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(Article begins on next page)

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

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

4  
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19  
20 **Keywords:** grapevine yellows, Imp, leafhopper, planthopper, transmission efficiency

21 **Running headline:** binding between phytoplasma Imp and insect proteins

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25

26 **Abstract**

27 **Aims:** In this study, binding between the immunodominant membrane protein Imp of the  
28 16SrV-D phytoplasma associated with Flavescence dorée disease (FD-Dp) and insect  
29 proteins of vectors and non-vectors of FD-Dp was tested.

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

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

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

48

## 49 **Introduction**

50 Phytoplasmas are phloem-limited wall-less plant pathogenic bacteria in the class Mollicutes  
51 causing hundreds of plant diseases worldwide and responsible for severe economic losses to  
52 agriculturally important plants (Strauss 2009; Marcone 2014). Although phytoplasmas  
53 represent a well-defined monophyletic clade in the family Acholeplasmataceae, they are still  
54 treated as indefinite taxa due to severe difficulties hampering *in vitro* cultivation.

55 Phytoplasma classification is based on *16S rRNA* and ribosomal protein (*rp*) gene sequences  
56 (Lee *et al.* 2000) and the ‘*Candidatus*’ species concept is applied for well characterized  
57 phytoplasmas (Murray and Stackebrandt 1995; IRPCM 2004). The known phytoplasma  
58 vectors are hemipteran insects belonging to the suborder Auchenorrhyncha (Fulgoromorpha  
59 and Cicadomorpha) and the family Psyllidae (suborder Sternorrhyncha) (Weintraub and  
60 Beanland 2006).

61 The 16SrV phytoplasma (16SrVp) phylogenetic group represents the third largest  
62 phytoplasma cluster (Lee *et al.* 2004) showing more than 98.6% 16S rRNA nucleotide  
63 identity among different strains (Lee *et al.* 1998a). However, this group is naturally clustered  
64 in several different ecological niches (Lee *et al.* 1998b) that could contribute to genetic drift.

65 The 16SrVp group comprises six described phytoplasma subgroups, four of which are  
66 described as ‘*Candidatus* Phytoplasma (*Ca. P.*)’ (Bertaccini *et al.* 2014): 16SrV-A (‘*Ca. P.*  
67 *ulmi*’, Lee *et al.* 2004), 16SrV-B (‘*Ca. P. ziziphi*’, Jung *et al.* 2003), 16SrV-E (‘*Ca. P. rubi*’,  
68 Malembic-Maher *et al.* 2011), and 16SrV-F (‘*Ca. P. balanitae*’, Harrison *et al.* 2014). The  
69 remaining two subgroups, 16SrV-C and -D, are assigned to the not formally described ‘*Ca. P.*  
70 *vitis*’ reported as an incidental citation (IRPCM 2004). The latter two genetically  
71 heterogeneous subgroups include well known harmful plant pathogens causing the  
72 Flavescence dorée (FD) disease associated mainly with grapevine, and other diseases  
73 associated with wild host plants. Analyses of single nucleotide polymorphisms (SNPs) in

74 ribosomal and non-ribosomal fragments have been carried out to characterize the relationship  
75 between closely related genotypes clustering in the clade of 16SrV-C and -D subgroups (e.g.  
76 Palatinate grapevine yellows, Alder yellows) (Angelini *et al.* 2001; Martini *et al.* 2002;  
77 Arnaud *et al.* 2007; Rossi *et al.*, 2019). An exclusive association between the FD-related  
78 genotypes, plant hosts and their insect vectors has been suggested (EFSA 2014). The  
79 leafhopper *Scaphoideus titanus* Ball (Cicadellidae: Deltocephalinae) is the only grapevine FD  
80 vector reported to have major epidemiological significance in the field. Other insect species  
81 have been recorded as competent vectors of FD or FD-related genotypes in experimental  
82 conditions but are suspected to play only minor epidemiological roles in spreading FD to  
83 grapevine, and are more likely associated with other epidemiological routes involving  
84 different plants (e.g. clematis and alder). These include *Anoplotettix fuscovenosus*,  
85 *Euscelidius variegatus*, *Euscelis incisus* (Bressan *et al.* 2006), *Orientus ishidae* (Lessio *et al.*  
86 2016), *Allygus* spp. (Malembic-Maher *et al.* 2017), *Dictyophara europaea* (Filippin *et al.*  
87 2009), and *Oncopsis alni* (Maixner *et al.* 2000; Malembic-Maher *et al.* 2017). The first five  
88 species belong to the same subfamily as *S. titanus*, whereas *O. alni* belongs to cicadellid  
89 subfamily Eurymelinae, and *D. europaea* to the phylogenetically distant planthopper family  
90 Dictyopharidae.

