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Evidence suggesting interactions between immunodominant membrane protein Imp of Flavescence dorée phytoplasma and protein extracts from distantly related insect species.

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Running headline: binding between phytoplasma Imp and insect proteins

Abstract

Aims: In this study, binding between the immunodominant membrane protein Imp of the 16SrV-D phytoplasma associated with Flavescence dorée disease (FD-Dp) and insect proteins of vectors and non-vectors of FD-Dp was tested.

Methods and Results: Six Auchenorrhyncha species, from distantly related groups were selected: *Scaphoideus titanus*, *Euscelidius variegatus*, *Macrosteles quadripunctulatus*, *Zyginidia pullula* (Cicadomorpha), *Ricania speculum* and *Metcalfa pruinosa* (Fulgoromorpha). The vector status of each species was retrieved from the literature or determined by transmission trials in this study. A His-tagged partial Imp protein and a rabbit polyclonal antibody were synthesized and used for Western and Far-Western dot Blot (FWdB) experiments. Total native and membrane proteins were extracted from entire bodies and organs (gut and salivary glands) of each insect species. FWdB showed a decreasing interaction intensities of Imp fusion protein with total proteins from entire bodies of *S. titanus*, *E. variegatus* (competent vectors) and *M. quadripunctulatus* (non-vector), while no interaction signal was detected with the other three species (non-vectors). A strong signal detected upon interaction of FD-D Imp and membrane proteins from guts of closely related insects supports the role of this organ as the first barrier to ensure successful transmission.

Conclusions: Our results showed that specific Imp binding, correlated with vector status, is involved in interactions between FD-Dp and insect proteins.

Significance and Impact of the Study: Integrating knowledge on host-pathogen protein-protein interactions and on insect phylogeny would help to identify the actual range of vectors of phytoplasma strains of economic importance.

Introduction

Phytoplasmas are phloem-limited wall-less plant pathogenic bacteria in the class Mollicutes causing hundreds of plant diseases worldwide and responsible for severe economic losses to agriculturally important plants (Strauss 2009; Marcone 2014). Although phytoplasmas represent a well-defined monophyletic clade in the family Acholeplasmataceae, they are still treated as indefinite taxa due to severe difficulties hampering *in vitro* cultivation. Phytoplasma classification is based on *16S rRNA* and ribosomal protein (*rp*) gene sequences (Lee *et al.* 2000) and the '*Candidatus*' species concept is applied for well characterized phytoplasmas (Murray and Stackebrandt 1995; IRPCM 2004). The known phytoplasma vectors are hemipteran insects belonging to the suborder Auchenorrhyncha (Fulgoromorpha and Cicadomorpha) and the family Psyllidae (suborder Sternorrhyncha) (Weintraub and Beanland 2006).

The 16SrV phytoplasma (16SrVp) phylogenetic group represents the third largest phytoplasma cluster (Lee *et al.* 2004) showing more than 98.6% 16S rRNA nucleotide identity among different strains (Lee *et al.* 1998a). However, this group is naturally clustered in several different ecological niches (Lee *et al.* 1998b) that could contribute to genetic drift. The 16SrVp group comprises six described phytoplasma subgroups, four of which are described as '*Candidatus* Phytoplasma (*Ca.* P.)' (Bertaccini *et al.* 2014): 16SrV-A ('*Ca.* P. ulmi', Lee *et al.* 2004), 16SrV-B ('*Ca.* P. ziziphi', Jung *et al.* 2003), 16SrV-E ('*Ca.* P. rubi', Malembic-Maher *et al.* 2011), and 16SrV-F ('*Ca.* P. balanitae', Harrison *et al.* 2014). The remaining two subgroups, 16SrV-C and -D, are assigned to the not formally described '*Ca.* P. vitis' reported as an incidental citation (IRPCM 2004). The latter two genetically heterogeneous subgroups include well known harmful plant pathogens causing the Flavescence dorée (FD) disease associated mainly with grapevine, and other diseases associated with wild host plants. Analyses of single nucleotide polymorphisms (SNPs) in

ribosomal and non-ribosomal fragments have been carried out to characterize the relationship between closely related genotypes clustering in the clade of 16SrV-C and -D subgroups (e.g. Palatinate grapevine yellows, Alder yellows) (Angelini *et al.* 2001; Martini *et al.* 2002; Arnaud *et al.* 2007; Rossi *et al.*, 2019). An exclusive association between the FD-related genotypes, plant hosts and their insect vectors has been suggested (EFSA 2014). The leafhopper *Scaphoideus titanus* Ball (Cicadellidae: Deltocephalinae) is the only grapevine FD vector reported to have major epidemiological significance in the field. Other insect species have been recorded as competent vectors of FD or FD-related genotypes in experimental conditions but are suspected to play only minor epidemiological roles in spreading FD to grapevine, and are more likely associated with other epidemiological routes involving different plants (e.g. clematis and alder). These include *Anoplotettix fuscovenosus, Euscelidius variegatus*, *Euscelis incisus* (Bressan *et al.* 2006), *Orientus ishidae* (Lessio *et al.* 2016), *Allygus* spp. (Malembic-Maher *et al.* 2017), *Dictyophara europaea* (Filippin *et al.* 2009), and *Oncopsis alni* (Maixner *et al.* 2000; Malembic-Maher *et al.* 2017). The first five species belong to the same subfamily as *S. titanus,* whereas *O. alni* belongs to cicadellid subfamily Eurymelinae, and *D. europaea* to the phylogenetically distant planthopper family Dictyopharidae.

The concepts of transmission specificity and efficiency include the existence of constraints (factors) acting between a specific pathogen and its carrier/s, that determine which species are capable of being vectors. Such factors can be intrinsic (e.g. species identity and intraspecific variation; Verbeek *et al.* 2010) or extrinsic (e.g. environmental constraints and biological relationships; Daugherty *et al.* 2009, Lopes *et al.* 2009), and affect pathogen-vector interactions at different spatio-temporal scales and biological organization levels. Some studies suggested the involvement of specific molecular phytoplasma-ligand interactions in transmission specificity with insect hosts (Suzuki *et al.* 2006; Galetto *et al.* 2011a; Arricau-

Bouvery *et al.* 2018). Different types of phytoplasma membrane proteins are in direct contact with the host environment and have been recognized to play an important role in promoting phytoplasma internalization in insect cells.The major membrane proteins of phytoplasmas are the immunodominant membrane proteins (IDPs), variable membrane proteins (Vmps), membrane transport proteins (e.g. SecY), adhesins, ATP-dependent proteases, as well as those encoded by potential mobile units and plasmids. However, a number of studies focused on the IDPs, since they are the most abundant proteins of the cell surface of phytoplasmas (for an overview see Konnerth *et al.* 2016). Previous studies showed that two (Immunodominant membrane protein [Imp] and Antigenic membrane protein [Amp]) of the three non-homologous immunodominant membrane proteins are involved in interactions between the phytoplasma cell surface and insect proteins (Suzuki *et al.* 2006; Galetto *et al.* 2011a; Siampour *et al.* 2011, 2013; Rashidi *et al.* 2015). In particular, *imp* has high genetic variability among phytoplasmas and is under strong positive selection pressure, a strong indicator of its role in interactions with the environment and the host (Kakizawa *et al.* 2009). Moreover, RNA-Seq studies revealed that *imp* is one of the most expressed genes in FD phytoplasma (Abbà *et al.* 2014).

We investigated the transmission specificity mediated by the immunodominant membrane protein Imp in the pathosystem of 16SrV-Dp and its insect hosts. A specific genotype in the 16SrV-D subgroup (hereafter FD-D), which is known as the most widespread phytoplasma strain causing the FD disease of grapevine, was selected (Arnaud *et al.* 2007; Filippin *et al.* 2009). Since available evidence suggested that related pathogens tend to be associated with particular lineages of leafhopper vectors (Hogenhout *et al.* 2008a; Perilla-Henao and Casteel 2016), the protein-protein interactions of vectors and non-vectors of FD-D phytoplasma (FD-Dp) selected from distantly related insect species, were tested. The insect species were chosen to represent a gradient of vector ability with respect to the acquisition and

transmission rate of FD-Dp, from highly competent vector to non-vector in two distantly related Auchenorrhyncha lineages.

