFSA J. 12, 3851. https://doi.org/10.2903/j.efsa.2014.3851.

Fernando, D.D., Fischer, K., 2020. Proteases and pseudoproteases in parasitic arthropods of clinical importance. FEBS J. 287, 4284–4299. https://doi.org/10.1111/febs.15546.

Filippin, L., Trivellone, V., Galetto, L., Marzachí, C., Elicio, V., Angelini, E., 2019. Development of an anti-Imp serological assay for the detection of "flavescence dorée" phytoplasmas in grapevine, insect vectors and host plants. Phyt. Moll. 9, 75. https://doi.org/10.5958/2249-4677.2019.00038.0.

Fuzita, F.J., Pinkse, M.W.H., Patane, J.S.L., Juliano, M.A., Verhaert, P.D.E.M., Lopes, A. R., 2015. Biochemical, Transcriptomic and Proteomic Analyses of Digestion in the Scorpion Tityus serrulatus: Insights into Function and Evolution of Digestion in an Ancient Arthropod. PLoS ONE 10, e0123841. https://doi.org/10.1371/journal.pone.0123841.

Galetto, L., Abbà, S., Rossi, M., Vallino, M., Pesando, M., Arricau-Bouvery, N., Dubrana, M.-P., Chitarra, W., Pegoraro, M., Bosco, D., Marzachì, C., 2018. Two phytoplasmas elicit different responses in the insect vector *Euscelidius variegatus* Kirschbaum. IAI.00042-18 Infect. Immun. 86. https://doi.org/10.1128/IAI.00042-18.

Galetto, L., Miliordos, D., Roggia, C., Rashidi, M., Sacco, D., Marzachì, C., Bosco, D., 2014. Acquisition capability of the grapevine Flavescence dorée by the leafhopper vector *Scaphoideus titanus* Ball correlates with phytoplasma titre in the source plant. J. Pest Sci. 87, 671–679. https://doi.org/10.1007/s10340-014-0593-3.

Galetto, L., Pegoraro, M., Marzachì, C., Rossi, E., Lucchi, A., Bosco, D., 2019. Potential role of the alien planthopper Ricania speculum as vector of Flavescence dorée phytoplasma. Eur. J. Plant Pathol. 154, 1103–1110. https://doi.org/10.1007/s10658-019-01731-0.

Gonella, E., Benelli, G., Arricau-Bouvery, N., Bosco, D., Duso, C., Dietrich, C.H., Galetto, L., Rizzoli, A., Jović, J., Mazzoni, V., Mori, N., Nieri, R., Roversi, P.F., Strauss, G., Thiéry, D., Trivellone, V., Virant-Doberlet, M., Lucchi, A., Alma, A., 2024. Scaphoideus titanus up-to-the-minute: biology, ecology, and role as a vector. entomologia 44, 481–496. https://doi.org/10.1127/entomologia/2023/2597.

Hartmann, D., Šíma, R., Konvičková, J., Perner, J., Kopáček, P., Sojka, D., 2018. Multiple legumain isoenzymes in ticks. Int. J. Parasitol. 48, 167–178. https://doi.org/10.1016/j.ijpara.2017.08.011.

Hatakeyama, T., Unno, H., 2023. Functional Diversity of Novel Lectins with Unique Structural Features in Marine Animals. Cells 12, 1814. https://doi.org/10.3390/cells12141814.

Jiang, S., Xiong, Y., Zhang, W., Zhu, J., Cheng, D., Gong, Y., Wu, Y., Qiao, H., Fu, H., 2022. A Novel Legumain-Like Protease in Macrobrachium nipponense: Identification, Characterization, and Function Analysis in Ovary Maturation. Front. Endocrinol. 13, 858726. https://doi.org/10.3389/fendo.2022.858726.

Kakizawa, S., Oshima, K., Ishii, Y., Hoshi, A., Maejima, K., Jung, H.-Y., Yamaji, Y., Namba, S., 2009. Cloning of immunodominant membrane protein genes of phytoplasmas and their *in planta* expression. FEMS Microbiol. Lett. 293, 92–101. https://doi.org/10.1111/j.1574-6968.2009.01509.x.

Katunuma, N., 2010. Posttranslational processing and modification of cathepsins and cystatins. J. Signal Transduct. 2010, 1–8. https://doi.org/10.1155/2010/375345.

Koinuma, H., Maejima, K., Tokuda, R., Kitazawa, Y., Nijo, T., Wei, W., Kumita, K., Miyazaki, A., Namba, S., Yamaji, Y., 2020. Spatiotemporal dynamics and quantitative analysis of phytoplasmas in insect vectors. Sci. Rep. 10, 4291. https://doi.org/10.1038/s41598-020-61042-x.