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

99 Bouvery *et al.* 2018). Different types of phytoplasma membrane proteins are in direct contact  
100 with the host environment and have been recognized to play an important role in promoting  
101 phytoplasma internalization in insect cells. The major membrane proteins of phytoplasmas are  
102 the immunodominant membrane proteins (IDPs), variable membrane proteins (Vmpps),  
103 membrane transport proteins (e.g. SecY), adhesins, ATP-dependent proteases, as well as  
104 those encoded by potential mobile units and plasmids. However, a number of studies focused  
105 on the IDPs, since they are the most abundant proteins of the cell surface of phytoplasmas  
106 (for an overview see Konnerth *et al.* 2016). Previous studies showed that two  
107 (Immunodominant membrane protein [Imp] and Antigenic membrane protein [Amp]) of the  
108 three non-homologous immunodominant membrane proteins are involved in interactions  
109 between the phytoplasma cell surface and insect proteins (Suzuki *et al.* 2006; Galetto *et al.*  
110 2011a; Siampour *et al.* 2011, 2013; Rashidi *et al.* 2015). In particular, *imp* has high genetic  
111 variability among phytoplasmas and is under strong positive selection pressure, a strong  
112 indicator of its role in interactions with the environment and the host (Kakizawa *et al.* 2009).  
113 Moreover, RNA-Seq studies revealed that *imp* is one of the most expressed genes in FD  
114 phytoplasma (Abbà *et al.* 2014).

115 We investigated the transmission specificity mediated by the immunodominant membrane  
116 protein Imp in the pathosystem of 16SrV-Dp and its insect hosts. A specific genotype in the  
117 16SrV-D subgroup (hereafter FD-D), which is known as the most widespread phytoplasma  
118 strain causing the FD disease of grapevine, was selected (Arnaud *et al.* 2007; Filippin *et al.*  
119 2009). Since available evidence suggested that related pathogens tend to be associated with  
120 particular lineages of leafhopper vectors (Hogenhout *et al.* 2008a; Perilla-Henao and Casteel  
121 2016), the protein-protein interactions of vectors and non-vectors of FD-D phytoplasma (FD-  
122 Dp) selected from distantly related insect species, were tested. The insect species were  
123 chosen to represent a gradient of vector ability with respect to the acquisition and

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

126

## 127 **Materials and Methods**

### 128 *Insect species selection and specimens' recruitment*

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



149 *Scaphoideus* and *Euscelidius* belong to the same cicadellid subfamily (Deltocephalinae) but  
150 are placed in unrelated tribes. One additional deltocephaline leafhopper species, *Macrosteles*  
151 *quadripunctulatus*, which belongs to another unrelated tribe, was selected because it is a  
152 known vector of other phytoplasmas, but not FD-Dp. The competence of *M.*  
153 *quadripunctulatus* was already reported in the literature for two phylogenetically related  
154 phytoplasmas, 16Sr-I and 16Sr-XII (for an overview see Trivellone 2019). According to a  
155 recent phylogenetic study of Membracoidea, the subfamily Typhlocybinae is only distantly  
156 related to Deltocephalinae, the group comprising most phytoplasma vectors (Dietrich *et al.*  
157 2017). Unlike Deltocephalinae, which feed preferentially on phloem, Typhlocybinae are  
158 thought to feed primarily on mesophyll (Backus *et al.* 2005). Therefore, the typhlocybine  
159 species *Zyginidia pullula* was selected for testing as a phylogenetically distantly related non-  
160 vector leafhopper.

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

170 Prior data on AR and TR were not available for *M. quadripunctulatus* and experiments using  
171 specimens collected in the field were published for *M. pruinosa*, so these parameters were  
172 evaluated on laboratory-reared specimens in this study with respect to FD-Dp and the vector  
173 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

199 experimental vineyard in Piedmont and allowed to feed on *V. faba* in the laboratory. This FD  
200 phytoplasma isolate was genetically identified as member of 16SrV-D subgroup based on  
201 DNA sequence analysis (Rossi *et al.* 2019). FD-Dp was then routinely maintained under  
202 controlled conditions with sequential transmissions from broad beans to broad beans by the  
203 experimental vector *E. variegatus*, continuously reared under laboratory conditions on oat.  
204 The same FD-D phytoplasma strain was also transmitted to *Catharanthus roseus* (periwinkle)  
205 by the experimental vector *E. variegatus*, and then routinely maintained under controlled  
206 conditions with sequential grafting on periwinkle.  
207 For FD-Dp acquisition and transmission trials by *M. quadripunctulatus* and *M. pruinosa*,  
208 about 100 nymphs (3<sup>rd</sup> and 4<sup>th</sup> instar) of the former species and about 50 nymphs of the latter  
209 were separately isolated on FD-D-infected broad beans for an acquisition access period of  
210 two weeks, and transferred to healthy broad beans for a latent period of two weeks. Groups of  
211 five FD-Dp exposed adults per plant were then used to inoculate healthy broad beans for  
212 seven days for an inoculation access period. After the inoculation period, insects were  
213 collected and DNA was extracted and analysed by qPCR for the presence of FD-Dp (see  
214 subsection below). Inoculated plants were inspected for symptom appearance twice a week,  
215 and their DNA was extracted one month after the inoculation period.