Materials and Methods

Insect species selection and specimens' recruitment

The specific protein-protein interaction between Imp of FD-Dp and proteins of putative insect vectors was tested using six insect species. The selected hemipteran species belong to two distantly related lineages of Auchenorrhyncha (Johnson *et al.* 2018). Four species of the infraorder Cicadomorpha, all in the family Cicadellidae were tested: *Scaphoideus titanus*, *Euscelidius variegatus*, *Macrosteles quadripunctulatus* (subfamily Deltocephalinae) and *Zyginidia pullula* (Typhlocybinae). Additionally, two species of infraorder Fulgoromorpha, *Ricania speculum* (family Ricanidae) and *Metcalfa pruinosa* (family Flatidae) were also tested. The vector status of the species was defined based on prior evidence of their ability to acquire and/or transmit FD-Dp. In particular, data on transmission efficiency tested in experimental conditions, as measured by Acquisition Rate (AR) and Transmission Rate (TR), were retrieved from the literature. *Scaphoideus titanus* and *E. variegatus*, are known as competent vectors of FDp (Schvester *et al.* 1963; Caudwell *et al.* 1972). For *S. titanus,* AR was 63% (N=60) using 16SrV-C infected *Vicia faba* as source plants and TR was 60% (N=10) on healthy micro-propagated *Vitis vinifera* (Miliordos *et al.* 2017). Since *S. titanus* was reported as an efficient vector both in the field and in laboratory in many countries (for a review see Trivellone 2019), it is considered here to be an efficient competent vector of FD-Dp. For *E. variegatus* AR was 67% (N=69) from FD-infected *V. faba* and TR was 62% (N=108) on healthy *V. faba* in the laboratory (Rashidi *et al.* 2014), although this species is not known to be a competent vector under field conditions. Thus, *E. variegatus* is here treated as an efficient competent vector of FD-Dp in laboratory conditions only (EFSA 2014).

Scaphoideus and *Euscelidius* belong to the same cicadellid subfamily (Deltocephalinae) but are placed in unrelated tribes. One additional deltocephaline leafhopper species, *Macrosteles quadripunctulatus*, which belongs to another unrelated tribe, was selected because it is a known vector of other phytoplasmas, but not FD-Dp. The competence of *M. quadripunctulatus* was already reported in the literature for two phylogenetically related phytoplasmas, 16Sr-I and 16Sr-XII (for an overview see Trivellone 2019). According to a recent phylogenetic study of Membracoidea, the subfamily Typhlocybinae is only distantly related to Deltocephalinae, the group comprising most phytoplasma vectors (Dietrich *et al.* 2017). Unlike Deltocephalinae, which feed preferentially on phloem, Typhlocybinae are thought to feed primarily on mesophyll (Backus *et al.* 2005). Therefore, the typhlocybine species *Zyginidia pullula* was selected for testing as a phylogenetically distantly related non-vector leafhopper.

Recently, Galetto *et al.* (2019) reported for the first time the capability of the ricaniid planthopper *R. speculum* to acquire FD-Dp from *V. faba* with AR of 53% (N=15); however, the inoculation trial resulted in TR of 0% in tests carried out on both *V. faba* (N=24) and *V. vinifera* (N=21). Therefore, with respect to the efficient competent vectors of FD-Dp, *R. speculum* was selected as a phylogenetically unrelated non-vector. For the flatid planthopper, *M. pruinosa*, FD-Dp infected adults were collected in infected vineyards in the field by Guadagnini *et al.* (2000), and further investigations using juveniles collected in the field reported the capability of *M. pruinosa* to acquire FDp from *V. faba* with AR of 54% (mean of two years; N=95), but not to transmit FDp to *V. faba* (Clair *et al.* 2001).

Prior data on AR and TR were not available for *M. quadripunctulatus* and experiments using specimens collected in the field were published for *M. pruinosa*, so these parameters were evaluated on laboratory-reared specimens in this study with respect to FD-Dp and the vector status was assigned accordingly (for further details see next section *Phytoplasma isolate and*

174 *transmission trials*). An overview of acquisition and transmission ability is reported in Table
175 1.
176 All reared specimens were obtained from the healthy colonies at the IPSP-CNR (Turin, Italy).
177 Laboratory-reared *S. titanus* and *M. pruinosa* neonates emerged from two-year-old branches
178 bearing insect eggs collected in infested vineyards (Piedmont region, Italy) during winter.
179 Branches were placed in screen houses (100 cm × 100 cm × 100 cm) and kept in a glasshouse
180 with natural light and temperature ranging from 20 to 30°C. Potted phytoplasma-free
181 grapevine cuttings together with potted phytoplasma-free broad beans (*V. faba*) were
182 introduced in the screen house and replaced every three weeks. Adults started to appear about
183 two months after the introduction of the branches in the cage. Healthy *M. quadripunctulatus*
184 and *E. variegatus* were reared on oat (*Avena sativa*) in climatic chambers with 20-25°C and
185 photoperiod 16:8 h (light:dark, L:D). Healthy *R. speculum* specimens were obtained from
186 eggs laid on twigs of *Clematis vitalba*, *Ligustrum lucidum* and *Rubus* spp. collected in
187 infested areas in Northern and central Italy (Galetto *et al.* 2019). Eggs were allowed to hatch
188 under laboratory conditions (21 ± 1°C, 60 ± 10% RH, and a photoperiod of 16:8 h L:D) and
189 maintained on healthy *V. faba*. Adults and plants were periodically confirmed to be PCR-
190 negative for phytoplasma presence and collected to be used for the experiments.
191 Adults of *Z. pullula* were collected on ruderal patches of graminoid species naturally
192 phytoplasma-free in the city of Turin (Italy).
193
194 *Phytoplasma isolate and transmission trials*
195 In this study, experimental trials were carried out to measure the ability of *M.*
196 *quadripunctulatus* and *M. pruinosa* to acquire and/or inoculate the FD-D phytoplasma. The
197 FD-Dp strain "FD-D CRA AT" was used for acquisition and transmission trials. The isolate
198 was obtained from infective *S. titanus* adults that were collected in 2015 from an

experimental vineyard in Piedmont and allowed to feed on *V. faba* in the laboratory. This FD phytoplasma isolate was genetically identified as member of 16SrV-D subgroup based on DNA sequence analysis (Rossi *et al.* 2019). FD-Dp was then routinely maintained under controlled conditions with sequential transmissions from broad beans to broad beans by the experimental vector *E. variegatus*, continuously reared under laboratory conditions on oat. The same FD-D phytoplasma strain was also transmitted to *Catharanthus roseus* (periwinkle) by the experimental vector *E. variegatus*, and then routinely maintained under controlled conditions with sequential grafting on periwinkle.

For FD-Dp acquisition and transmission trials by *M. quadripunctulatus* and *M. pruinosa*, about 100 nymphs (3rd and 4th instar) of the former species and about 50 nymphs of the latter were separately isolated on FD-D-infected broad beans for an acquisition access period of two weeks, and transferred to healthy broad beans for a latent period of two weeks. Groups of five FD-Dp exposed adults per plant were then used to inoculate healthy broad beans for seven days for an inoculation access period. After the inoculation period, insects were collected and DNA was extracted and analysed by qPCR for the presence of FD-Dp (see subsection below). Inoculated plants were inspected for symptom appearance twice a week, and their DNA was extracted one month after the inoculation period.

