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

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3 **species.**

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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*, *Macrosteles 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 (Vmps),

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

8

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 μl volume.

228   Final concentrations were 300 and 200 nmol l^{-1} for primers and probe, respectively, and

229   cycling conditions were as indicated in the original paper (Pelletier *et al.* 2009). Samples

230   were run in duplicate in a CFX Connect Real-Time PCR Detection System (Bio-Rad).

231   The concentration of FD-Dp in the unique positive *M. pruinosa* head sample was quantified

232   by qPCR using two primer pairs: FdSecyFw/FdSecyRv, targeting FD-Dp *sec*Y gene (Roggia

233   *et al.* 2014), and MqFw/MqRv, targeting insect *18S* ribosomal gene (Marzachí and Bosco

234   2005). The sample was diluted to 10 ng $μl^{-1}$ and 1 μl was added to a qPCR reaction mix of 10

235   μl volume, containing 1x iTaq Universal SYBR Green Supermix (Bio-Rad) and 300 nmol l^{-1}

236   of each primer. The sample was then run in triplicate in a CFX Connect Real-Time PCR

237   Detection System (Bio-Rad). Cycling conditions were as detailed in the original paper

238   (Roggia *et al.* 2014). For each primer pair a standard curve, based on serial dilutions of either

239   a plasmid harboring FD-Dp *sec*Y gene or total DNA of a healthy insect sample, was run.

240   Mean starting quantity was automatically calculated by CFX Maestro™ Software (Bio-Rad)

241   and used to express FD-Dp amount as Genome Unite (GU) ng^{-1} insect DNA.

242

243   *Fusion proteins and antibody production*

244   The *dnaD-imp-pyrG* genomic fragment of FD-D strain was amplified from FD-D infected

245   vine leaves by nested PCR using primer pair DnaDFDf1/PyrGFDr1 (5'-TAG AGA GAA

246   TTT TAG GCC ACG-3' forward primer, 5'-AAT AAT GAA GAA CAA TTA CCT G-3'

247   reverse primer), generating a 940 bp amplicon, followed by primer pair

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} NaH_2PO_4, 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} Na_2HPO_4/KH_2PO_4, 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} $MgCl_2$, 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 μl of the supernatant were added to 10 μl of

275   Laemmli sample buffer 3x (Laemmli 1970) and 10 μl 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)

13

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 µg 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.

14

348

Results

Vector status and transmission trials

For *S. titanus*, *E. variegatus*, and *R. speculum* detailed data about their acquisition and

transmission ability of the FD-Dp have been already published (Schvester *et al.* 1963;

Caudwell *et al.* 1972; Galetto *et al.* 2019), and here summarized in Table 1.

Acquisition and inoculation experiments were also performed in this study to assess the

vector status of two species, *M. quadripunctulatus* and *M. pruinosa*, for which no detailed

data on AR and TR were available in the literature. For *M. quadripunctulatus*, following

isolation on FD-D-infected broad bean plants, all insects (N=19) and all inoculated plants

(N=5) tested PCR negative for the presence of FD-Dp. Following isolation of *M. pruinosa* on

FD-D-infected broad bean plants, five out of 12 body samples and one out of 11 head

samples tested PCR positive for the presence of FD-Dp. The pathogen amount in the unique

positive head sample was 134 FD GU ng^{-1} of insect DNA. All plants (N=5) inoculated by *M.*

pruinosa adults exposed to FD-Dp tested PCR negative for the presence of FD phytoplasma

and were asymptomatic two months after the inoculation. *Macrosteles quadripunctulatus* and

M. pruinosa were therefore considered here as phylogenetically related and unrelated non-

vectors of FD-Dp, respectively, as compared to the primary competent vector, *S. titanus*.

Fusion antigen and antibody specificity

The predicted number of FD-D Imp amino acids was 162, with a molecular weight of 18052

Dalton and pI is 9.70. TMHMM identified a transmembrane domain corresponding to the N-

terminal hydrophobic region between 21th and 43th residues. Residues 44-162 were predicted

to be exposed outside the phytoplasma membrane. Structure and predicted membrane

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   *Emposca 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

21

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

543   **Ethical approval**

544   Not required.

545   **Conflict of interest**

546   No conflict of interest declared.

547

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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.