216

#### 217 *DNA extraction, phytoplasma detection and quantification*

218 Total DNA was extracted from single heads and bodies of *M. quadripunctulatus* and *M.*  
219 *pruinosa*, with cethyl-trimethyl-ammonium bromide (CTAB) buffer, as described in Rashidi  
220 *et al.* (2014). Total DNA was also extracted from plant samples (1 g of leaf tissues) with  
221 CTAB buffer, as described in Pelletier *et al.* (2009). Insect and plant samples were  
222 resuspended in 50 and 100  $\mu\text{l}$  of 0.01 mol l<sup>-1</sup> 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  $\mu$ l volume.  
228 Final concentrations were 300 and 200 nmol l<sup>-1</sup> 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 *secY* gene (Roggia  
233 *et al.* 2014), and MqFw/MqRv, targeting insect *18S* ribosomal gene (Marzachi and Bosco  
234 2005). The sample was diluted to 10 ng  $\mu$ l<sup>-1</sup> and 1  $\mu$ l was added to a qPCR reaction mix of 10  
235  $\mu$ l volume, containing 1x iTaq Universal SYBR Green Supermix (Bio-Rad) and 300 nmol l<sup>-1</sup>  
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 *secY* 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<sup>-1</sup> 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

248 DnaDFDF2/PyrGFD<sub>r</sub>2 (5'-ATA GAA AAT AAC GAT AAA GCA G-3' forward primer, 5'-  
249 TCA AGA CCT TTT AAA CCA CAC CC-3 reverse primer), amplifying a 830 bp genomic  
250 region (Italian patent no. 1429213; *imp* sequence Genbank MK614707). The obtained  
251 sequence was analysed by TMHMM server 2.0 (Sonnhammer *et al.* 1998; Krogh *et al.* 2001)  
252 to exclude its transmembrane domain from further cloning.

253 The His-tagged Imp protein was synthesized via the PCR product of the *imp* FD-D gene  
254 region encoding the C-terminus external domain of the FD-D Imp protein. This portion was  
255 expressed as a fusion protein with a N-terminal 10x His tag by cloning the corresponding  
256 gene fragment in p2N expression vector (Primm srl, Milano, Italy). The FD-D Imp fusion  
257 protein was solubilized in 6 mol l<sup>-1</sup> Urea, 0.02 mol l<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mol l<sup>-1</sup> NaCl, 0.5 mol l<sup>-1</sup>  
258 Imidazole. A rabbit polyclonal antibody was produced by using the His tagged FD-D Imp  
259 fusion protein as recombinant antigen (European patent no. 2918685, Italian patent no.  
260 1429213). The affinity chromatography purified IgG were prepared *in-house* for Western and  
261 Far-Western blot experiments. The IgG were purified on protein A column (r-ProtA  
262 Sepharose Fast Flow; GE Healthcare, 17-1279-01), using Phosphate Buffered Saline (PBS,  
263 0.137 mol l<sup>-1</sup> NaCl, 0.10 mol l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, 0.027 mol l<sup>-1</sup> KCl, pH 7.0) as binding  
264 buffer and 0.1 mol l<sup>-1</sup> glycine (pH 3.0) as elution buffer, with a flow rate of 2 ml min<sup>-1</sup>.

265  
266 *Western blot*

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

273 five adult insects crushed in 0.5 ml of the same buffer. The homogenates were sonicated 1  
274 min and centrifuged 1 min at 13,000 g. Twenty  $\mu$ l of the supernatant were added to 10  $\mu$ l of  
275 Laemmli sample buffer 3x (Laemmli 1970) and 10  $\mu$ l per lane were loaded onto 15%  
276 polyacrylamide gels, together with prestained molecular weight standards (Sharpmass VII,  
277 Euroclone). Gels were either stained with colloidal Coomassie stain (Candiano *et al.* 2004) or  
278 blotted on a polyvinylidene difluoride (PVDF) membrane. The membrane was first incubated  
279 under slow agitation for 30 min in blocking solution (3% bovine serum albumin Tris-buffered  
280 saline with 0.1% Tween, BSA-TBST) and then overnight at 4°C with primary polyclonal  
281 antibody diluted 1:1000 in BSA-TBST. The membrane was then washed four times with  
282 BSA-TBST, incubated for 2 h at room temperature with anti-rabbit horseradish peroxidase  
283 (HRP)-conjugated secondary antibody developed in goat (A0545 GAR-HRP Sigma) diluted  
284 1:15000 in BSA-TBST, washed four times with TBST, and finally developed with West Pico  
285 SuperSignal chemiluminescent substrate (Pierce) in a VersaDoc 4000 MP system (Bio-Rad).

