

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

Journal:	<i>Applied Microbiology</i>
Manuscript ID	JAM-2019-0804.R2
Journal Name:	Journal of Applied Microbiology
Manuscript Type:	JAM - Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Trivellone, Valeria; University of Illinois at Urbana-Champaign, Illinois Natural History Survey; CREA, Research Centre for Viticulture and Enology Ripamonti, Matteo; National Research Council, Institute for Sustainable Plant Protection; Università degli Studi di Torino, Dipartimento di Scienze Agrarie, Forestali ed Alimentari Angelini, Elisa; CREA, Research Centre for Viticulture and Enology Filippin, Luisa; CREA, Research Centre for Viticulture and Enology Rossi, Marika; National Research Council, Institute for Sustainable Plant Protection Marzachì, Cristina; National Research Council, Institute for Sustainable Plant Protection Galletto, Luciana; National Research Council, Institute for Sustainable Plant Protection
Key Words:	Agriculture, Plant diseases, Proteomics, Diseases, Membrane

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

4
5 V. Trivellone^{1,2*}, M. Ripamonti^{3,4}, E. Angelini², L. Filippin², M. Rossi³, C. Marzachi³, L.
6 Galetto³

7 *¹ Illinois Natural History Survey, Prairie Research Institute, University of Illinois at Urbana-*
8 *Champaign, Champaign, 61820, United State of America*

9 *² Council for Agricultural Research and Economics (CREA), Research Centre for Viticulture*
10 *and Enology, Viale XXVIII aprile 26, 31015 Conegliano (TV), Italy*

11 *³ Institute for Sustainable Plant Protection, National Research Council, Strada delle Cacce*
12 *73, 10135 Turin, Italy*

13 *⁴ Dipartimento di Scienze Agrarie, Forestali ed Alimentari DISAFA, Università degli Studi di*
14 *Torino, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy*

15
16 *Corresponding author: Valeria Trivellone, Illinois Natural History Survey, Prairie Research
17 Institute, University of Illinois at Urbana-Champaign, Champaign, 61820, United State of
18 America. E-mail: valeria.trivellone@gmail.com

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

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

22

23

24

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 (3rd and 4th 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 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 μ l 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 *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 μ l⁻¹ and 1 μ l was added to a qPCR reaction mix of 10
235 μ l 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 *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⁻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

248 DnaDFDF2/PyrGFDr2 (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⁻¹ Urea, 0.02 mol l⁻¹ NaH₂PO₄, 0.5 mol l⁻¹ NaCl, 0.5 mol l⁻¹
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⁻¹ NaCl, 0.10 mol l⁻¹ Na₂HPO₄/KH₂PO₄, 0.027 mol l⁻¹ KCl, pH 7.0) as binding
264 buffer and 0.1 mol l⁻¹ glycine (pH 3.0) as elution buffer, with a flow rate of 2 ml min⁻¹.

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⁻¹ KCl, 3
272 mmol l⁻¹ NaCl, 3.5 mmol l⁻¹ MgCl₂, 1.25 mmol l⁻¹ EGTA, 1.25 mmol l⁻¹ 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 μ l of the supernatant were added to 10 μ l of
275 Laemmli sample buffer 3x (Laemmli 1970) and 10 μ 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 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)

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 μg from gut and 0.8 μg 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 μg of BSA were spotted as positive and negative controls, respectively. Each sample
331 spotted onto the PVDF membrane consisted in 20 μl . 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⁻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 21th and 43th 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

534 **Acknowledgements**

535 We thank Lilia Formica (Agritest, Italy) for helpful discussion and technical help in the use
536 of the polyclonal antiserum. We thank Andrea Lucchi and Elisabetta Rossi for providing
537 *Ricania speculum* nymphs.

538

539 **Funding information**

540 This research was supported by the Swiss National Science Foundation (P2NEP3_168526 /
541 3) and partially by TROPICSAFE project (contract No. 727459), and by Fondazione Cassa di
542 Risparmio di Torino, Project FOotSTEP (RF = 2018-0678).

543 **Ethical approval**

544 Not required.

545 **Conflict of interest**

546 No conflict of interest declared.

547

548 **References**

549 Abbà, S., Galetto, L., Carle, P., Carrère, S., Delledonne, M., Foissac, X., Palmano, S., Veratti,
550 F. and Marzachi, C. (2014) RNA-Seq profile of flavescence dorée phytoplasma in
551 grapevine. *BMC genomics* **15** (1), 1.

552 Alegria-Schaffer, A., Lodge, A. and Vatter K. (2009) Performing and optimizing Western
553 blots with an emphasis on chemiluminescent detection. In *Methods in enzymology*,
554 vol. 463, pp. 573-599. Academic Press.

555 Angelini, E., Clair, D., Borgo, M., Bertaccini, A. and Boudon-Padieu, E. (2001) Flavescence
556 dorée in France and Italy-Occurrence of closely related phytoplasma isolates and their
557 near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma.
558 *Vitis* **40** (2), 79-86.

559 Arnaud, G., Malembic-Maher, S., Salar, P., Bonnet, P., Maixner, M., Marcone, C., Boudon-
560 Padieu, E. and Foissac, X. (2007) Multilocus sequence typing confirms the close
561 genetic interrelatedness of three distinct flavescence dorée phytoplasma strain clusters
562 and group16SrV phytoplasmas infecting grapevine and alder in Europe. *Appl Environ*
563 *Microbiol* **73**, 4001–4010.