DNA extraction, phytoplasma detection and quantification

Total DNA was extracted from single heads and bodies of *M. quadripunctulatus* and *M. pruinosa*, with cethyl-trimethyl-ammonium bromide (CTAB) buffer, as described in Rashidi *et al.* (2014). Total DNA was also extracted from plant samples (1 g of leaf tissues) with CTAB buffer, as described in Pelletier *et al.* (2009). Insect and plant samples were resuspended in 50 and 100 µl of 0.01 mol l^{-1} Tris-Cl pH 8.0, respectively. Concentration and

223 purity of extracted total DNAs were checked with a Nanodrop 2000 UV-visible
224 spectrophotometer (Thermofisher).
225 Primers mapFD-F/mapFD-R and TaqMan probe mapFD-FAM (Pelletier *et al.* 2009) were
226 used to detect FD-Dp presence in phytoplasma-exposed insects and inoculated plants by
227 qPCR, using 1x iTaq Universal Probe Supermix (Bio-Rad) in a reaction mix of 10 µl volume.
228 Final concentrations were 300 and 200 nmol l^{-1} for primers and probe, respectively, and
229 cycling conditions were as indicated in the original paper (Pelletier *et al.* 2009). Samples
230 were run in duplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad).
231 The concentration of FD-Dp in the unique positive *M. pruinosa* head sample was quantified
232 by qPCR using two primer pairs: FdSecyFw/FdSecyRv, targeting FD-Dp *sec*Y gene (Roggia
233 *et al.* 2014), and MqFw/MqRv, targeting insect *18S* ribosomal gene (Marzachí and Bosco
234 2005). The sample was diluted to 10 ng $µl^{-1}$ and 1 µl was added to a qPCR reaction mix of 10
235 µl volume, containing 1x iTaq Universal SYBR Green Supermix (Bio-Rad) and 300 nmol l^{-1}
236 of each primer. The sample was then run in triplicate in a CFX Connect Real-Time PCR
237 Detection System (Bio-Rad). Cycling conditions were as detailed in the original paper
238 (Roggia *et al.* 2014). For each primer pair a standard curve, based on serial dilutions of either
239 a plasmid harboring FD-Dp *sec*Y gene or total DNA of a healthy insect sample, was run.
240 Mean starting quantity was automatically calculated by CFX Maestro™ Software (Bio-Rad)
241 and used to express FD-Dp amount as Genome Unite (GU) ng^{-1} insect DNA.
242
243 *Fusion proteins and antibody production*
244 The *dnaD-imp-pyrG* genomic fragment of FD-D strain was amplified from FD-D infected
245 vine leaves by nested PCR using primer pair DnaDFDf1/PyrGFDr1 (5'-TAG AGA GAA
246 TTT TAG GCC ACG-3' forward primer, 5'-AAT AAT GAA GAA CAA TTA CCT G-3'
247 reverse primer), generating a 940 bp amplicon, followed by primer pair

DnaDFDf2/PyrGFDr2 (5'-ATA GAA AAT AAC GAT AAA GCA G-3' forward primer, 5'-TCA AGA CCT TTT AAA CCA CAC CC-3 reverse primer), amplifying a 830 bp genomic region (Italian patent no. 1429213; *imp* sequence Genbank MK614707). The obtained sequence was analysed by TMHMM server 2.0 (Sonnhammer *et al.* 1998; Krogh *et al.* 2001) to exclude its transmembrane domain from further cloning.

The His-tagged Imp protein was synthesized via the PCR product of the *imp* FD-D gene region encoding the C-terminus external domain of the FD-D Imp protein. This portion was expressed as a fusion protein with a N-terminal 10x His tag by cloning the corresponding gene fragment in p2N expression vector (Primm srl, Milano, Italy). The FD-D Imp fusion protein was solubilized in 6 mol l^{-1} Urea, 0.02 mol l^{-1} NaH_2PO_4, 0.5 mol l^{-1} NaCl, 0.5 mol l^{-1} Imidazole. A rabbit polyclonal antibody was produced by using the His tagged FD-D Imp fusion protein as recombinant antigen (European patent no. 2918685, Italian patent no. 1429213). The affinity chromatography purified IgG were prepared *in-house* for Western and Far-Western blot experiments. The IgG were purified on protein A column (r-ProtA Sepharose Fast Flow; GE Healthcare, 17-1279-01), using Phosphate Buffered Saline (PBS, 0.137 mol l^{-1} NaCl, 0.10 mol l^{-1} Na_2HPO_4/KH_2PO_4, 0.027 mol l^{-1} KCl, pH 7.0) as binding buffer and 0.1 mol l^{-1} glycine (pH 3.0) as elution buffer, with a flow rate of 2 ml min^{-1}.

Western blot

In order to check the functionality of the polyclonal antibody developed against recombinant FD-D Imp fusion protein, a Western blot assay was performed on 100 ng of FD-D Imp fusion protein, as well as on healthy and FD-D infected plants (broad beans and periwinkles) and insects (*E. variegatus*). For each sample, proteins were extracted from 200 mg of leaf tissue crushed with mortar and pestle in 2 ml of Rx Buffer (0.1% Triton-X 100, 0.1 mol l^{-1} KCl, 3 mmol l^{-1} NaCl, 3.5 mmol l^{-1} $MgCl_2$, 1.25 mmol l^{-1} EGTA, 1.25 mmol l^{-1} Hepes) and from

five adult insects crushed in 0.5 ml of the same buffer. The homogenates were sonicated 1 min and centrifuged 1 min at 13,000 *g*. Twenty µl of the supernatant were added to 10 µl of Laemmli sample buffer 3x (Laemmli 1970) and 10 µl per lane were loaded onto 15% polyacrylamide gels, together with prestained molecular weight standards (Sharpmass VII, Euroclone). Gels were either stained with colloidal Coomassie stain (Candiano *et al.* 2004) or blotted on a polyvinylidene difluoride (PVDF) membrane. The membrane was first incubated under slow agitation for 30 min in blocking solution (3% bovine serum albumin Tris-buffered saline with 0.1% Tween, BSA-TBST) and then overnight at 4°C with primary polyclonal antibody diluted 1:1000 in BSA-TBST. The membrane was then washed four times with BSA-TBST, incubated for 2 h at room temperature with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody developed in goat (A0545 GAR-HRP Sigma) diluted 1:15000 in BSA-TBST, washed four times with TBST, and finally developed with West Pico SuperSignal chemiluminescent substrate (Pierce) in a VersaDoc 4000 MP system (Bio-Rad).

Far-Western dot Blots

To test the specific interaction of FD-D Imp fusion peptide with proteins of the tested species, a Far-Western dot Blot (FWdB) analysis was used. This analysis reveals the interaction between prey proteins (insect proteins) and a bait protein (His tagged FD-D Imp fusion peptide). FWdB was applied using six protein extracts for each insect species: total and membrane proteins from the entire insect bodies, total and membrane proteins from insect guts and total and membrane proteins extracted from insect salivary glands. Total proteins were tested to cover all the insect peptides (cytosolic and membrane fractions) potentially interacting with FD-D Imp. Membrane proteins were further investigated to determine their actual role at the interface of the pathogen/vector relationship.

In total, in the experiments 36 samples were analysed (six species x six sample types).

298 Protein extraction. For entire body samples, total and membrane proteins were extracted from
299 batches of all insect species (five *S. titanus*, five *E. variegatus*, 10 *M. quadripunctulatus*,
300 three *R. speculum*, three *M. pruinosa*, and 20 *Z. pullula*). The number of specimens for each
301 species pool was defined in a preliminary experiment using Bradford reagent (Bio-Rad) with
302 the aim to load comparable amounts of total and membrane proteins extracted from entire
303 bodies and organs respectively. The similar quantities of the extracted total proteins were
304 verified by spotting the samples onto a membrane stained with Ponceau S (Sigma-Aldrich)
305 Solution (Fig. S1A). Total Native Proteins (TNP) from the entire insect bodies were extracted
306 following homogenization in 300 µl of RX buffer (Suzuki *et al.* 2006), and centrifuged for 1
307 min at 13,000 *g*; 250 µl of proteins in the supernatant were transferred in a new tube and
308 preserved at -80°C until the FWdB procedure. Membrane Proteins (MP) from the entire
309 insect bodies were extracted according to the protocol described in Galetto *et al.* (2011a) and
310 20 µl of collected proteins were added with 6 µl of Base Buffer (0.15 mol l^{-1} NaCl, 0.01 mol
311 l^{-1} Tris, pH 7.4). The correct extraction of TNP and MP fractions were verified by SDS-
312 PAGE on the experimental vector *Euscelidius variegatus* (Fig. S1B).
313 A series of samples from total and membrane proteins was also extracted from two type of
314 organs of the selected species, the gut and the salivary glands. For each species, organs were
315 dissected from freshly euthanized insects (CO_2 flushed) on a soft dark support of wax using
316 handle pins, and then pooled in a tube containing 50 µl of Buffer (Rx or Base for total or
317 membrane proteins, respectively) added with EDTA-free antiprotease cocktail Complete I
318 (Roche). After dissection, the proteins were extracted following the same protocol used for
319 the entire insect bodies.
320 FWdB procedure. FD-D Imp partial fusion protein was used as bait in FWdB experiments
321 against the 36 insect protein samples. For the entire insect body, 40 µg of total proteins and
322 10 µg of membrane proteins were spotted onto polyvinyl difluoride (PVDF, Bio-Rad)