286

### 287 *Far-Western dot Blots*

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

297 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  $\mu\text{l}$  of RX buffer (Suzuki *et al.* 2006), and centrifuged for 1  
307 min at 13,000 g; 250  $\mu\text{l}$  of proteins in the supernatant were transferred in a new tube and  
308 preserved at  $-80^{\circ}\text{C}$  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  $\mu\text{l}$  of collected proteins were added with 6  $\mu\text{l}$  of Base Buffer (0.15 mol  $\text{l}^{-1}$  NaCl, 0.01 mol  
311  $\text{l}^{-1}$  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 ( $\text{CO}_2$  flushed) on a soft dark support of wax using  
316 handle pins, and then pooled in a tube containing 50  $\mu\text{l}$  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  $\mu\text{g}$  of total proteins and  
322 10  $\mu\text{g}$  of membrane proteins were spotted onto polyvinyl difluoride (PVDF, Bio-Rad)



323 membranes with a 96-well Minifold dot-blotter (model I SRC 96D; Schleicher and Schuell,  
324 Dassel, Germany). For dissected insect organs, total (1.6  $\mu\text{g}$  from gut and 0.8  $\mu\text{g}$  from  
325 salivary glands) as well as membrane proteins were spotted onto PVDF membranes.  
326 Undiluted samples of membrane proteins from organs were spotted as these samples were  
327 always below the limit of Bradford detection. All samples were serially diluted 1:10 and  
328 1:100 in PBS (pH 7.4) added with EDTA-free antiprotease cocktail Complete I (Roche) and  
329 spotted onto PVDF membranes as well. One hundred ng of FD-D Imp partial fusion protein  
330 and 40  $\mu\text{g}$  of BSA were spotted as positive and negative controls, respectively. Each sample  
331 spotted onto the PVDF membrane consisted in 20  $\mu\text{l}$ . Each membrane was blocked, incubated  
332 and washed in glass Petri dish (9 cm diameter) with 10 ml of appropriate solution. Two sets  
333 of membranes for each experiment were blocked for 1 h at 4°C with BSA-TBST and then  
334 incubated overnight at 4°C either with the bait recombinant FD-D Imp fusion protein diluted  
335 in BSA-TBST (0.4  $\mu\text{g ml}^{-1}$ ) mixed with EDTA-free antiprotease cocktail Complete I (Roche)  
336 or with buffer only (no bait control) as negative control. The PVDF membranes were washed  
337 for 1 h with BSA-TBST and for 10 min with TBST at room temperature, then incubated 4 h  
338 at RT with anti-FD-D Imp polyclonal antibody, washed 3 times in BSA-TBST, incubated 2 h  
339 with goat anti-rabbit HRP-conjugated secondary antibody (A0545, Sigma-Aldrich), and  
340 washed three times with TBST. Primary and secondary antibodies were diluted in BSA-  
341 TBST to 1:1000 and 1:15000, respectively. Detection was performed with West Pico  
342 SuperSignal chemiluminescent substrate (Pierce) and a VersaDoc 4000 MP (Bio-Rad). Each  
343 experiment was repeated three times, each with freshly extracted insect proteins.  
344 For each sample, the results of the overall interaction were summarized based on the signals  
345 present in the three dilutions (undiluted starting quantity, 1:10, 1:100). Categorical codes  
346 were assigned as follows: +, signal present only in undiluted starting quantity; ++ and +++,  
347 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<sup>-1</sup> 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<sup>th</sup> and 43<sup>th</sup> 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-

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

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

384

#### 385 *Analysis of protein-protein binding*

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

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

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

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

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

406

## 407 **Discussion**

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

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

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

447 Two distantly related phloem feeding planthopper species, *Ricania speculum* (Rs) and  
448 *Metcalfa pruinosa* (Mp), were also considered here as non-vector of FD-Dp, as both are able  
449 to acquire FD-Dp, but not to transmit it to healthy plants (Clair *et al.* 2001; Galetto *et al.*  
450 2019; this study). A similar acquisition rate of FD-Dp from *V. faba* was reported for the two  
451 planthoppers, 53% for Rs (Galetto *et al.* 2019), 54% (Clair *et al.* 2001) and 42% (this study)  
452 for Mp, and such results are consistent with their phylogenetic relatedness. In our  
453 experiments with *V. faba*, FD-Dp was detected in more than 40% of Mp analysed bodies, but  
454 its presence within the salivary glands dropped to less than 10%, and the only infected  
455 salivary gland had a low load of FD-Dp.