564 Arricau-Bouvery, N., Duret, S., Dubrana, M.P., Batailler, B., Desqué, D., Béven, L., Danet,
565 J.L., Monticone, M., Bosco, D., Malembic-Maher, S. and Foissac, X. (2018) Variable
566 membrane protein A of flavescence dorée phytoplasma binds the midgut
567 perimicrovillar membrane of *Euscelidius variegatus* and promotes adhesion to its
568 epithelial cells. *Appl Environ Microbiol* **84** (8), e02487–17.

569 Backus, E.A. (1994) History, development, and applications of the AC electronic monitoring
570 system for insect feeding. *Entomol Soc Am* Lanham, MD, 1–51.

- 571 Backus, E.A., Serrano, M.S. and Ranger, C.M. (2005) Mechanisms of hopperburn: an
572 overview of insect taxonomy, behavior, and physiology. *Annu Rev Entomol* **50**, 125–
573 151.
- 574 Bertaccini, A., Duduk, B., Paltrinieri, S. and Contaldo, N. (2014) Phytoplasmas and
575 phytoplasma diseases: a severe threat to agriculture. *Am J Plant Sci* **5** (12), 1763–
576 1788.
- 577 Boonrod, K., Munteanu, B., Jarausch, B., Jarausch, W. and Krczal, G. (2012) An
578 immunodominant membrane protein (Imp) of ‘*Candidatus Phytoplasma mali*’ binds to
579 plant actin. *Mol Plant Microbe Interact* **25** (7), 889–895.
- 580 Bosco, D., Galetto, L., Leoncini, P., Saracco, P., Raccah, B. and Marzachi, C. (2007)
581 Interrelationships between “*Candidatus Phytoplasma asteris*” and its leafhopper
582 vectors (Homoptera: Cicadellidae). *J Econ Entomol* **100** (5), 1504-1511.
- 583 Bressan, A., Clair, D., Sémétey, O. and Boudon-Padieu, E. (2006) Insect injection and
584 artificial feeding bioassays to test the vector specificity of Flavescence dorée
585 phytoplasma. *Phytopathology* **96** (7), 790–796.
- 586 Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G. M., Carnemolla, B.,
587 Orecchia, P., Zardi, L. and Righetti, P. G. (2004) Blue silver: A very sensitive
588 colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* **25**, 1327–
589 1333.
- 590 Caudwell, A., Kuszla, C., Larrue, J. and Bachelier, J. C. (1972) Transmission de la
591 flavescence dorée de la feve a la feve par des cicadelles des genres *Euscelis* et
592 *Euscelidius*: Intervention possible de ces insectes dans l'epidemiologie du bois noir en
593 Bourgogne. *Annales de phytopathologie*, 181–189.
- 594 Clair, D., Larrue, J., Boudon-Padieu, E. and Lozzia, C. (2001) Evaluation of vectoring ability
595 of phytoplasmas by *Metcalfa pruinosa* Say (Homoptera: Flatidae) recently introduced

- 596 in Europe. In Proceedings of the IOBC-WPRS working group "integrated control in
597 viticulture" at Ponte de Lima, Portugal, March 2–7 2001, *Bulletin-OILB-SROP* **24**
598 (7), 195–197.
- 599 Dietrich, C.H., Allen, J.M., Lemmon, A.R., Lemmon, E.M., Takiya, D.M., Evangelista, O.,
600 Walden, K.K., Grady, P.G., Johnson, K.P. and Wiegmann, B. (2017) Anchored hybrid
601 enrichment-based phylogenomics of leafhoppers and treehoppers (Hemiptera:
602 Cicadomorpha: Membracoidea). *Insect Syst Divers* **1** (1), 57-72.
- 603 Daugherty, M.P., Bosco, D. and Almeida, R.P.P. (2009). Temperature mediates vector
604 transmission efficiency: inoculum supply and plant infection dynamics. *Ann Appl Biol*
605 **155** (3), 361–369.
- 606 Drobnjakovic, T., Peric, P., Marcic, D., Picciau, L., Alma, A., Mitrovic, J., Duduk, B. and
607 Bertaccini, A. (2010). Leafhoppers and cixiids in phytoplasma-infected carrot fields:
608 species composition and potential phytoplasma vectors. *Pestic Phyto-med* **25** (4), 311-
609 318.
- 610 EFSA Panel on Plant Health (PLH) 2014 Scientific Opinion on pest categorisation of
611 Grapevine Flavescence dorée. *EFSA Journal* **12** (10), 3851, 31 pp.
612 doi:10.2903/j.efsa.2014.3851
- 613 Fabre, A., Danet, J.-L. and Foissac, X. (2011) The “stolbur” phytoplasma antigenic
614 membrane protein gene stamp is submitted to diversifying positive selection. *Gene*
615 **472**, 37–41.
- 616 Filippin, L., Jović, J., Cvrković, T., Forte, V., Clair, D., Toševski, I., Boudon-Padieu, E.,
617 Borgo, M. and Angelini, E. (2009) Molecular characteristics of phytoplasmas
618 associated with Flavescence dorée in clematis and grapevine and preliminary results
619 on the role of *Dictyophara europaea* as a vector. *Plant Pathology* **58** (5), 826–837.