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

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

800   **Figure Legends**

801   **Figure 1.** Full length native FD-D Imp protein structure (A) and structure of the His-tagged

802   recombinant fusion FD-D Imp protein used as antigen for the production of the polyclonal

803   antiserum (B). INd= inner domain; OTd= outer domain; TMd= transmembrane domain; N-

804   ter= N-terminus; C-ter= C-terminus; HisT= histidine tag.

805   **Figure 2.** SDS-Poly-acrylamide gel (A) and Western blots (B) of total proteins extracted

806   from healthy (Healthy) and FD-D-infected (FD-D) *Euscelidius variegatus* (lanes 1), *Vicia*

807   *faba* (broad bean, lanes 2), and *Catharanthus roseus* (periwinkle, lanes 3). Western blots

808   were developed with the polyclonal antiserum against recombinant FD-D Imp fusion protein.

809   M: prestained molecular weight standards (Sharpmass VII, Euroclone); K+: 100 ng of

810   purified recombinant fusion FD-D Imp (15.7 kDa).

811   **Figure 3.** *In vitro* interaction of phytoplasma immunodominant membrane protein with

812   competent vector and non-vector insect proteins analysed by Far-Western dot blots. Total

813   native proteins (TNP) and membrane proteins (MP) from vector and non-vector insect

814   species were blotted onto polyvinyl difluoride membranes with fusion Flavescence dorée

815   (FD) immunodominant membrane proteins (Imp) as a positive control (IMP test) and

816   negative control with buffer devoid of protein bait (No Bait Control). St: *Scaphoideus titanus*,

817   Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp:

818   *Metcalfa pruinosa*, Zp: *Zyginidia pullula*. C: Controls (Imp positive control (A) and BSA

819   negative control (B)). For each species, three serial dilutions were spotted: undiluted starting

820   quantity sample (1), 1:10 (2) and 1:100 dilutions (3). The Anti-FD-D Imp polyclonal

821   antibody was used to detect bound phytoplasma recombinant proteins, and horseradish

822   peroxidase conjugated to goat antirabbit secondary antibody was used for chemiluminescent

823   detection.