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

463 In vector-pathogen associations, it was previously demonstrated that several factors can affect  
464 transmission efficiency. For example, environmental constraints (e.g. temperature) can act at  
465 short-term temporal scale (Daugherty *et al.* 2009), while synergistic biological interactions  
466 (Heck 2018) and molecular matching can evolve at medium- to long-term temporal scales  
467 (Frago *et al.* 2012). Host-pathogen interactions occur through different nested levels of  
468 biological organization. Since phytoplasmas are intracellular bacterial pathogens, interactions  
469 at the molecular level represent the crucial stage governing invasion success in phytoplasma-  
470 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

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

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

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

507 As far as concerns the overall comparison between signals produced by total and membrane  
508 fractions, the total native proteins of entire insect and salivary gland of vector species  
509 interacted with phytoplasma recombinant peptide. On the other hand, except for a weak  
510 signal in *S. titanus* salivary gland sample, no signal was produced by corresponding  
511 membrane fractions. This might probably indicate that cytosolic vector peptides are more  
512 likely to be involved in interaction with FD-D Imp or that putative interacting membrane  
513 proteins were scarcely represented in these fractions and therefore unable to generate a signal.  
514 Conversely in vector gut samples, both total and membrane portions showed strong signals  
515 suggesting that interaction with FD-D Imp is mediated by membrane proteins. Interestingly,  
516 FD-D Imp interaction signals were obtained with concentrated proteins of Mq (entire insect  
517 and gut), in contrast with its inability to transmit this phytoplasma. As previously discussed,  
518 the non-vector status of this species is probably due to low phloem feeding of Mq on *V. faba*  
519 (not its preferred host plant). Other discrepancies were observed in samples from Rs and Mp  
520 (membrane fractions of entire insects and salivary glands), as well as in samples from the

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

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

533

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538

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#### 543 **Ethical approval**

544 Not required.

#### 545 **Conflict of interest**



546 No conflict of interest declared.

547

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- 789

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

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

*Zyginidia pullula*

(Zp)

**no**                      **no**                      -                      -                      -                      -                      ++                      -

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For Peer Review

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: *Macrostes 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

## 825 Supporting Information

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

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

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

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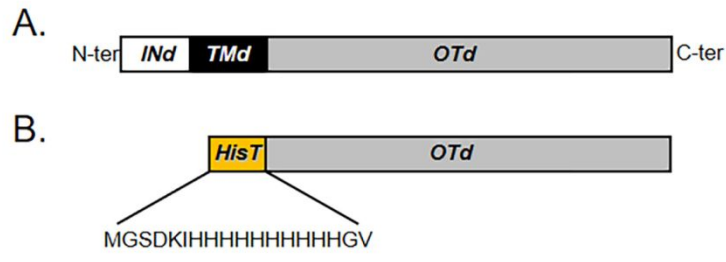
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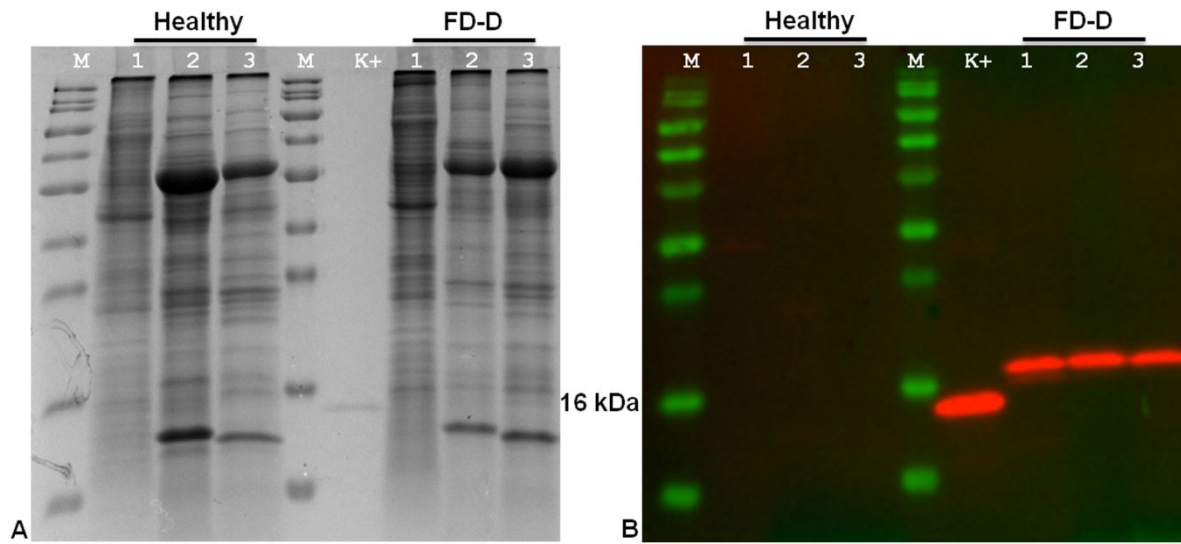