membranes with a 96-well Minifold dot-blotter (model I SRC 96D; Schleicher and Schuell, Dassel, Germany). For dissected insect organs, total (1.6 µg from gut and 0.8 µg from salivary glands) as well as membrane proteins were spotted onto PVDF membranes. Undiluted samples of membrane proteins from organs were spotted as these samples were always below the limit of Bradford detection. All samples were serially diluted 1:10 and 1:100 in PBS (pH 7.4) added with EDTA-free antiprotease cocktail Complete I (Roche) and spotted onto PVDF membranes as well. One hundred ng of FD-D Imp partial fusion protein and 40 µg of BSA were spotted as positive and negative controls, respectively. Each sample spotted onto the PVDF membrane consisted in 20 µl. Each membrane was blocked, incubated and washed in glass Petri dish (9 cm diameter) with 10 ml of appropriate solution. Two sets of membranes for each experiment were blocked for 1 h at 4°C with BSA-TBST and then incubated overnight at 4°C either with the bait recombinant FD-D Imp fusion protein diluted in BSA-TBST (0.4 µg ml^{-1}) mixed with EDTA-free antiprotease cocktail Complete I (Roche) or with buffer only (no bait control) as negative control. The PVDF membranes were washed for 1 h with BSA-TBST and for 10 min with TBST at room temperature, then incubated 4 h at RT with anti-FD-D Imp polyclonal antibody, washed 3 times in BSA-TBST, incubated 2 h with goat anti-rabbit HRP-conjugated secondary antibody (A0545, Sigma-Aldrich), and washed three times with TBST. Primary and secondary antibodies were diluted in BSA-TBST to 1:1000 and 1:15000, respectively. Detection was performed with West Pico SuperSignal chemiluminescent substrate (Pierce) and a VersaDoc 4000 MP (Bio-Rad). Each experiment was repeated three times, each with freshly extracted insect proteins.

For each sample, the results of the overall interaction were summarized based on the signals present in the three dilutions (undiluted starting quantity, 1:10, 1:100). Categorical codes were assigned as follows: +, signal present only in undiluted starting quantity; ++ and +++, signal present in 1:10 and 1:100 dilutions, respectively.

348

349 **Results**

350 *Vector status and transmission trials*

351 For *S. titanus*, *E. variegatus*, and *R. speculum* detailed data about their acquisition and
352 transmission ability of the FD-Dp have been already published (Schvester *et al.* 1963;
353 Caudwell *et al.* 1972; Galetto *et al.* 2019), and here summarized in Table 1.
354 Acquisition and inoculation experiments were also performed in this study to assess the
355 vector status of two species, *M. quadripunctulatus* and *M. pruinosa*, for which no detailed
356 data on AR and TR were available in the literature. For *M. quadripunctulatus*, following
357 isolation on FD-D-infected broad bean plants, all insects (N=19) and all inoculated plants
358 (N=5) tested PCR negative for the presence of FD-Dp. Following isolation of *M. pruinosa* on
359 FD-D-infected broad bean plants, five out of 12 body samples and one out of 11 head
360 samples tested PCR positive for the presence of FD-Dp. The pathogen amount in the unique
361 positive head sample was 134 FD GU ng^{-1} of insect DNA. All plants (N=5) inoculated by *M.*
362 *pruinosa* adults exposed to FD-Dp tested PCR negative for the presence of FD phytoplasma
363 and were asymptomatic two months after the inoculation. *Macrosteles quadripunctulatus* and
364 *M. pruinosa* were therefore considered here as phylogenetically related and unrelated non-
365 vectors of FD-Dp, respectively, as compared to the primary competent vector, *S. titanus*.

366

367 *Fusion antigen and antibody specificity*

368 The predicted number of FD-D Imp amino acids was 162, with a molecular weight of 18052
369 Dalton and pI is 9.70. TMHMM identified a transmembrane domain corresponding to the N-
370 terminal hydrophobic region between 21^{th} and 43^{th} residues. Residues 44-162 were predicted
371 to be exposed outside the phytoplasma membrane. Structure and predicted membrane
372 localization of the native full-length Imp FD-D are depicted in Figs 1A and B. The His-

tagged recombinant fusion Imp FD-D protein, used as an antigen for the production of the rabbit polyclonal antiserum, contained the complete C-terminus domain predicted to be exposed out of the phytoplasma cell (Fig. 1C).

The FD-D Imp fusion protein was successfully expressed with the expected molecular weight, purified for antibody production (Fig. S2) and properly detected by the IgG fraction of the polyclonal antiserum (Fig. 2). In particular, the IgG fraction recognised 100 ng of purified recombinant Imp FD-D fusion antigen (theoretical mass 15.7 kDa), as well as the native full-length FD Imp (theoretical mass 18.0 kDa) from infected periwinkle, broad bean and *E. variegatus* proteins, comigrating with the 16 kDa prestained molecular mass marker (Fig. 2). Although very weak signals were detected in plant healthy samples, no signal was evident in insect healthy samples.

Analysis of protein-protein binding

The total number of specimens and the protein concentration for each sample were estimated by the Bradford assay (Table S1).

The FWdB assays showed an interaction of FD-D Imp fusion protein with TNP extracted from the entire body of *S. titanus*, *E. variegatus* and *M. quadripunctulatus*, and with MP extracted from the entire body of *R. speculum* and *M. pruinosa* (Fig. 3). No signal was detected in the other samples.

Results for proteins extracted from guts showed a clear gradient of the signal intensities of the interaction between FD-D Imp fusion protein and insect proteins, both in undiluted TNP and MP samples. Indeed, in MP fraction the signals varied from strong (*S. titanus*) to weak (*M. quadripunctulatus*), while no signal was observed for *R. speculum*, *M. pruinosa* and *Z. pullula* samples.

Similarly, a gradient of signal intensities of protein-protein interaction was observed for salivary gland TNP samples, varying from medium (*S. titanus*) to weak (*E. variegatus*) signals in undiluted samples, and from weak to very weak signal in 1:10 diluted *S. titanus* and *E. variegatus* samples, respectively. No interaction signal was observed for salivary gland proteins extracted from *M. quadripunctulatus*, *R. speculum*, and *M. pruinosa* in TNP. However, unexpectedly strong signals in *Z. pullula* TNP (undiluted and 1:10 diluted) and weak signals in *R. speculum* and *M. pruinosa* MP (undiluted) samples were detected.

The results of FWdB assays are summarized in Table 1. The same results were obtained in all the three replicates of the experiment.

Discussion

Previous studies suggested that in pathosystems with a persistent-propagative transmission mode the vector-pathogen interaction tends to evolve toward a highly specific relationship (Robert and Bourdin 2001). It was also suggested that ability of Hemiptera to transmit plant pathogens varies across phylogenetic lineages, and non-random patterns of transmission specificity have been detected at different taxonomic levels; e.g. between specific orders of pathogens and hemipteran superfamilies (Perilla-Henao and Casteel 2016), genera of viruses and hemipteran families (Nault 1997), species of bacteria and hemipteran subfamilies (Frazier 1965), and species of bacteria and hemipteran species (Severin 1945; Nielson 1968). In persistent and propagative transmission, once acquired by the vector a phytoplasma must cross the midgut membrane, spread in the hemolymph, migrate to the salivary glands and enter the saliva in order to be transmitted to another plant (Hogenhout *et al.* 2008b). This suggests that, like the associations between obligate bacterial endosymbionts and insects (Hansen and Moran 2014), persistent phytoplasma-vector associations involve a considerable amount of co-evolution. In our study, *Scaphoideus titanus* (St) and *Euscelidius variegatus*

(Ev), belonging to the Deltocephalinae subfamily, were selected as competent and efficient FDp vectors. Our tests of the phylogenetically related species *Macrosteles quadripunctulatus* (Mq) provided experimental evidence that this species is a non-vector of FD-Dp, despite its ability to transmit other phytoplasmas as already reported in the literature (for an overview see Trivellone 2019). In particular, although Mq is a highly efficient vector of 16SrI-B phytoplasma to daisy (Bosco *et al.* 2007), this species not only is unable to acquire the same pathogen from infected *Vicia faba* but infective specimens, after acquisition on infected daises, are also unable to transmit 16SrI-B phytoplasma to *V. faba* (D. Bosco, pers. comm.). Because an association between Mq and FD-Dp has never been detected in the field (Trivellone 2019), we speculate that the feeding behavior in Mq is host-plant dependent, making a tri-trophic relationship with FD-Dp and its host plants unlikely.

