An aphid effector promotes barley susceptibility through suppression of defence gene expression

Escudero-Martinez, Carmen[1,2], Rodriguez, Patricia A.[1♯], Santos, Pablo A.[2], Stephens, Jennifer[1], Bos, Jorunn I.B.[1,2]

[1]Cell and Molecular Sciences, The James Hutton Institute, Dundee, DD2 5DA, UK.
[2] Division of Plant Sciences, School of Life Sciences, University of Dundee, Dundee DD2 5DA, UK
♯Present address: Helmholtz Zentrum München, Institute of Network Biology (INET), Munich, Germany

*Corresponding Author:
Jorunn I.B. Bos, PhD
Division of Plant Sciences, School of Life Sciences, University of Dundee
Cell and Molecular Sciences, James Hutton Institute
Invergowrie, Dundee
DD2 5DA
United Kingdom
Phone: +44 (0)3449285428
Email: j.bos@dundee.ac.uk

Abstract

Aphids secrete diverse repertoires of effectors into their hosts to promote the infestation process. While “omics”-approaches facilitated the identification and comparison of effector repertoires from a number of aphid species, the functional characterization of these proteins has been limited to dicot (model) plants. The bird cherry-oat aphid *Rhopalosiphum padi* is a pest of cereal crops, including barley. Here, we extended efforts to characterize aphid effectors with regards to their role in promoting susceptibility to the *R. padi*-barley interaction. We selected 3 *R. padi* effectors based on sequences similarity to previously characterized *M. persicae* effectors and assessed their subcellular localisation, expression, and role in promoting plant susceptibility. Expression of *R. padi* effectors RpC002 and Rp1 in transgenic barley lines enhanced plant susceptibility to *R. padi* but not *M. persicae*, for which barley is a poor host. Characterization of Rp1 transgenic barley lines revealed reduced gene expression of plant hormone signalling genes relevant to plant-aphid interactions, indicating this effector enhances susceptibility by suppressing plant defences in barley. Our data suggests that some aphid effectors specifically function when expressed in host species, and feature activities that benefit their corresponding aphid species.

28 **Introduction**

29

30 Similar to plant pathogens, aphids form close associations with their hosts and secrete
31 effector molecules to modulate host cell processes to their benefit. Over the past decade,
32 a combination of genomics- and proteomics-based approaches allowed the identification
33 of putative effectors from different aphid species, including economically important pests of
34 both monocot and dicot crops (Nicholson *et al.*, 2012) (Rao *et al.*, 2013) (Cooper *et al.*,
35 2011) (Atamian *et al.*, 2013) (Vandermoten *et al.*, 2014) (Thorpe *et al.*, 2016) (Zhang *et al.*,
36 2017) (Thorpe *et al.*, 2018). Comparative analyses of aphid effector repertoires across
37 species has revealed core and diverse sets, provided insight into effector diversity, and
38 evidence for a shared transcriptional control mechanism driving their expression (Thorpe
39 *et al.*, 2016) (Boulain *et al.*, 2018) (Thorpe *et al.*, 2018). Moreover, functional
40 characterization of aphid effectors increased our understanding of how these proteins may
41 function to enhance plant susceptibility during infestation (as reviewed by (Yates and
42 Michel, 2018) and (Nalam *et al.*, 2019)), and pointed to host-specific effector activities
43 (Pitino and Hogenhout, 2013) (Elzinga *et al.*, 2014) (Rodriguez *et al.*, 2017b).

44 The C002 salivary protein was first described as an effector in *Acyrthosiphon pisum*, and
45 promotes host susceptibility to aphids (Mutti *et al.*, 2008). However, while expression of
46 MpC002 (*M. persicae* C002) in host species *Arabidopsis* and *N. benthamiana* enhances
47 susceptibility to *M. persicae*, expression of ApC002 from *A. pisum* in these same plant
48 species has no visible impact on the host interaction with *M. persicae* (Pitino and
49 Hogenhout, 2013). The difference in effector activity was attributed to a motif sequence
50 (NDQGEE) in the N-terminal region of MpC002, which is lacking in ApC002 (Pitino and
51 Hogenhout, 2013). In addition, several effectors from the broad host range pest *M.*
52 *persicae* have been implicated in promoting host susceptibility, including Mp1 and Mp58
53 (Pitino and Hogenhout, 2013) (Elzinga *et al.*, 2014) (Rodriguez *et al.*, 2017b). However,

54 the underlying mechanisms by which these effectors impact susceptibility remain largely
55 unknown. We previously described that Mp1 associates with the host trafficking protein
56 VPS52 (Vacuolar Sorting Associated Protein 52) to promote plant susceptibility (Rodriguez
57 *et al.*, 2017a). Using different combinations of Mp1 and VPS52 variants from different plant
58 and aphid species, respectively, we showed that the Mp1-VPS52 association is highly
59 specific to the broad host range pest *M. persicae* and its hosts, and likely shaped by plant-
60 aphid co-evolution. Critically, effector-host protein interactions correlate with effector
61 virulence activities (Rodriguez *et al.*, 2017a). The Mp1 and Mp58 effectors and their
62 putative orthologs are genetically linked across the genomes of at least 5 different aphid
63 species (Thorpe *et al.*, 2018). Although a functional link between Mp1 and Mp58 remains
64 to be elucidated, Mp58 was previously implicated in plant-aphid interactions. For example,
65 Elzinga *et al.* 2014 observed a decrease in *M. persicae* performance when Mp58 was
66 ectopically expressed in *Nicotiana tabacum* or transgenic Arabidopsis lines. In contrast,
67 the Mp58-like effector from *M. euphorbiae* (also called Me10) enhances tomato and *N.*
68 *benthamiana* susceptibility to *M. euphorbiae* and *M. persicae* (Atamian *et al.*, 2013). Me10
69 was recently reported to interact with tomato 14-3-3 isoform 7 (TFT7), which contributes to
70 defence against aphids (Chaudhary *et al.*, 2019).