850

851 **Figure 1.**

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For Peer Review



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855 **Figure 2.**

For Peer Review



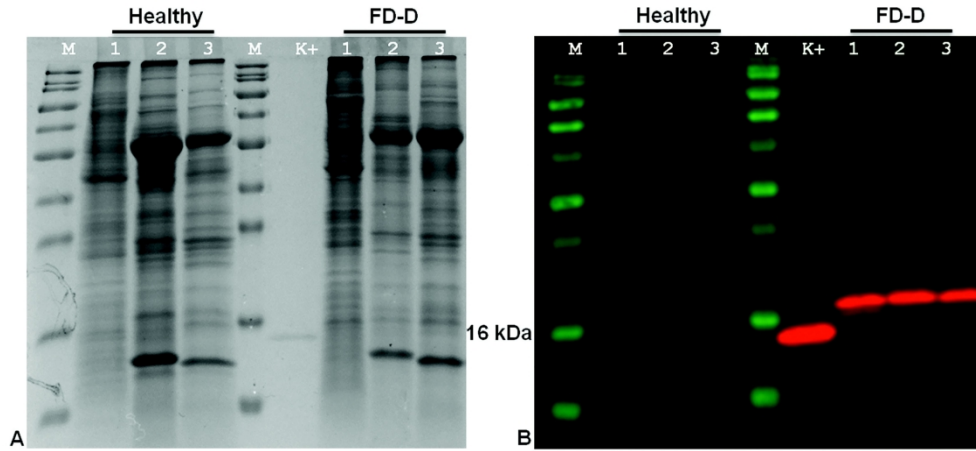


Figure 2. SDS-Poly-acrylamide gel (A) and Western blots (B) of total proteins extracted from healthy (Healthy) and FD-D-infected (FD-D) *Euscelidius variegatus* (lanes 1), *Vicia faba* (broad bean, lanes 2), and *Catharanthus roseus* (periwinkle, lanes 3). Western blots were developed with the polyclonal 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).

202x94mm (300 x 300 DPI)

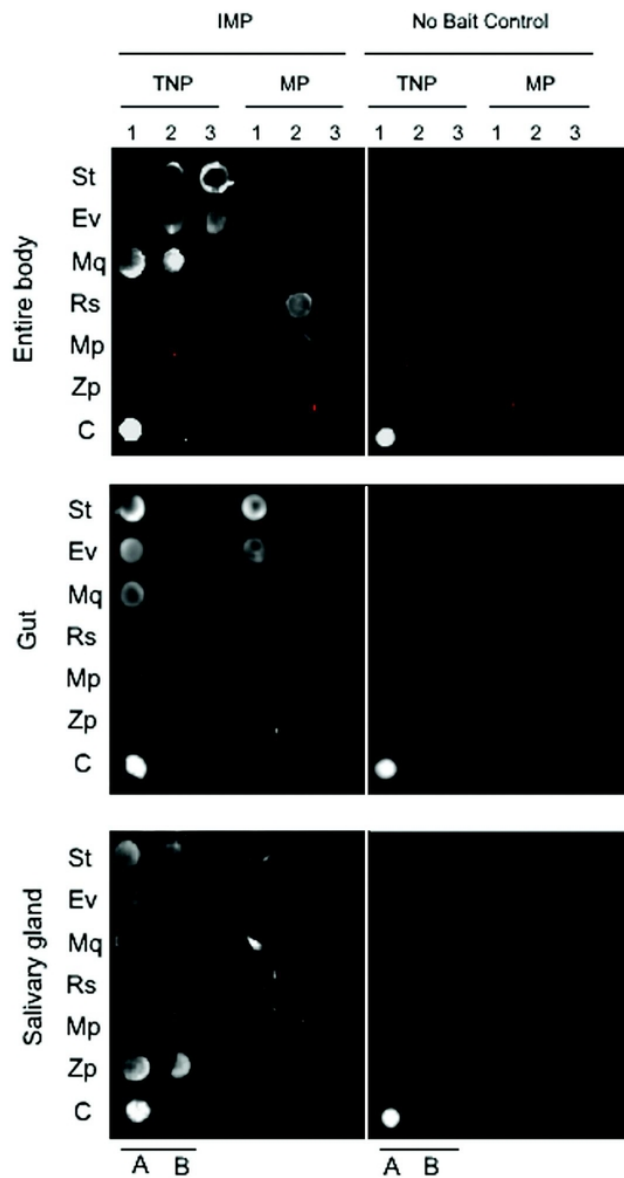


Figure 3. In vitro interaction of phytoplasma immunodominant membrane protein with competent vector and non-vector insect proteins analysed by Far-Western dot blots. Total native proteins (TNP) and membrane proteins (MP) from vector and non-vector insect species were blotted onto polyvinyl difluoride 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: *Macrostelus 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)