824

Supporting Information

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

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

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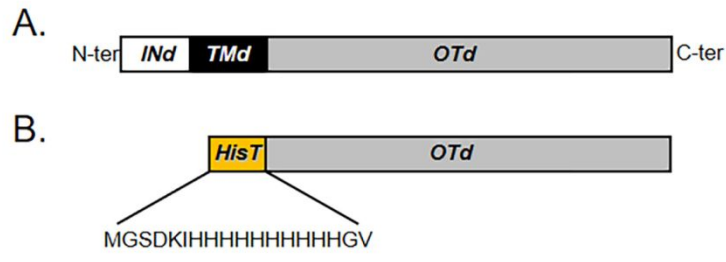
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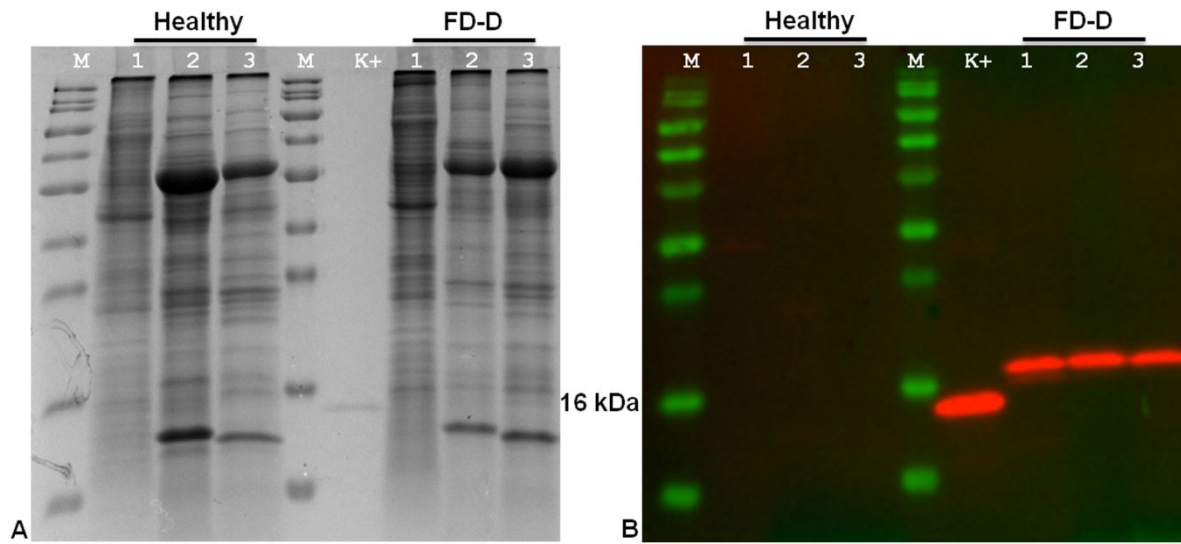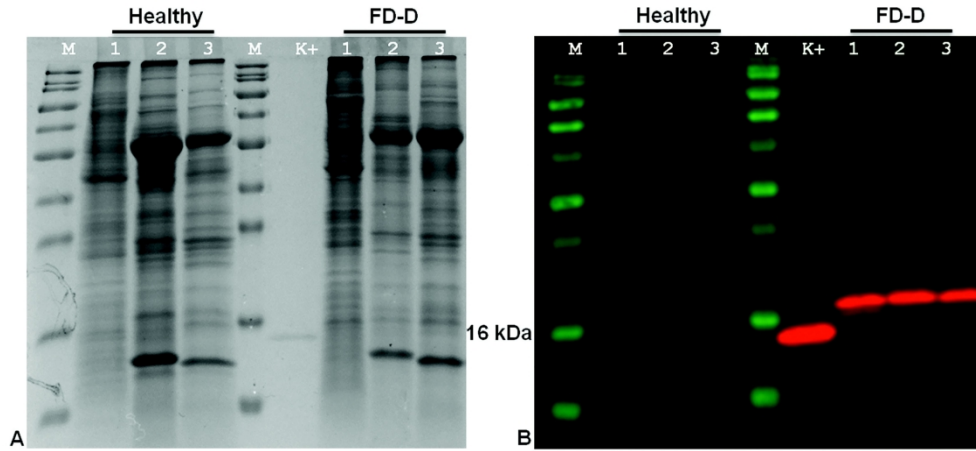