- 620 Frago, E., Dicke, M. and Godfray, H.C.J. (2012) Insect symbionts as hidden players in
621 insect–plant interactions. *Trends Ecol Evol* **27** (12), 705-711.
- 622 Frazier, N.W. (1965) Xylem viruses and their insect vectors. In Proceedings International
623 Conference on Virus and Vector on Perennial Hosts, with Special Reference to Vitis
624 ed. Hewitt, W.B. 6-10 September 1965, Davies, CA. University of California Press,
625 Davis, pp. 91–99.
- 626 Galetto, L., Bosco, D., Balestrini, R., Genre, A., Fletcher, J. and Marzachi, C. (2011a) The
627 major antigenic membrane protein of “*Candidatus Phytoplasma asteris*” selectively
628 interacts with ATP synthase and actin of leafhopper vectors. *PLoS One* **6** (7), e22571.
- 629 Galetto, L., Marzachi, C., Demichelis, S. and Bosco, D. (2011b) Host plant determines the
630 phytoplasma transmission competence of *Empoasca decipiens* (Hemiptera:
631 Cicadellidae). *J Econ Entomol* **104** (2), 360–366.
- 632 Galetto, L., Rashidi M., Yamchi, A., Veratti, F. and Marzachi, C. (2014) *In vitro* expression
633 of phytoplasma immunodominant membrane proteins. In Phytoplasmas and
634 phytoplasma diseases management: how to reduce their economic impact ed.
635 Bertaccini, A. pp. 272–279. Bologna: IPWG - International Phytoplasma
636 Working Group.
- 637 Galetto, L., Pegoraro, M., Marzachi, C., Rossi, E., Lucchi, A. and Bosco, D. (2019) Potential
638 role of the alien planthopper *Ricania speculum* as vector of Flavescence dorée
639 phytoplasma. *Eur J Plant Pathol* DOI: 10.1007/s10658-019-01731-0.
- 640 Guadagnini, M., Mori, N., Alberghini, S., Carturan, E., Girolami, V. and Bertaccini, A.
641 (2000) Molecular evidence of phytoplasma transmission to grapevine by *Metcalfa*
642 *pruinosa* (Say) in Italy. In 13th Meeting of the International Council for the study of
643 Virus-like diseases of the Grapevine (ICVG) pp. 99–100. March 12-18, 2000,
644 Adelaide, Australia.

- 645 Hansen, A.K. and Moran, N.A. (2014) The impact of microbial symbionts on host plant
646 utilization by herbivorous insects. *Molecular ecology* **23** (6), 1473–1496.
- 647 Harrison, N.A., Davis, R.E., Oropeza, C., Helmick, E.E., Narvaez, M., Eden-Green, S.,
648 Dollet, M., Dickinson, M. and Konan, J.L. (2014) 'Candidatus phytoplasma
649 palmicola': a novel taxon associated with a lethal yellowing-type disease (LYD) of
650 coconut (*Cocos nucifera* L.) in Mozambique. *Int J Syst Evol Microbiol* **64** (6), 1890-
651 1899.
- 652 Heck, M. (2018) Insect Transmission of Plant Pathogens: a Systems Biology Perspective.
653 *mSystems* **3** (2), e00168–17.
- 654 Hogenhout, S.A., Ammar, E.D., Whitfield, A.E. and Redinbaugh, M.G. (2008a) Insect vector
655 interactions with persistently transmitted viruses. *Annu Rev Phytopathol* **46**, 327–359.
- 656 Hogenhout, S.A., Oshima, K., Ammar, E.D., Kakizawa, S., Kingdom, H.N. and Namba, S.
657 (2008b) Phytoplasmas: bacteria that manipulate plants and insects. *Mol Plant Pathol* **9**
658 (4), 403–423.
- 659 Johnson, K.P., Dietrich, C.H., Friedrich, F., Beutel, R.G., Wipfler, B., Peters, R.S., Allen,
660 J.M., Petersen, M., Donath, A., Walden, K.K. and Kozlov, A.M. (2018)
661 Phylogenomics and the evolution of hemipteroid insects. *Proceedings of the National*
662 *Academy of Sciences* **115** (50), 12775-12780.
- 663 Jović, J., Cvrković, T., Mitrović, M., Krnjajić, S., Petrović, A., Redinbaugh, M.G., Pratt,
664 R.C., Hogenhout, S.A. and Toševski, I. (2009) Stolbur phytoplasma transmission to
665 maize by *Reptalus panzeri* and the disease cycle of maize redness in Serbia.
666 *Phytopathology* **99** (9), 1053-1061.
- 667 Kakizawa, S., Oshima, K., Ishii, Y., Hoshi, A., Maejima, K., Jung, H.Y., Yamaji, Y. and
668 Namba, S. (2009) Cloning of immunodominant membrane protein genes of
669 phytoplasmas and their in planta expression. *FEMS Microbiol Lett* **293** (1), 92–101.

- 670 Konnerth, A., Krczal, G. and Boonrod, K. (2016) Immunodominant membrane proteins of
671 phytoplasmas. *Microbiology* **162** (8), 1267–1273.
- 672 Krogh, A., Larsson, B., Von Heijne, G. and Sonnhammer, E.L. (2001) Predicting
673 transmembrane protein topology with a hidden Markov model: application to
674 complete genomes. *Journal of molecular biology* **305** (3), 567-580
- 675 IRPCM Phytoplasma/Spiroplasma Working Team (2004) Phytoplasma taxonomy group:
676 '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that
677 colonize plant phloem and insects. *Int J Syst Evol Microbiol* **54**, 1243–1255.
- 678 Laemmli, U.K. 1970 Cleavage of structural proteins during the assembly of the head of
679 bacteriophage T4. *Nature* **227** (5259), 680.
- 680 Lee, I.M., Gundersen-Rindal, D.E., Davis, R.E. and Bartoszyk, I.M. (1998a) Revised
681 classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and
682 ribosomal protein gene sequences. *Int J Syst Evol Microbiol* **48** (4), 1153–1169.
- 683 Lee, I.M., Gundersen-Rindal, D.E. and Bertaccini, A. (1998b) Phytoplasma: ecology and
684 genomic diversity. *Phytopathology* **88** (12), 1359–1366.
- 685 Lee, I.M., Davis, R.E. and Gundersen-Rindal, D.E. (2000) Phytoplasma: Phytopathogenic
686 mollicutes. *Annu Rev Microbiol* **54**: 221–255.
- 687 Lee, M., Martini, M., Marcone, C. and Zhu, S.F. (2004) Classification of phytoplasma strains
688 in the elm yellows group (16SrV) and proposal of '*Candidatus* Phytoplasma ulmi' for
689 the phytoplasma associated with elm yellows. *Int J Syst Evol Microbiol* **54** (2), 337–
690 347.
- 691 Lessio, F., Picciau, L., Gonella, E., Tota, F., Mandrioli, M. and Alma, A. (2016) The mosaic
692 leafhopper *Orientalus ishidae*: host plants, spatial distribution, infectivity, and
693 transmission of 16SrV phytoplasmas to vines. *Bull Insectology* **69**, 277–289.