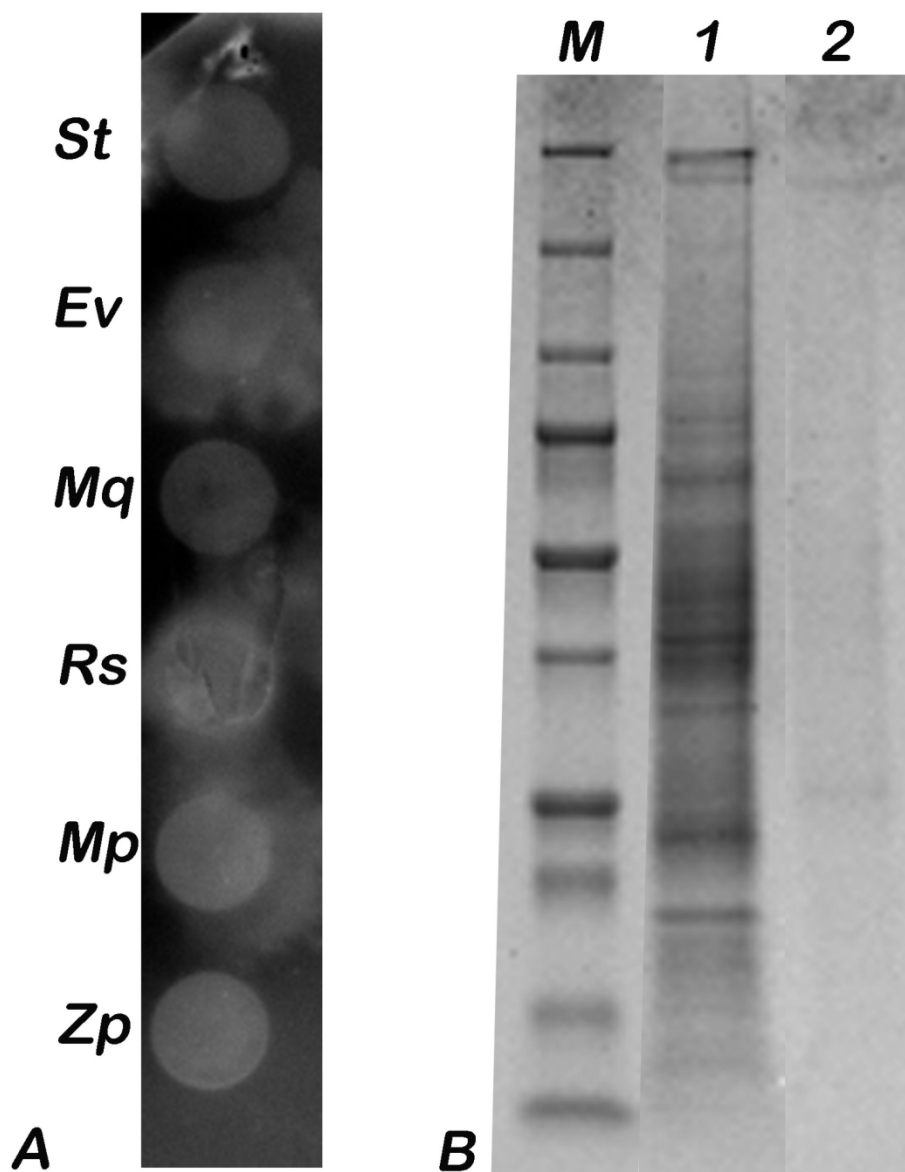


Figure S1: Total native proteins from entire insect bodies of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) spotted onto a membrane stained with Ponceau S Solution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

141x178mm (300 x 300 DPI)