Originally developed by McLean and Kinsey (1964), the electrical penetration graph (EPG) technique was a major breakthrough in the investigation of feeding behavior and feeding site selection of sucking insects. Advanced electronic monitoring systems have been previously applied to study hopperburning leafhopper species (most belonging Typhlocybinae), especially the *Empoasca*-legume model (Backus *et al.* 2005). Analyses with EPG indicates that feeding behavior may be affected by the host plant, e.g. Backus (1994) showed that *Emposca fabae* switches from phloem to nonvascular tissue when feeding on broad beans. Galetto and collaborators (2011b) demonstrated that *Empoasca decipiens* is able to acquire and transmit 16SrI-B phytoplasmas from daisy to daisy with low efficiency but it cannot acquire phytoplasmas from broad bean, suggesting a host plant-dependent feeding site shift from mesophyll to phloem. This evidence was collected on the monophyletic group Typhlocybinae. Although, similar studies have not been conducted on Deltocephalinae (which includes Mq), we infer that Mq also switches among tissues when feeding on the non-preferred food plant.

Two distantly related phloem feeding planthopper species, *Ricania speculum* (Rs) and *Metcalfa pruinosa* (Mp), were also considered here as non-vector of FD-Dp, as both are able to acquire FD-Dp, but not to transmit it to healthy plants (Clair *et al.* 2001; Galetto *et al.* 2019; this study). A similar acquisition rate of FD-Dp from *V. faba* was reported for the two planthoppers, 53% for Rs (Galetto *et al.* 2019), 54% (Clair *et al.* 2001) and 42% (this study) for Mp, and such results are consistent with their phylogenetic relatedness. In our experiments with *V. faba*, FD-Dp was detected in more than 40% of Mp analysed bodies, but its presence within the salivary glands dropped to less than 10%, and the only infected salivary gland had a low load of FD-Dp.

Finally, the leafhopper *Zyginidia pullula* (Zp) was considered in this study as a non-vector belonging to a group of typhlocybines, so called stipplers, which feed preferentially on mesophyll (Backus *et al.* 2005). Moreover, other studies reported that specimens of Zp collected from the field tested negative for the presence of phytoplasmas (Jović *et al.* 2009; Drobnjakovic *et al.* 2010), and there were no interaction signal results from previous investigations on the '*Ca*. P. asteris' membrane protein Amp and proteins from Zp samples (Galetto *et al.* 2011a).

In vector-pathogen associations, it was previously demonstrated that several factors can affect transmission efficiency. For example, environmental constraints (e.g. temperature) can act at short-term temporal scale (Daugherty *et al.* 2009), while synergistic biological interactions (Heck 2018) and molecular matching can evolve at medium- to long-term temporal scales (Frago *et al.* 2012). Host-pathogen interactions occur through different nested levels of biological organization. Since phytoplasmas are intracellular bacterial pathogens, interactions at the molecular level represent the crucial stage governing invasion success in phytoplasma-host interplay (Suzuki *et al.* 2006; Galetto *et al.* 2011a).

471 The phytoplasma membrane protein Imp used in this study as a candidate for interactions
472 with vector proteins is suspected to be the ancestor of the other immunodominant membrane
473 proteins (Kakizawa *et al.* 2009). Moreover, previous studies revealed that Imp may interact
474 with vector (Siampour *et al.* 2011) and host plant proteins (Boonrod *et al.* 2012). Indeed,
475 evidence for strong positive selection has been provided by sequence analyses of the highly
476 variable extracellular domain of Imp homologues of 16SrX (Morton *et al.* 2003) and 16SrII
477 (Siampour *et al.* 2013) phytoplasmas. Interestingly, positive selection acting on phytoplasma
478 membrane proteins has been suggested as a key feature in the adaptation of the pathogen to
479 different hosts (Fabre *et al.* 2011). Strong antigenic properties, previously described for
480 extracellular domains of IDPs (Konnerth *et al.* 2016), were also confirmed here for FD-D
481 Imp. Indeed, preliminary unpublished data confirm that the polyclonal serum, raised against
482 the Imp recombinant domain in this study, resulted in specific recognition of the
483 corresponding native full-length protein in field samples (E. Angelini, pers. comm.).

484 Our study showed a specific interaction between FD-D Imp fusion protein and insect
485 proteins. We also provided evidence for a continuum in interaction with FD-D phytoplasma
486 varying among phylogenetically unrelated insect species. Indeed, decreasing interaction
487 intensities with the fusion Imp peptide were evident between total native proteins of efficient
488 and competent vectors (St and Ev) and those of non-vector insect species. Similarly, the clear
489 gradient of interaction intensities with the FD-D Imp peptide and both total and membrane
490 proteins from guts of St, Ev, and Mq, all belonging to the leafhopper subfamily
491 Deltocephalinae, correlated with their status as ‘natural’, ‘laboratory’ and ‘non -vector’
492 species, respectively. No interaction was detected for the non-vectors Rs, Mp (Fulgoroidea)
493 and Zp (Typhlocybinae) in membrane protein fractions from gut samples. However, the
494 possibility that insect proteins interacting with FD-D Imp might be expressed at different
495 levels among vector and non-vector species, resulting therefore in stronger or weaker

interaction signals, cannot be ruled out. Indeed, the technique used in this study does not allow the identification of insect putative ligands of FD-D Imp and the previous hypothesis cannot be further investigated.

The strong signal detected upon interaction of FD-D Imp and membrane proteins from vector guts supports the role of this organ as the first barrier where a specific interaction is required upon acquisition to ensure successful transmission. Moreover, the weak interaction signals between total proteins of Rs and Mp guts were absent when the corresponding membrane protein fractions were analysed, indicating that the FD-D Imp fusion protein interactome could also include soluble proteins in addition to membrane proteins.

A weak gradient of interaction signals was also observed with salivary gland total protein samples, consistent with the vector status summarized in Table 1.

As far as concerns the overall comparison between signals produced by total and membrane fractions, the total native proteins of entire insect and salivary gland of vector species interacted with phytoplasma recombinant peptide. On the other hand, except for a weak signal in *S. titanus* salivary gland sample, no signal was produced by corresponding membrane fractions. This might probably indicate that cytosolic vector peptides are more likely to be involved in interaction with FD-D Imp or that putative interacting membrane proteins were scarcely represented in these fractions and therefore unable to generate a signal.

Conversely in vector gut samples, both total and membrane portions showed strong signals suggesting that interaction with FD-D Imp is mediated by membrane proteins. Interestingly, FD-D Imp interaction signals were obtained with concentrated proteins of Mq (entire insect and gut), in contrast with its inability to transmit this phytoplasma. As previously discussed, the non-vector status of this species is probably due to low phloem feeding of Mq on *V. faba* (not its preferred host plant). Other discrepancies were observed in samples from Rs and Mp (membrane fractions of entire insects and salivary glands), as well as in samples from the

mesophyll feeder Zp (total proteins of salivary glands). The unexpected signals not consistent with vector status suggest that, beyond the well-supported role for FD-D Imp in molecular relationship with vector proteins, a complex of other key factors might be involved in determining phytoplasma-vector specificity. Further discordances emerged when a signal was present in diluted samples and absent in corresponding undiluted ones (i.e., total protein fractions from entire bodies of St and Ev, and membrane fractions from entire bodies of Rs and Mp). Capturing on the blot an excessive amount of secondary antibody HRP-conjugated, the overloading of insect proteins might have depleted the substrate and caused the signal to quickly fade (Alegria-Schaffer *et al.* 2009).