- 694 Lopes, J.R., Daugherty, M.P. and Almeida, R.P. (2009) Context-dependent transmission of a
695 generalist plant pathogen: host species and pathogen strain mediate insect vector
696 competence. *Entomol Exp Appl* **131** (2), 216–224.
- 697 Jung, H.Y., Sawayanagi, T., Kakizawa, S., Nishigawa, H., Wei, W., Oshima, K., Miyata, S.I.,
698 Ugaki, M., Hibi, T. and Namba, S. (2003) ‘*Candidatus Phytoplasma ziziphi*’, a novel
699 phytoplasma taxon associated with jujube witches'-broom disease. *Int J Syst Evol*
700 *Microbiol* **53**(4), 1037-1041.
- 701 Maixner, M., Reinert, W. and Darimont, H. (2000) Transmission of grapevine yellows by
702 *Oncopsis alni* (Schrank) (Auchenorrhyncha: Macropsinae). *Vitis* **39** (2), 83-84.
- 703 Malembic-Maher, S., Salar, P., Filippin, L., Carle, P., Angelini, E. and Foissac, X. (2011)
704 Genetic diversity of European phytoplasmas of the 16SrV taxonomic group and
705 proposal of ‘*Candidatus Phytoplasma rubi*’. *Int J Syst Evol Microbiol* **61** (9), 2129–
706 2134.
- 707 Malembic-Maher, S., Desque, D., Khalil, D., Salar, P., Danet, JL, Bergey, B., Duret, S.,
708 Beven, L., Arricau-Bouvery, N., Jović, J., Krnjajić, S., Angelini, E., Filippin, L.,
709 Ember, I., Kölber, M., Della Bartola, M., Materazzi, A., Lang, F., Jarausch, B.,
710 Maixner, M. and Foissac, X. (2017) When a Palearctic bacterium meets a Nearctic
711 insect vector: genetic and ecological insights into the emergence of the grapevine
712 Flavescence dorée epidemics in Europe. In Book of Abstracts of International
713 Meeting “Future IPM 3.0 towards a sustainable agriculture” pp. 211-213. Riva del
714 Garda (VR, Italy).
- 715 Marccone, C. (2014) Molecular biology and pathogenicity of phytoplasmas. *Ann Appl Biol*
716 **165** (2), 199–221.

- 717 Martini, M., Botti, S., Marcone, C., Marzachi, C., Casati, P., Bianco, P.A., Benedetti, R. and
718 Bertaccini, A. (2002) Genetic variability among Flavescence dorée phytoplasmas
719 from different origins in Italy and France. *Mol Cell Probes* **16** (3), 197–208.
- 720 Marzachi, C. and Bosco, D. (2005) Relative quantification of chrysanthemum yellows (16Sr
721 I) phytoplasma in its plant and insect host using real-time polymerase chain reaction.
722 *Molecular Biotechnology* **30** (2), 117–128.
- 723 McLean, D.L. and Kinsey, M.G. (1964) A technique for electronically recording aphid
724 feeding and salivation. *Nature* **202** (4939), 1358.
- 725 Miliordos, D.E., Galetto, L., Ferrari, E., Pegoraro, M., Marzachi, C. and Bosco, D. (2017)
726 Acibenzolar-S-methyl may prevent vector-mediated flavescence dorée phytoplasma
727 transmission, but is ineffective in inducing recovery of infected grapevines. *Pest*
728 *Manag Sci* **73** (3), 534–540.
- 729 Morton, A., Davies, D.L., Blomquist, C.L. and Barbara, D.J. (2003) Characterization of
730 homologues of the apple proliferation immunodominant membrane protein gene from
731 three related phytoplasmas. *Mol Plant Pathol* **4** (2), 109–114.
- 732 Murray, R.E. and Stackebrandt, E. (1995) Taxonomic Note: Implementation of the
733 Provisional Status *Candidatus* for Incompletely Described Prokaryotes. *Int J Syst*
734 *Bacteriol* **45**, 186–187.
- 735 Nault, L.R. (1997) Arthropod transmission of plant viruses: a new synthesis. *Ann Entomol*
736 *Soc Am* **90** (5), 521–541.
- 737 Nielson, M.W. (1968) The leafhopper vectors of phytopathogenic viruses (Homoptera,
738 Cicadellidae): taxonomy, biology, and virus transmission, Technical Bulletin No.
739 1382, Agricultural Research Service, US Department of Agriculture, Washington,
740 D.C.