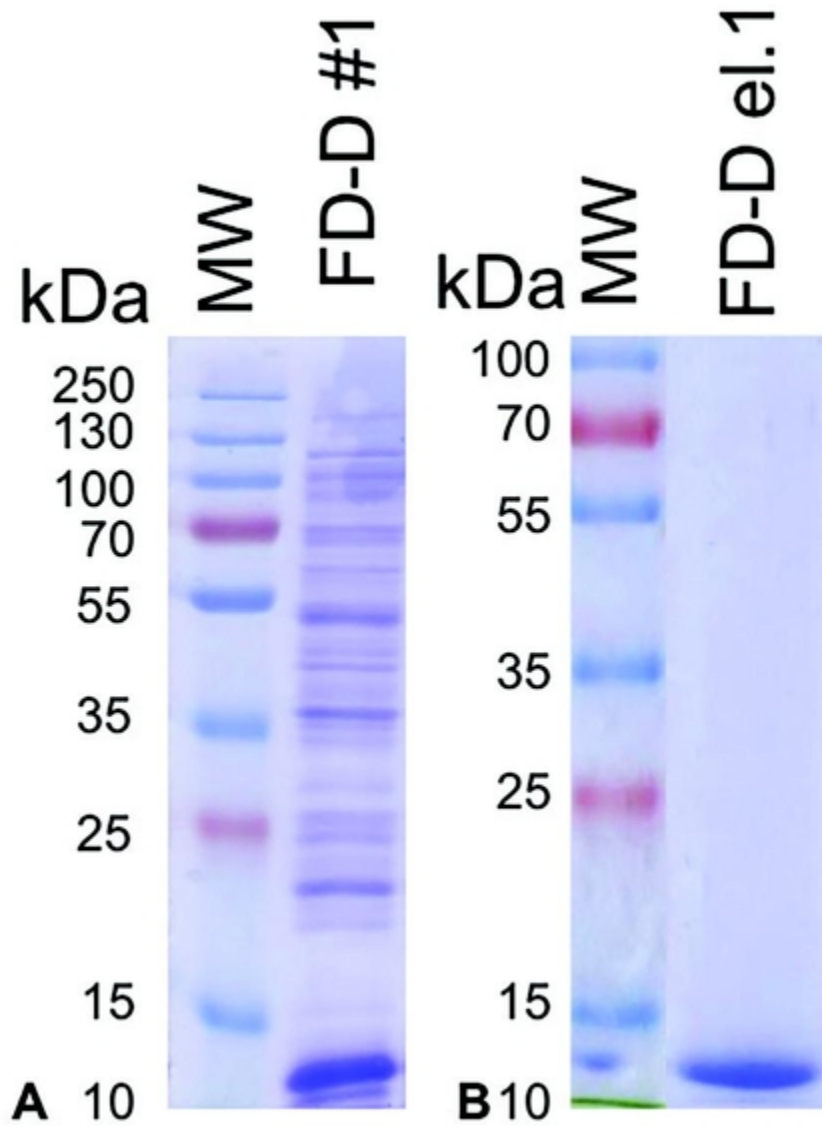
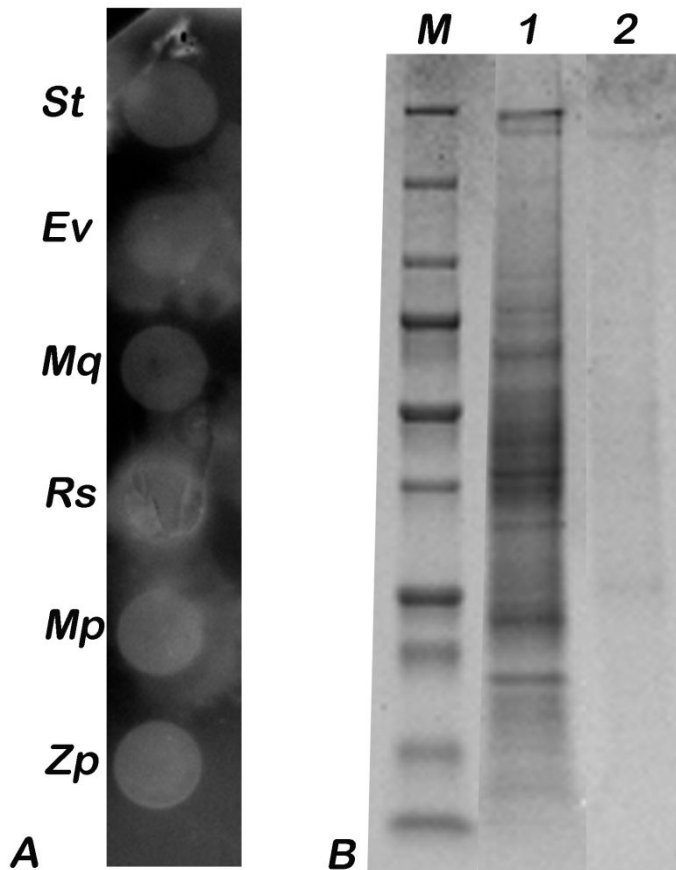
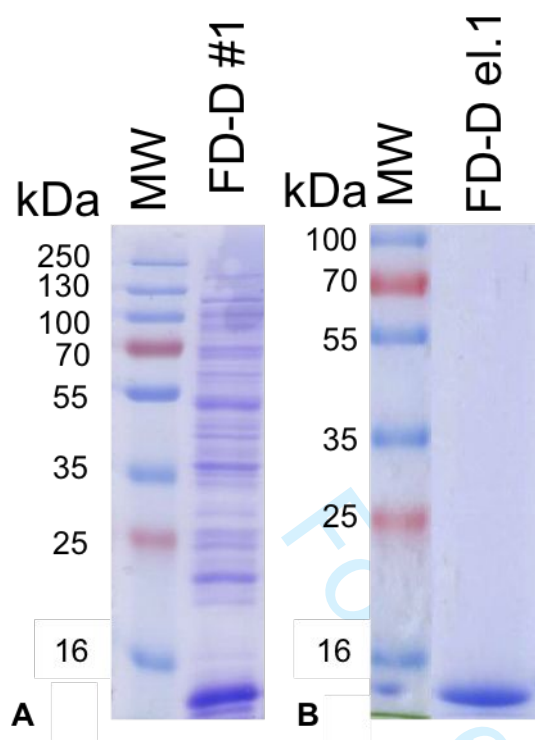


Figure S2: SDS-Poly-acrylamide gels of (A) *Escherichia coli* crude cell extract following recombinant expression of FD-D Imp (FD-D clone #1) and (B) corresponding eluted purified protein (5  $\mu$ g) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

35x48mm (300 x 300 DPI)



**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-Polyacrylamide 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  $\mu$ g) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

**Supplementary Table S1:** Concentration [ $\mu\text{g } \mu\text{l}^{-1}$ ] 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.

Species	Number of specimens	Entire Body		Gut	Salivary gland
		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