Taken together, our results showed that specific Imp binding is involved in interactions between FD-D phytoplasma and insect vector proteins and that this molecular recognition probably occurs at the gut barrier level.

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Ethical approval

Not required.

Conflict of interest

546 No conflict of interest declared.

547

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Table 1. Results of transmission specificity for the six species selected in this study. The first two columns of the table report two parameters of the vector status (acquisition and inoculation ability) with respect to the FD phytoplasma, defined using data from literature and results from our experiments (*). Far Western dot Blot (FWdB) results are summarized in the last six columns. The results of the overall interaction are based on the signals observed with three sample dilutions (undiluted starting quantity, 1:10, 1:100, see Fig. 3), and coded as +, ++, and +++, respectively. -, no signal; TNP, total native proteins; MP, membrane proteins.

	Vector status		**FWdB analyses**					
			Entire body		**Gut**		**Salivary gland**	
Species	**Acquisition ability**	**Inoculation ability**	**TNP**	**MP**	**TNP**	**MP**	**TNP**	**MP**
Scaphoideus titanus (St)	**yes**	**yes**	+++	-	++	+	++	+
Euscelidius variegatus (Ev)	**yes**	**yes**	+++	-	+	+	+	-
Macrosteles quadripunctulatus (Mq) *	**no**	**no**	++	-	++	+	-	-
Ricania speculum (Rs)	**yes**	**no**	-	++	+	-	-	+
Metcalfa pruinosa (Mp) *	**yes**	**no**	-	++	+	-	-	+

Zyginidia pullula (Zp)	**no**	**no**	**-**	**-**	**-**	**-**	++	**-**

800 **Figure Legends**

801 **Figure 1.** Full length native FD-D Imp protein structure (A) and structure of the His-tagged
802 recombinant fusion FD-D Imp protein used as antigen for the production of the polyclonal
803 antiserum (B). INd= inner domain; OTd= outer domain; TMd= transmembrane domain; N-
804 ter= N-terminus; C-ter= C-terminus; HisT= histidine tag.

805 **Figure 2.** SDS-Poly-acrylamide gel (A) and Western blots (B) of total proteins extracted
806 from healthy (Healthy) and FD-D-infected (FD-D) *Euscelidius variegatus* (lanes 1), *Vicia*
807 *faba* (broad bean, lanes 2), and *Catharanthus roseus* (periwinkle, lanes 3). Western blots
808 were developed with the polyclonal antiserum against recombinant FD-D Imp fusion protein.
809 M: prestained molecular weight standards (Sharpmass VII, Euroclone); K+: 100 ng of
810 purified recombinant fusion FD-D Imp (15.7 kDa).

811 **Figure 3.** *In vitro* interaction of phytoplasma immunodominant membrane protein with
812 competent vector and non-vector insect proteins analysed by Far-Western dot blots. Total
813 native proteins (TNP) and membrane proteins (MP) from vector and non-vector insect
814 species were blotted onto polyvinyl difluoride membranes with fusion Flavescence dorée
815 (FD) immunodominant membrane proteins (Imp) as a positive control (IMP test) and
816 negative control with buffer devoid of protein bait (No Bait Control). St: *Scaphoideus titanus*,
817 Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp:
818 *Metcalfa pruinosa*, Zp: *Zyginidia pullula*. C: Controls (Imp positive control (A) and BSA
819 negative control (B)). For each species, three serial dilutions were spotted: undiluted starting
820 quantity sample (1), 1:10 (2) and 1:100 dilutions (3). The Anti-FD-D Imp polyclonal
821 antibody was used to detect bound phytoplasma recombinant proteins, and horseradish
822 peroxidase conjugated to goat antirabbit secondary antibody was used for chemiluminescent
823 detection.
824

Supporting Information

Figure S1: Total native proteins from entire insect bodies of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) spotted onto a membrane stained with Ponceau S Solution (A). SDS-Poly-acrylamide gel of total native (1) and membrane (2) proteins from guts of *Euscelidius variegatus* (B); M: prestained molecular weight standards.

Figure S2: SDS-Poly-acrylamide gels of (A) *Escherichia coli* crude cell extract following recombinant expression of FD-D Imp (FD-D clone #1) and (B) corresponding eluted purified protein (5 µg) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

Table S1: Concentration [µg μl^{-1}] of the total (TNP) and membrane (MP) proteins extracted from pools of entire bodies and organs of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) tested in Far-Western dot Blot analysis. The amount of proteins was measured with Bradford reagent (Bio-Rad). The amounts of MP extracted from insect organs were below the limit of detection of Bradford assay, and were not quantified.

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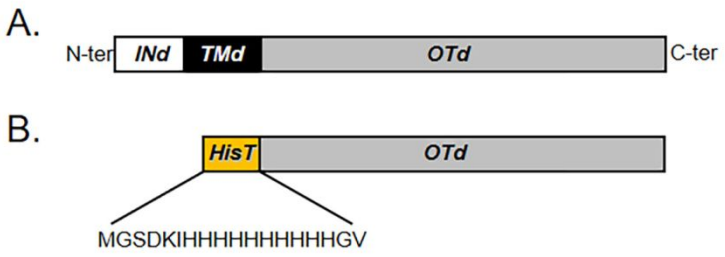
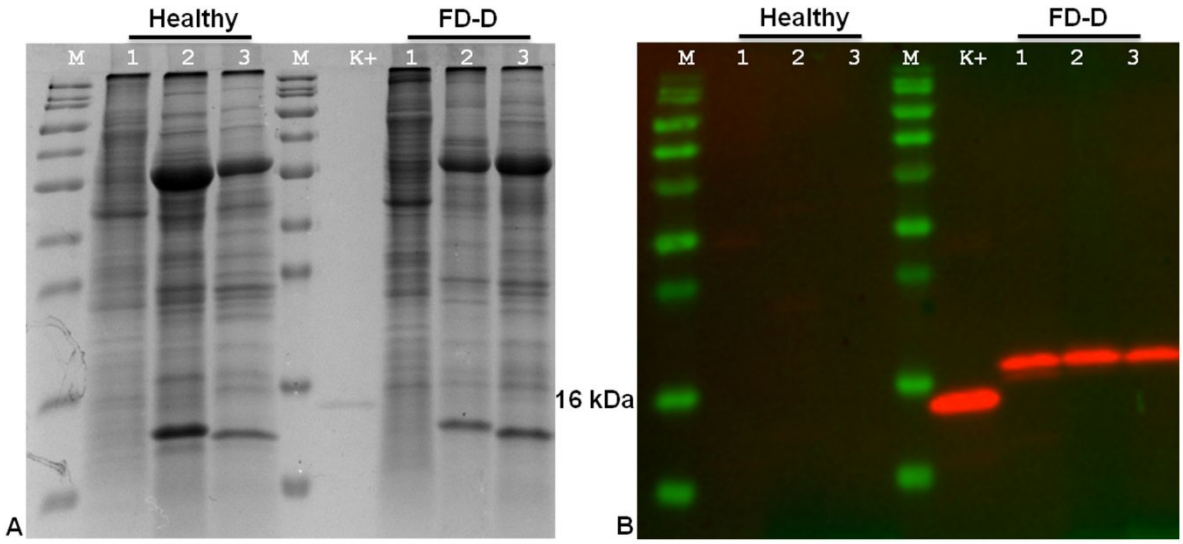