- 741 Pelletier, C., Salar, P., Gillet, J., Cloquemin, G., Very, P., Foissac, X. and Malembic-Maher,
742 S. (2009) Triplex real-time PCR assay for sensitive and simultaneous detection of
743 grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous
744 analytical control. *Vitis* **48** (2), 87–95.
- 745 Perilla-Henao, L.M. and Casteel, C.L. (2016). Vector-borne bacterial plant pathogens:
746 interactions with hemipteran insects and plants. *Front Plant Sci* **7**, 1163.
- 747 Rashidi, M., D'amelio, R., Galetto, L., Marzachi, C. and Bosco, D. (2014) Interactive
748 transmission of two phytoplasmas by the vector insect. *Ann Appl Biol* **165** (3), 404–
749 413.
- 750 Rashidi, M., Galetto, L., Bosco, D., Bulgarelli, A., Vallino, M., Veratti, F. and Marzachi, C.
751 (2015) Role of the major antigenic membrane protein in phytoplasma transmission by
752 two insect vector species. *BMC Microbiol* **15** (1), 193.
- 753 Robert, Y. and Bourdin, D. (2001) Aphid transmission of potato viruses. In Virus and virus-
754 like diseases of potatoes and production of seed-potatoes ed. Loebenstein, G., Berger,
755 P.H., Brunt, A.A. and Lawson, R.H. pp. 195–225. Dordrecht: Springer.
- 756 Roggia, C., Caciagli, P., Galetto, L., Pacifico, D., Veratti, F., Bosco, D. and Marzachi, C.
757 (2014) Flavescence dorée phytoplasma titre in field-infected Barbera and Nebbiolo
758 grapevines. *Plant Pathology* **63** (1), 31-41.
- 759 Rossi, M., Pegoraro, M., Ripamonti, M., Abbà, S., Beal, D., Giraudo, A., Veratti, F.,
760 Malembic-Maher, S., Salar, P., Bosco, D. and Marzachi, C. (2019) Genetic diversity
761 of Flavescence dorée phytoplasmas at vineyard scale. *Appl Environ Microbiol* **85**
762 (10), e03123-18.
- 763 Schvester, D., Carle, P. and Moutous, G. (1963) Transmission de la flavescence dorée de la
764 vigne par *Scaphoideus littoralis* Ball. *Annales des Epiphyties* **14**, 175–198.

- 765 Severin, H. (1945) Evidence of nonspecific transmission of California aster-yellows virus by
766 leafhoppers. *Hilgardia* **17** (1), 21–59.
- 767 Siampour, M., Galetto, L., Bosco, D., Izadpanah, K. and Marzachi, C. (2011) In vitro
768 interactions between immunodominant membrane protein of lime witches' broom
769 phytoplasma and leafhopper vector proteins. *Bull Insectology* **64** (Supplement), S149–
770 S150.
- 771 Siampour, M., Izadpanah, K., Galetto, L., Salehi, M. and Marzachi, C. (2013) Molecular
772 characterization, phylogenetic comparison and serological relationship of the Imp
773 protein of several '*Candidatus* Phytoplasma aurantifolia' strains. *Plant Pathology* **62**
774 (2), 452–459.
- 775 Sonnhammer, E.L., Von Heijne, G. and Krogh, A. (1998) A hidden Markov model for
776 predicting transmembrane helices in protein sequences. *Ismb* **6**, 175–182.
- 777 Strauss, E. (2009). Phytoplasma research begins to bloom. *Science* **325**, 388–390.
- 778 Suzuki, S., Oshima, K., Kakizawa, S., Arashida, R., Jung, H.Y., Yamaji, Y., Nishigawa, H.,
779 Ugaki, M. and Namba, S. (2006) Interaction between the membrane protein of a
780 pathogen and insect microfilament complex determines insect-vector specificity.
781 *Proceedings of the National Academy of Sciences* **103** (11), 4252–4257.
- 782 Trivellone, V. (2019) An online global database of Hemiptera-Phytoplasma-Plant biological
783 interactions. *Biodivers Data J* **7**, e32910. <https://bdj.pensoft.net/article/32910/>
- 784 Verbeek, M., Piron, P.G.M., Dullemans, A.M., Cuperus, C. and van Der Vlugt, R.A.A.
785 (2010) Determination of aphid transmission efficiencies for N, NTN and Wilga strains
786 of Potato virus Y. *Ann Appl Biol* **156** (1), 39–49.
- 787 Weintraub, P.G. and Beanland, L. (2006) Insect vectors of phytoplasmas. *Annu Rev Entomol*
788 **51**, 91–111.
- 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

-

-

-

-

++

-

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: *Macrostelus 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.