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N., Mathey-Prevot, B., 2006. Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. Nat. Methods 3, 833–838. https://doi.org/10.1038/nmeth935.

Li, Y., Yang, W., Sun, J., Lian, X., Li, X., Zhao, X., Liu, Y., Wang, L., Song, L., 2024. A DM9-containing protein from crab Eriocheir sinensis functions as a novel multipotent pattern recognition receptor. Fish. Shellfish Immunol. 145, 109356. https://doi.org/10.1016/j.fsi.2023.109356.

Liu, Y., Wang, W., Sun, J., Li, Y., Wu, S., Li, Q., Dong, M., Wang, L., Song, L., 2023. *Cg* DM9CP-5-Integrin-MAPK Pathway Regulates the Production of *Cg* IL-17s and *Cg* defensins in the Pacific Oyster, *Crassostrea gigas*. J. Immunol. 210, 245–258. https://doi.org/10.4049/jimmunol.2200016.

Malembic-Maher, S., Desqué, D., Khalil, D., Salar, P., Bergey, B., Danet, J.-L., Duret, S., Dubrana-Ourabah, M.-P., Beven, L., Ember, I., Acs, Z., Della Bartola, M., Materazzi, A., Filippin, L., Krnjajic, S., Krstić, O., Toševski, I., Lang, F., Jarausch, B., Kölber, M., Jović, J., Angelini, E., Arricau-Bouvery, N., Maixner, M., Foissac, X., 2020. When a Palearctic bacterium meets a Nearctic insect vector: genetic and ecological insights into the emergence of the grapevine Flavescence dorée epidemics in Europe. PLoS Pathog. 16, e1007967. https://doi.org/10.1371/journal.ppat.1007967.

Marcone, C., 2014. Molecular biology and pathogenicity of phytoplasmas. Ann. Appl. Biol. 165, 199–221. https://doi.org/10.1111/aab.12151.

Molina-Cruz, A., Canepa, G.E., Alves e Silva, T.L., Williams, A.E., Nagyal, S., Yenkoidiok-Douti, L., Nagata, B.M., Calvo, E., Andersen, J., Boulanger, M.J., Barillas-Mury, C., 2020. *Plasmodium falciparum* evades immunity of anopheline mosquitoes by interacting with a Pfs47 midgut receptor. Proc. Natl. Acad. Sci. USA 117, 2597–2605. https://doi.org/10.1073/pnas.1917042117.

Molina-Cruz, A., Canepa, G.E., Kamath, N., Pavlovic, N.V., Mu, J., Ramphul, U.N., Ramirez, J.L., Barillas-Mury, C., 2015. *Plasmodium* evasion of mosquito immunity and global malaria transmission: the lock-and-key theory. Proc. Natl. Acad. Sci. U. S. A. 112, 15178–15183. https://doi.org/10.1073/pnas.1520426112.

Orlovskis, Z., Singh, A., Kliot, A., Huang, W., Hogenhout, S.A., 2024. Phytoplasma targeting of MADS-box factor svp suppresses leaf responses to insect vector males. Promot. Female Attract. Colon. https://doi.org/10.1101/2024.05.09.593434.

Pan, L., Bai, P., Weng, X., Liu, J., Chen, Y., Chen, S., Ma, X., Hu, K., Sun, A., Ge, J., 2022. Legumain is an endogenous modulator of integrin αvβ3 triggering vascular degeneration, dissection, and rupture. Circulation 145, 659–674. https://doi.org/10.1161/CIRCULATIONAHA.121.056640.

Pelletier, C., Salar, P., Gillet, J., Cloquemin, G., Very, P., Foissac, X., Malembic-Maher, S., 2009. Triplex real-time PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. Vitis 48, 87–95.

Rahmeh, A.A., Morin, B., Schenk, A.D., Liang, B., Heinrich, B.S., Brusic, V., Walz, T., Whelan, S.P.J., 2012. Critical phosphoprotein elements that regulate polymerase architecture and function in vesicular stomatitis virus. Proc. Natl. Acad. Sci. U. S. A. 109, 14628–14633. https://doi.org/10.1073/pnas.1209147109.

Ramphul, U.N., Garver, L.S., Molina-Cruz, A., Canepa, G.E., Barillas-Mury, C., 2015. *Plasmodium falciparum* evades mosquito immunity by disrupting JNK-mediated apoptosis of invaded midgut cells. Proc. Natl. Acad. Sci. U. S. A. 112, 1273–1280. https://doi.org/10.1073/pnas.1423586112.

Rossi, M., Galetto, L., Bodino, N., Beltramo, J., Gamalero, S., Pegoraro, M., Bosco, D., Marzachì, C., 2023. Competition among flavescence dorée phytoplasma strains in the experimental insect vector euscelidius variegatus. Insects 14, 575. https://doi.org/10.3390/insects14070575.