850

851 **Figure 1.**

852



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855 **Figure 2.**

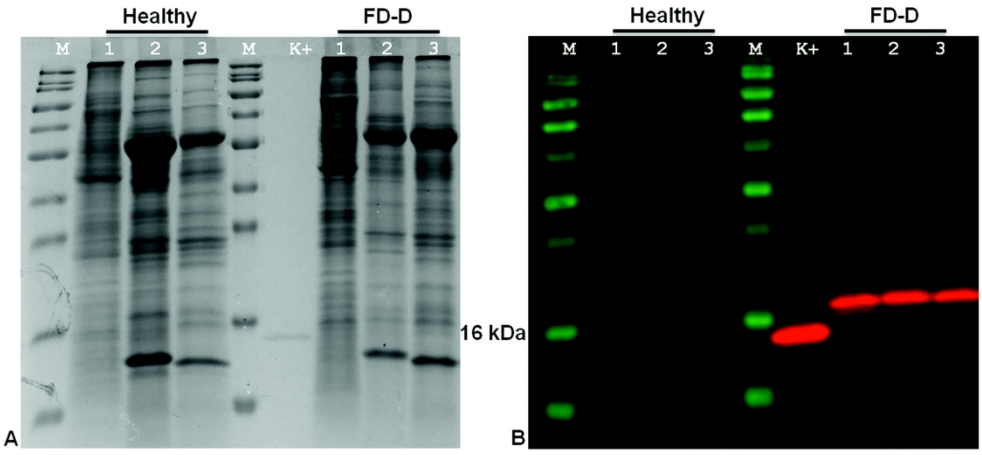

Figure 2. SDS-Poly-acrylamide gel (A) and Western blots (B) of total proteins extracted from healthy (Healthy) and FD-D-infected (FD-D) *Euscelidius variegatus* (lanes 1), *Vicia faba* (broad bean, lanes 2), and *Catharanthus roseus* (periwinkle, lanes 3). Western blots were developed with the polyclonal antiserum against recombinant FD-D Imp fusion protein. M: prestained molecular weight standards (Sharpmass VII, Euroclone); K+: 100 ng of purified recombinant fusion FD-D Imp (15.7 kDa).

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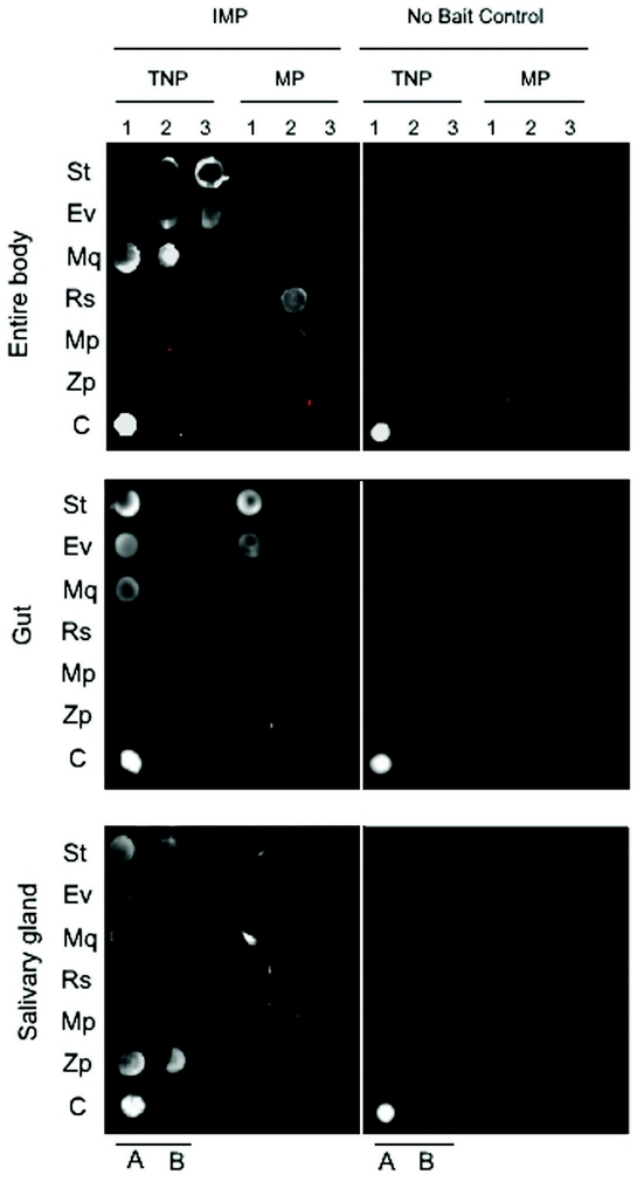

Figure 3. In vitro interaction of phytoplasma immunodominant membrane protein with competent vector and non-vector insect proteins analysed by Far-Western dot blots. Total native proteins (TNP) and membrane proteins (MP) from vector and non-vector insect species were blotted onto polyvinyl difluoride membranes with fusion Flavescence dorée (FD) immunodominant membrane proteins (Imp) as a positive control (IMP test) and negative control with buffer devoid of protein bait (No Bait Control). St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*. C: Controls (Imp positive control (A) and BSA negative control (B)). For each species, three serial dilutions were spotted: undiluted starting quantity sample (1), 1:10 (2) and 1:100 dilutions (3). The Anti-FD-D Imp polyclonal antibody was used to detect bound phytoplasma recombinant proteins, and horseradish peroxidase conjugated to goat antirabbit secondary antibody was used for chemiluminescent detection.

48x86mm (300 x 300 DPI)

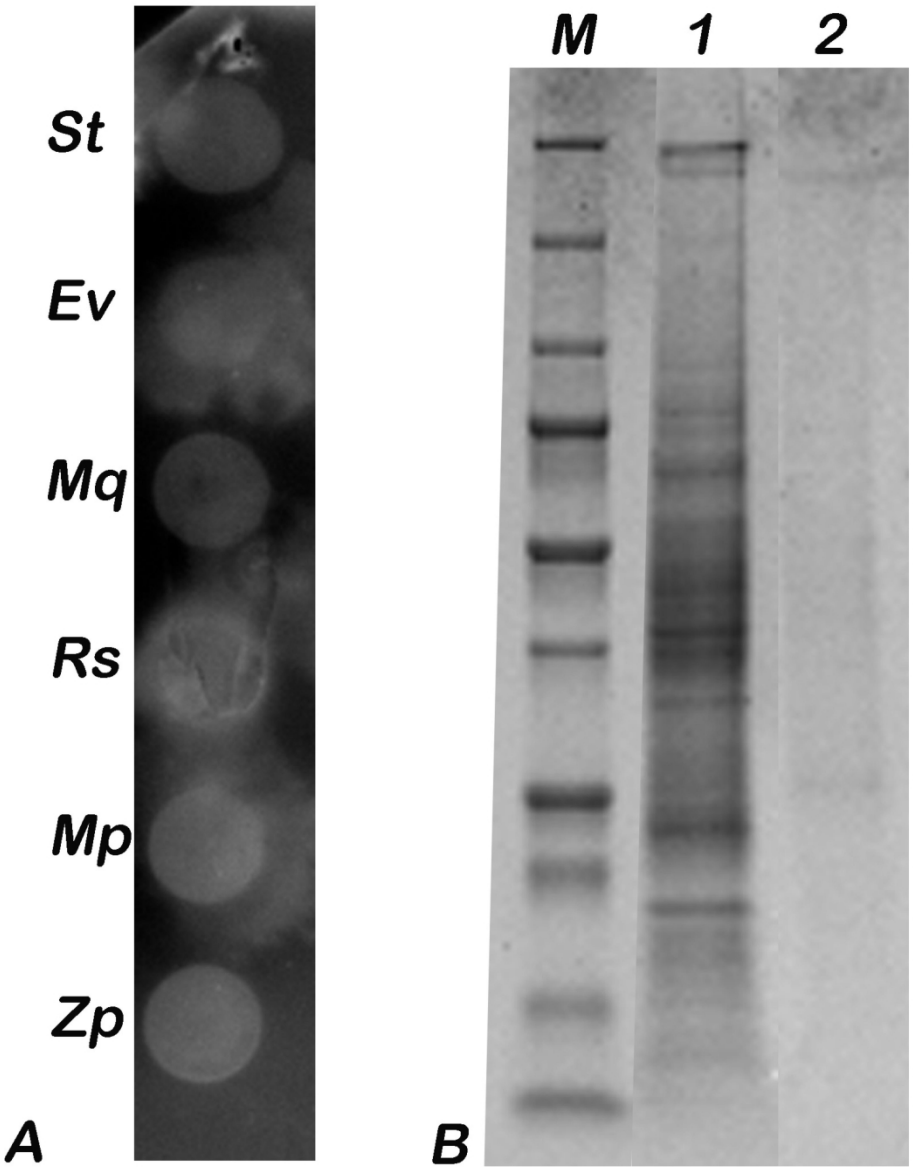

Figure S1: Total native proteins from entire insect bodies of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) spotted onto a membrane stained with Ponceau S Solution (A). SDS-Poly-acrylamide gel of total native (1) and membrane (2) proteins from guts of *Euscelidius variegatus* (B); M: prestained molecular weight standards.