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851 **Figure 1.**

852

854

855 **Figure 2.**

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

202x94mm (300 x 300 DPI)
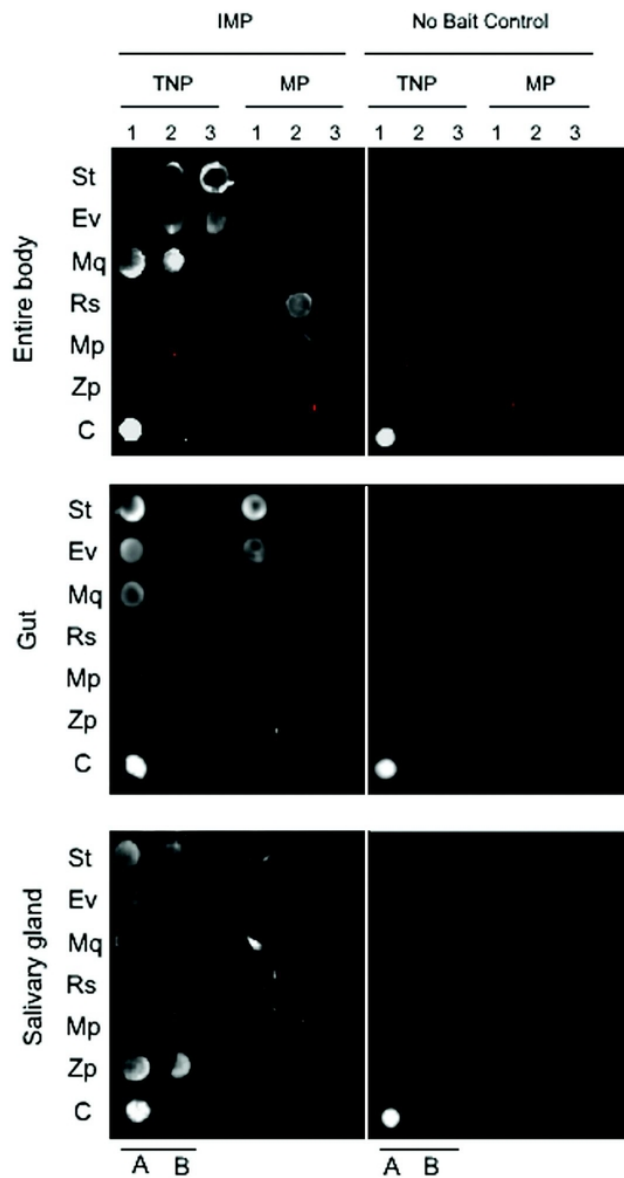
Figure 3. In vitro interaction of phytoplasma immunodominant membrane protein with competent vector and non-vector insect proteins analysed by Far-Western dot blots. Total native proteins (TNP) and membrane proteins (MP) from vector and non-vector insect species were blotted onto polyvinyl difluoride 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: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*. C: Controls (Imp positive control (A) and BSA negative control (B)). For each species, three serial dilutions were spotted: undiluted starting quantity sample (1), 1:10 (2) and 1:100 dilutions (3). The Anti-FD-D Imp polyclonal antibody was used to detect bound phytoplasma recombinant proteins, and horseradish peroxidase conjugated to goat antirabbit secondary antibody was used for chemiluminescent detection.
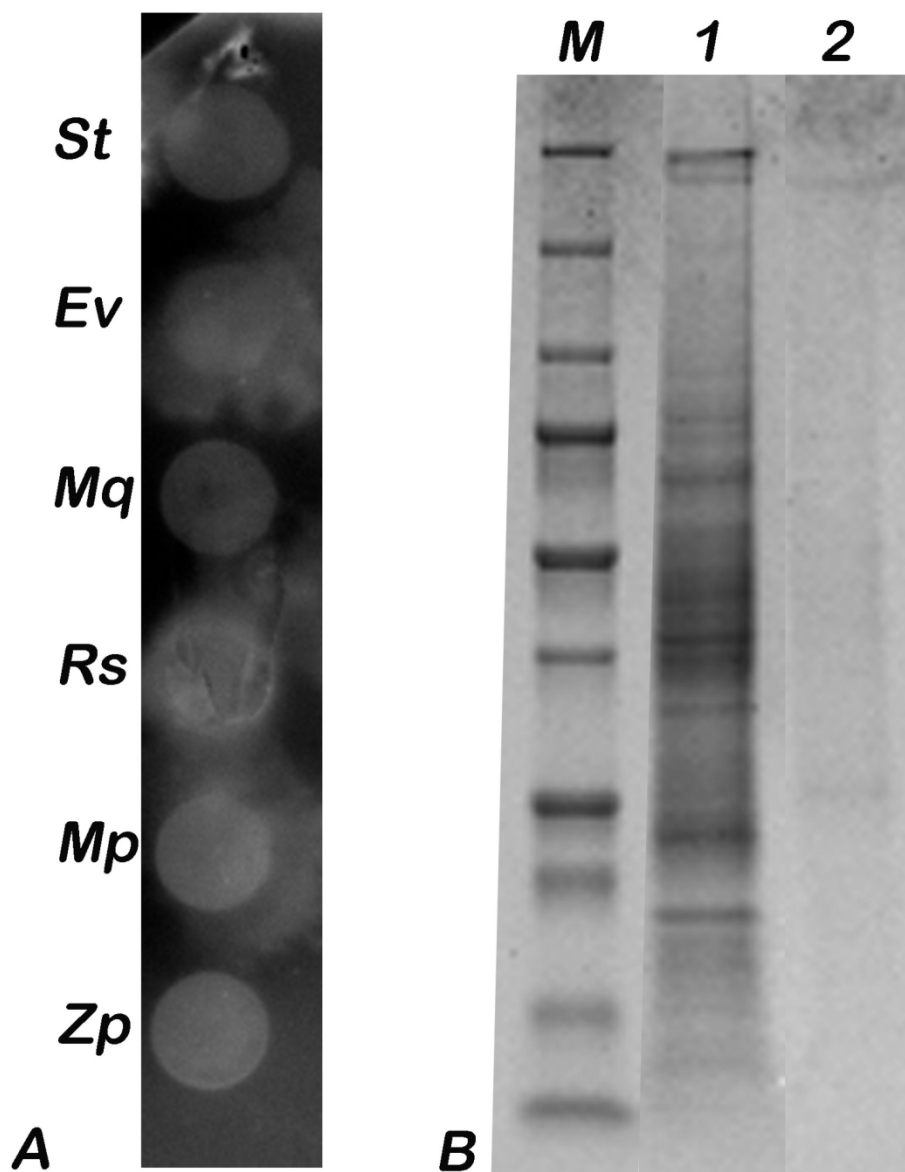
48x86mm (300 x 300 DPI)