842

843

844

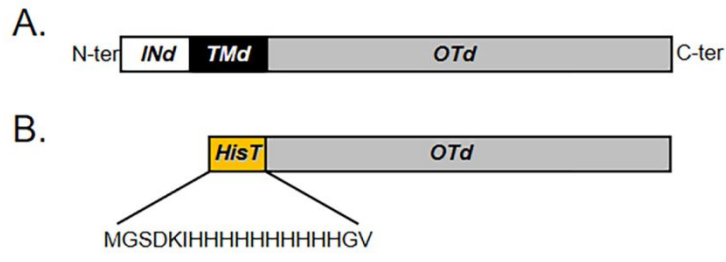
845

846

847

848

849

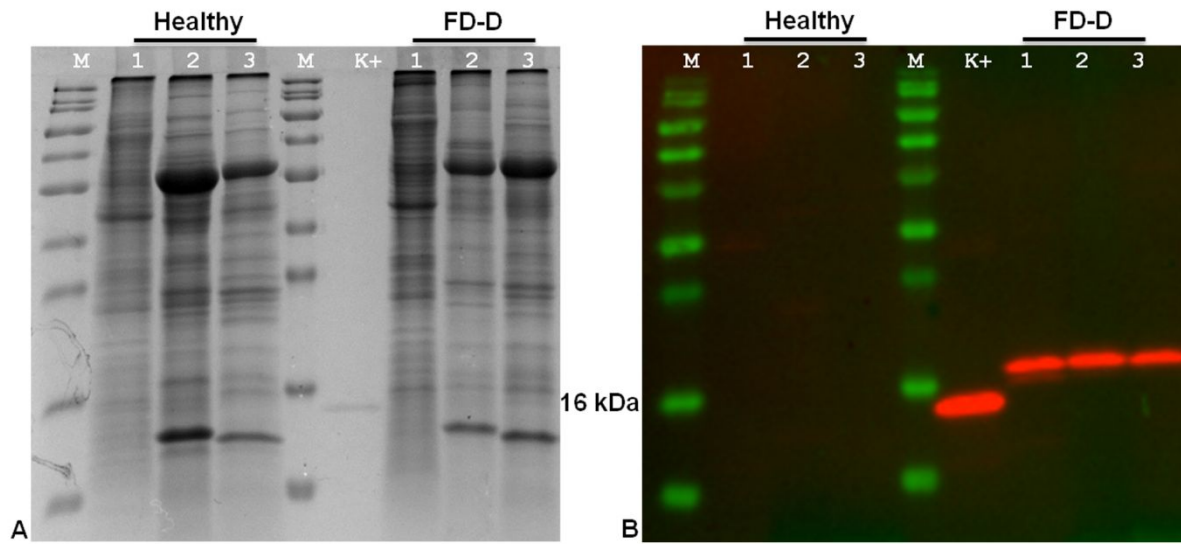


850

851 **Figure 1.**

852

For Peer Review



854

855 **Figure 2.**

For Peer Review

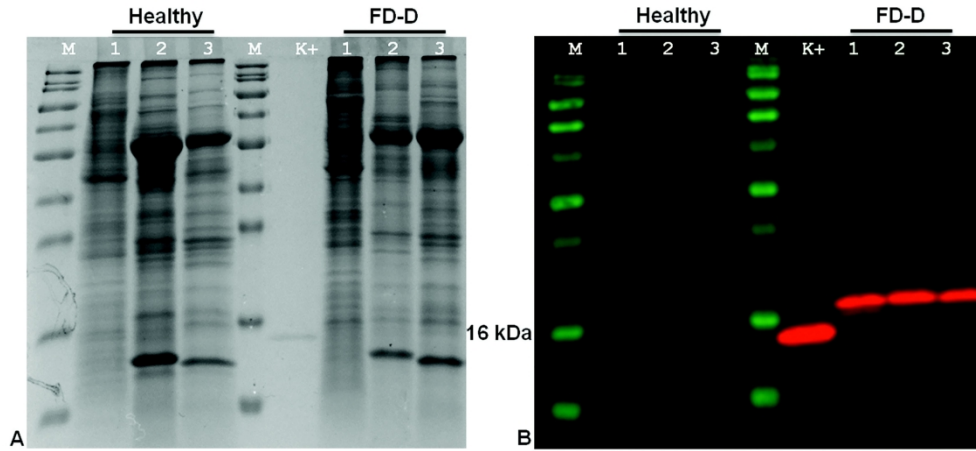


Figure 2. SDS-Poly-acrylamide gel (A) and Western blots (B) of total proteins extracted from healthy (Healthy) and FD-D-infected (FD-D) *Euscelidius variegatus* (lanes 1), *Vicia faba* (broad bean, lanes 2), and *Catharanthus roseus* (periwinkle, lanes 3). Western blots were developed with the polyclonal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

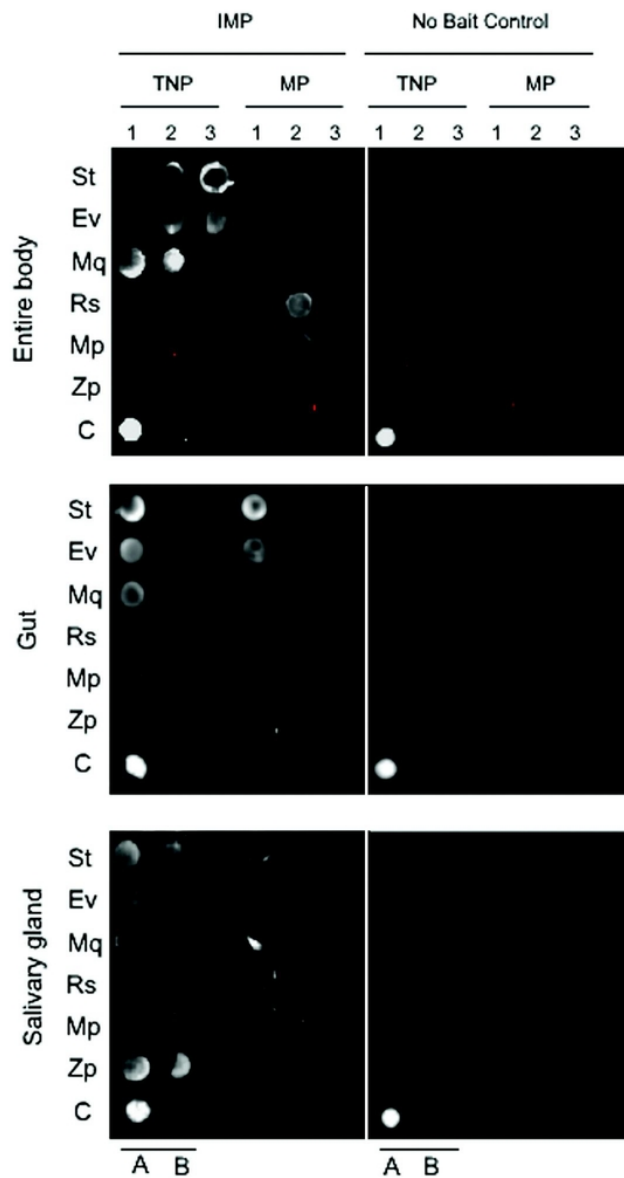


Figure 3. In vitro interaction of phytoplasma immunodominant membrane protein with competent vector and non-vector insect proteins analysed by Far-Western dot blots. Total native proteins (TNP) and membrane proteins (MP) from vector and non-vector insect species were blotted onto polyvinyl difluoride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