Rossi, M., Pegoraro, M., Ripamonti, M., Abbà, S., Beal, D., Giraudo, A., Veratti, F., Malembic-Maher, S., Salar, P., Bosco, D., Marzachì, C., 2019a. Genetic diversity of Flavescence dorée phytoplasmas at the vineyard scale. e03123-18, /aem/85/10/AEM.03123-18.atom Appl. Environ. Microbiol. 85. https://doi.org/10.1128/AEM.03123-18.

Rossi, M., Samarzija, I., Šeruga-Musić, M., Galetto, L., 2019b. Diversity and functional importance of phytoplasma membrane proteins. In: Bertaccini, A., Oshima, K., Kube, M., Rao, G.P. (Eds.), Phytoplasmas: Plant Pathogenic Bacteria - III. Springer Singapore, Singapore, pp. 69–88. https://doi.org/10.1007/978-981-13-9632-8_5.

Saikhedkar, N., Summanwar, A., Joshi, R., Giri, A., 2015. Cathepsins of lepidopteran insects: aspects and prospects. Insect Biochem. Mol. Biol. 64, 51–59. https://doi.org/10.1016/j.ibmb.2015.07.005.

Santamaría, M.E., González-Cabrera, J., Martínez, M., Grbic, V., Castañera, P., Díaz, L., Ortego, F., 2015. Digestive proteases in bodies and faeces of the two-spotted spider mite, tetranychus urticae. J. Insect Physiol. 78, 69–77. https://doi.org/10.1016/j.jinsphys.2015.05.002.

Santamaría, M.E., Hernández-Crespo, P., Ortego, F., Grbic, V., Grbic, M., Diaz, I., Martinez, M., 2012. Cysteine peptidases and their inhibitors in Tetranychus urticae: a comparative genomic approach. BMC Genom. 13, 307. https://doi.org/10.1186/1471-2164-13-307.

Schicht, S., Qi, W., Poveda, L., Strube, C., 2013. The predicted secretome and transmembranome of the poultry red mite Dermanyssus gallinae. Parasites Vectors 6, 259. https://doi.org/10.1186/1756-3305-6-259.

Shaw, W.R., Catteruccia, F., 2018. Vector biology meets disease control: using basic research to fight vector-borne diseases. Nat. Microbiol 4, 20–34. https://doi.org/10.1038/s41564-018-0214-7.

Siampour, M., Galetto, L., Bosco, D., Izadpanah, K., Marzachi, C., 2011. In vitro interactions between immunodominant membrane protein of lime witches' broom phytoplasma and leafhopper vector proteins. Bull. Insect 64, S149–S150.

Siampour, M., Izadpanah, K., Galetto, L., Salehi, M., Marzachí, C., 2013. Molecular characterization, phylogenetic comparison and serological relationship of the Imp protein of several '*Candidatus* Phytoplasma aurantifolia' strains: *imp* gene in '*Candidatus* Phytoplasma aurantifolia'. Plant Pathol. 62, 452–459. https://doi.org/10.1111/j.1365-3059.2012.02662.x.

Silver, K., Cooper, A.M., Zhu, K.Y., 2021. Strategies for enhancing the efficiency of RNA interference in insects. Pest Manag Sci. 77, 2645–2658. https://doi.org/10.1002/ps.6277.

Solberg, R., Lunde, N.N., Forbord, K.M., Okla, M., Kassem, M., Jafari, A., 2022. The mammalian cysteine protease legumain in health and disease. IJMS 23, 15983. https://doi.org/10.3390/ijms232415983.

Transdecoder, 2016. ⟨https://transdecoder.github.io/⟩.

Trivellone, V., Dietrich, C.H., 2021. Evolutionary diversification in insect vector–phytoplasma–plant associations. Ann. Èntomol. Soc. Am. 114, 137–150. https://doi.org/10.1093/aesa/saaa048.

Trivellone, V., Ripamonti, M., Angelini, E., Filippin, L., Rossi, M., Marzachí, C., Galetto, L., 2019. Evidence suggesting interactions between immunodominant membrane protein Imp of Flavescence dorée phytoplasma and protein extracts from distantly related insect species. J. Appl. Microbiol. 127, 1801–1813. https://doi.org/10.1111/jam.14445.

Waniek, P.J., Pacheco Costa, J.E., Jansen, A.M., Costa, J., Araújo, C.A.C., 2012. Cathepsin L of *Triatoma brasiliensis* (Reduviidae, Triatominae): sequence characterization, expression pattern and zymography. J. Insect Physiol. 58, 178–187. https://doi.org/10.1016/j.jinsphys.2011.11.008.