Figure S1: Total native proteins from entire insect bodies of the six species (St: *Scaphoideus titanus*, Ev: *Euscelidius variegatus*, Mq: *Macrosteles quadripunctulatus*, Rs: *Ricania speculum*, Mp: *Metcalfa pruinosa*, Zp: *Zyginidia pullula*) spotted onto a membrane stained with Ponceau S Solution (A). SDS-Poly-acrylamide gel of total native (1) and membrane (2) proteins from guts of *Euscelidius variegatus* (B); M: prestained molecular weight standards.
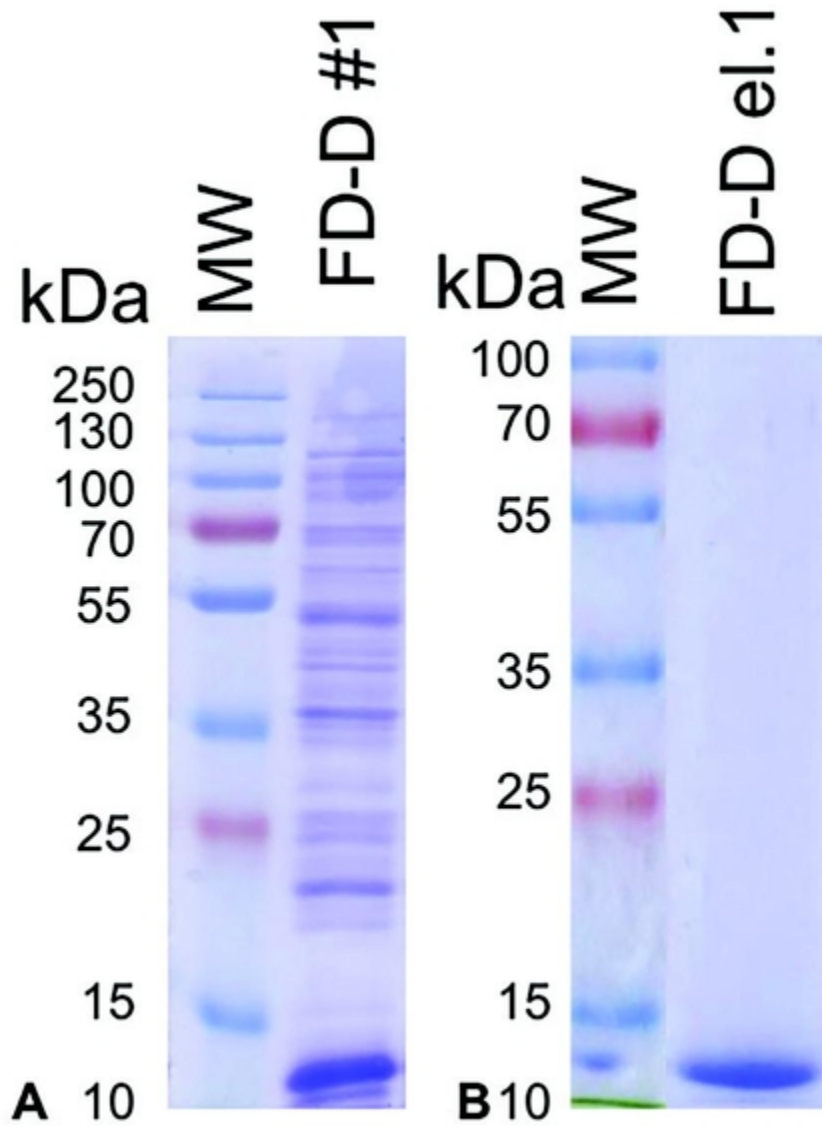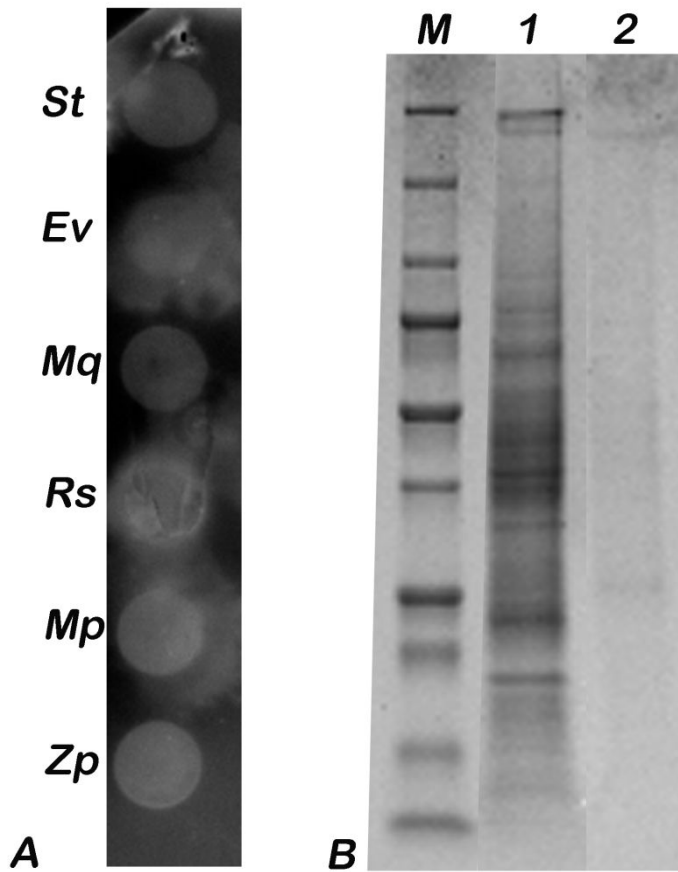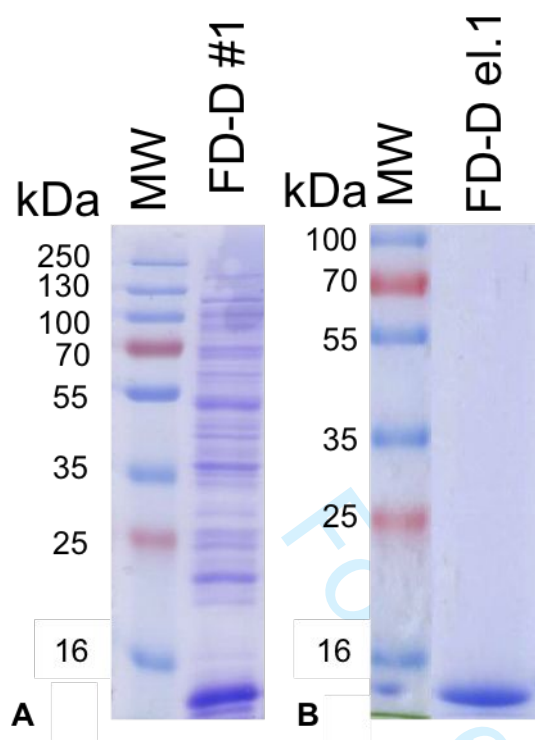
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Figure S2: SDS-Poly-acrylamide gels of (A) *Escherichia coli* crude cell extract following recombinant expression of FD-D Imp (FD-D clone #1) and (B) corresponding eluted purified protein (5 μg) after purification procedure (FD-D el.1: elution of FD-D #1). MW: prestained molecular weight standards.

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-Poly-acrylamide gel of total native (1) and membrane (2) proteins from guts of *Euscelidius variegatus* (B); M: prestained molecular weight standards.

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

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