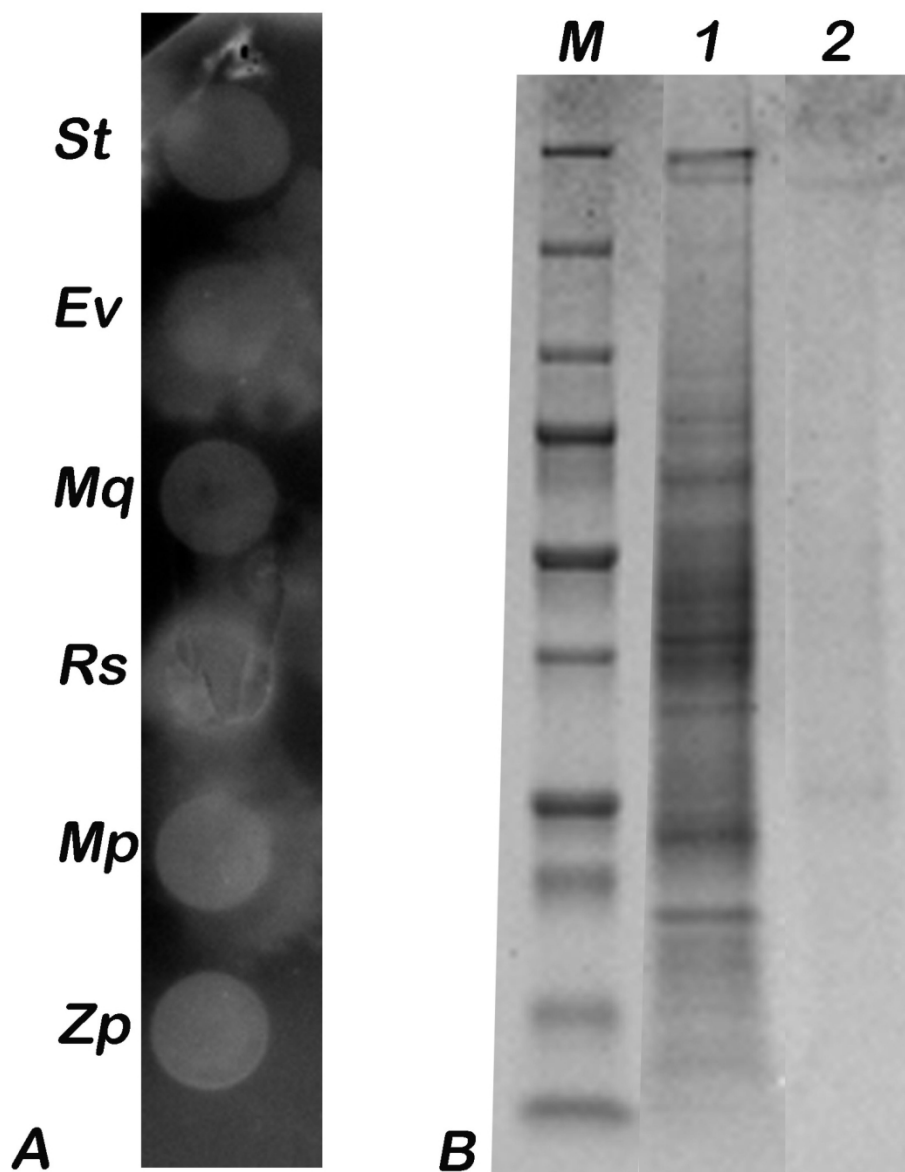


Figure S1: Total native proteins from entire insect bodies of the six species (*St*: *Scaphoideus titanus*, *Ev*: *Euscelidius variegatus*, *Mq*: *Macrosteles quadripunctulatus*, *Rs*: *Ricania speculum*, *Mp*: *Metcalfa pruinosa*, *Zp*: *Zyginidia pullula*) spotted onto a membrane stained with Ponceau 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)