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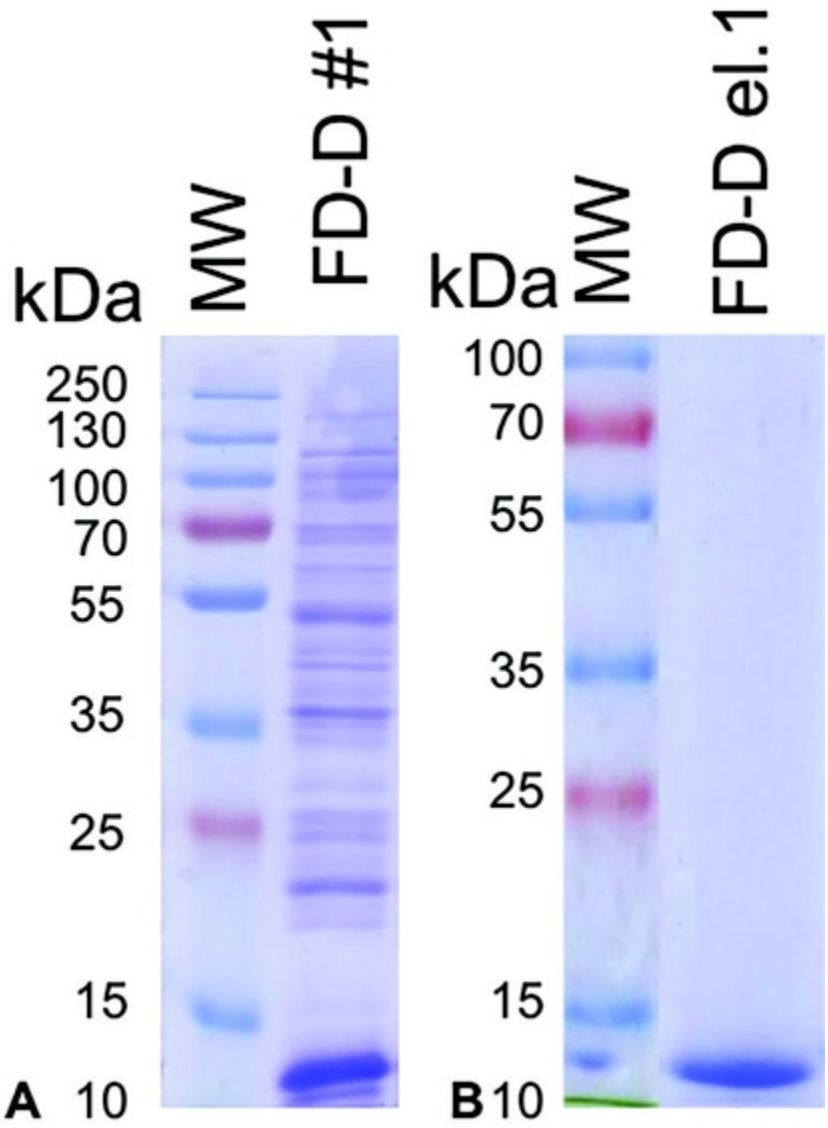

Figure S2: SDS-Poly-acrylamide gels of (A) *Escherichia coli* crude cell extract following recombinant expression of FD-D Imp (FD-D clone #1) and (B) corresponding eluted purified protein (5 µg) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

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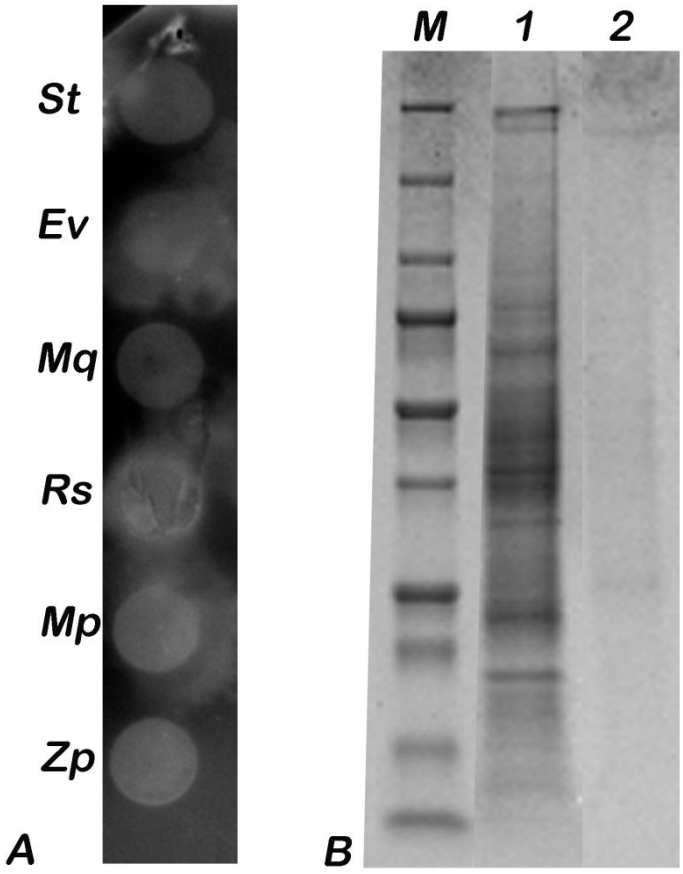
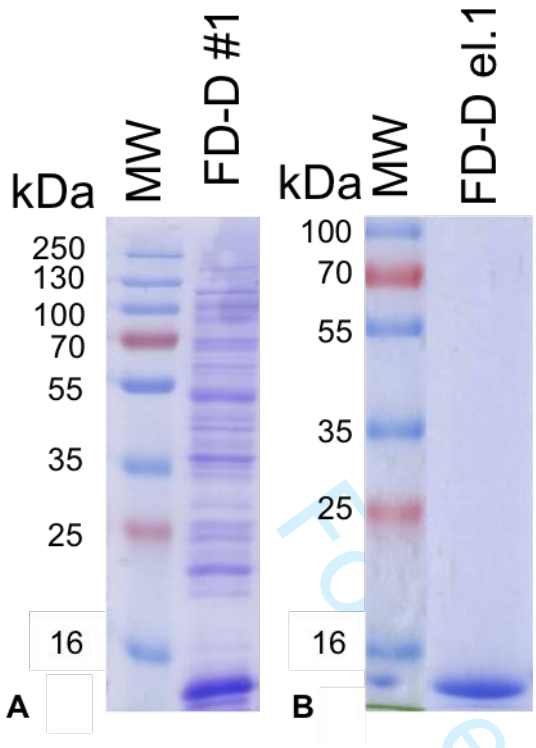


Supplementary Figure S1: Total native proteins from entire insect bodies of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) spotted onto a membrane stained with Ponceau S Solution (A). SDS-Poly-acrylamide gel of total native (1) and membrane (2) proteins from guts of *Euscelidius variegatus* (B); M: prestained molecular weight standards.



Supplementary Figure S2: SDS-Poly-acrylamide gels of (A) *Escherichia coli* crude cell extract following recombinant expression of FD-D Imp (FD-D clone #1) and (B) corresponding eluted purified proteins (5 µg) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

Supplementary Table S1: Concentration [µg µl^{-1}] of the total (TNP) and membrane (MP) proteins extracted from pools of entire bodies and organs of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) tested in Far-Western dot Blot analysis. The amount of proteins was measured with Bradford reagent (Bio-Rad). The amounts of MP extracted from insect organs were below the limit of detection of Bradford assay, and were not quantified.

		Entire Body		Gut	Salivary gland
Species	**Number of specimens**	**TNP**	**MP**	**TNP**	**TNP**
St	5	4.30	1.68	0.50	0.36
Ev	5	2.70	0.50	0.30	0.30
Mq	10	2.70	0.91	0.08	0.07
Rs	3	6.30	5.18	1.52	0.32
Mp	3	4.60	1.54	0.34	0.23
Zp	20	2.00	1.00	0.11	0.04