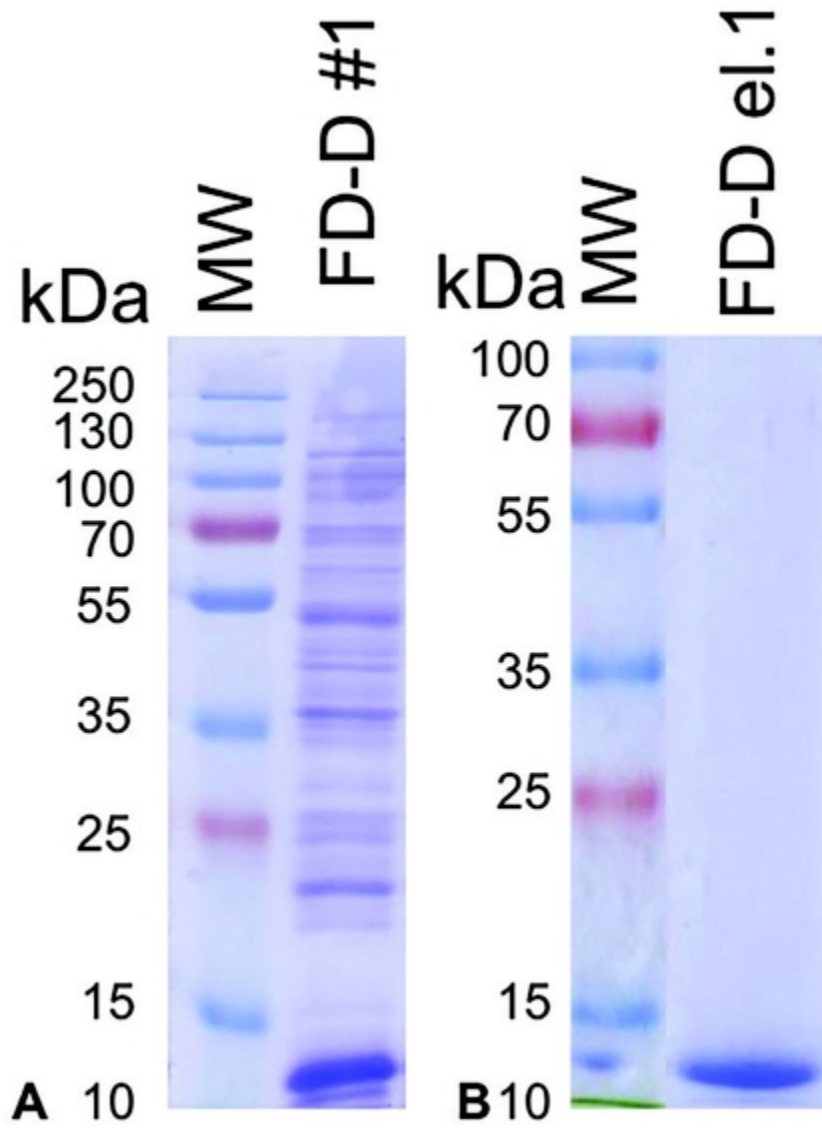
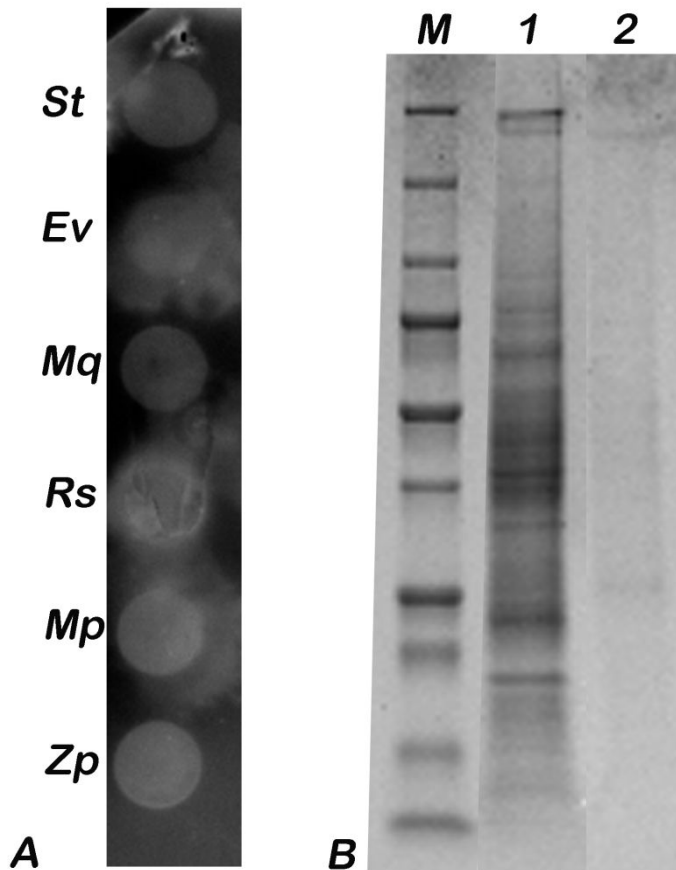
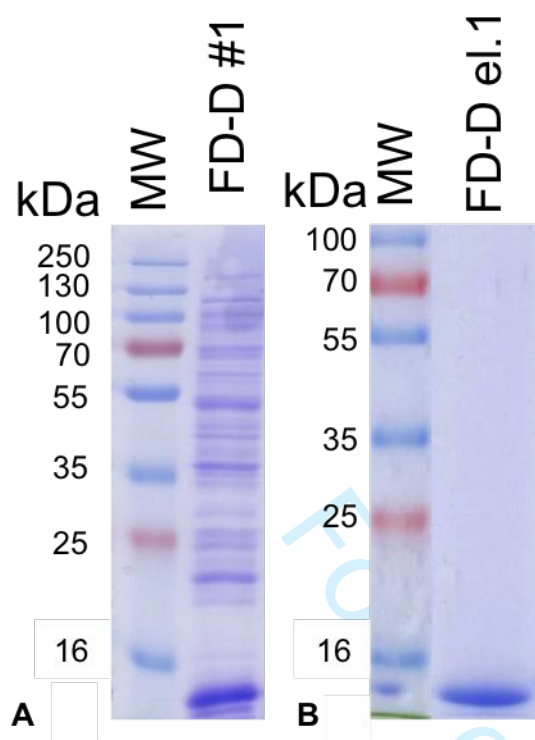


Figure S2: SDS-Poly-acrylamide gels of (A) *Escherichia coli* crude cell extract following recombinant expression of FD-D Imp (FD-D clone #1) and (B) corresponding eluted purified protein (5 μ g) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

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 μ 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