71 *Rhopalosiphum padi* is an aphid species with a narrow host range, which includes grass
72 species, such as barley, oats and wheat (Blackman and Eastop, 2000). This aphid is an
73 important pest of cereal crops that causes feeding damage and transmits some of the
74 most destructive viral diseases of cereals, such as *Barley Yellow Dwarf Virus* (BYDV).
75 Whilst *R. padi* is highly specialized on cereals, other species, like *M. persicae,* feature an
76 exceptionally broad host range that includes more than 4000 different plant species
77 (Blackman and Eastop, 2000), including the model plants Arabidopsis or *N. benthamiana*.
78 Despite its broad host range, *M. persicae* is not a pest of barley and performs poorly on
79 this plant species (Escudero-Martinez *et al.*, 2017). Recently, *M. persicae* and *R. padi*

effector repertoires were identified and compared, allowing the extension of effector characterization studies to cereal pests (Thorpe *et al.*, 2016; Thorpe *et al.*, 2018). Functional characterization of aphid effectors across different plant species, including cereals is important to gain insight into sequence variation among effector repertoires impacts host susceptibility.

Here, we characterized 3 *R. padi* effectors with regards to their subcellular localization, gene expression, and contribution to susceptibility in host barley and non-host *N. benthamiana* plants. We found that expression of the *R. padi* effectors Rp1 and RpC002 in transgenic barley lines enhances plant susceptibility to *R. padi* (host interaction) but not to *M. persicae* (poor-host interaction), highlighting the importance of these effectors for barley colonization in an aphid species-specific manner. Further characterization of Rp1 transgenic barley lines revealed reduced expression of several markers of plant hormone signalling pathways relevant to plant-aphid interactions, suggesting this effector may enhance susceptibility by suppressing plant defences.

Material & methods

Aphid cultures

Aphids used for the experiments were raised inside cages under controlled conditions in growth chambers (18°C, 16h light). *R. padi* was raised on *Hordeum vulgare* L. cv. Optic and *M. persicae* (genotype O) was reared on *Brassica napus*. The aphid species used were kindly provided by Alison Karley (JHI, UK) and Gaynor Malloch (JHI, UK).

Identification of putative effector orthologs and plasmid construction

Effector annotation and identification of orthologs was performed as described by Thorpe *et al.* 2016. Similarity searches were performed by reciprocal best BLAST hit analysis between *R. padi* and *M. persicae* transcriptomes with the minimum thresholds of 70 %

104 identity and 50 % query coverage. Pair-wise sequence analysis was performed in Jalview
105 2.10.4 (Waterhouse *et al.*, 2009) with T-coffee and default parameters. Signal peptide
106 sequences were predicted with SignalP 4.1 (Petersen *et al.*, 2011). Coding sequences
107 were amplified from *R. padi* and *M. persicae* cDNAs, without the region encoding for the
108 signal peptide, and verified by sequencing (Primers in Supplementary Table S1). The
109 resulting amplicons were cloned by Gateway technology into pDONR201, pDONR207 or
110 pENTR_D-TOPO (Gateway®, Invitrogen). Sequence verified inserts were cloned into
111 different destination vectors by LR reaction. Destination vectors pB7WGF2 (35S promoter,
112 N-terminal GFP) and pB7WG2 (35S promoter, no tag) (Karimi *et al.*, 2002) were used for
113 transient overexpression in *N. benthamiana*, and pBRACT214m (maize ubiquitin promoter,
114 no tag), kindly provided by Abdellah Barakate (JHI) (Colas *et al.*, 2019) , was used for
115 generating transgenic barley lines.

116 **Effector gene expression in aphids exposed to host-, non-/poor-host plants and**
117 **artificial diet**

118 The experimental set-up for determining aphid effector gene expression in aphids exposed
119 to the different feeding environments is explained in detail in Thorpe et al., (Thorpe *et al.*,
120 2018). Briefly, aphids were exposed to an artificial diet, host, poor/nonhost plant for 3h and
121 24h and collected for RNA samples preparation and their transcriptome was sequenced by
122 RNAseq. More specifically, *R. padi* was exposed to barley (host) and Arabidopsis (non-
123 host), and *M. persicae* was exposed to Arabidopsis (host) and barley (poor-host). Both
124 aphids were also exposed to artificial diet for 3h and 24h. A total of five independent
125 replicates were used for this experiment and differential expression (DE) analyses was
126 performed as described (Thorpe *et al.*, 2018). For each selected effector (Rp1, RpC002,
127 Rp58, Mp1, MpC002, and Mp58), we performed BLAST searches against the RNAseq
128 datasets described in Thorpe *et al.* (2018) to identify their corresponding gene models.

129 Transcripts were normalized by the fragments per kilo-base of exon per million reads
130 mapped (TMM-FPKM) method, which normalized the gene counts to the gene length and
131 the library size (Conesa *et al.*, 2016).

132 **Effector localization**

133 Effectors were cloned into pB7WGF2 and the constructs were transformed into
134 *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cells were harvested by
135 centrifugation (8min, 6000rpm) and resuspended in infiltration buffer (acetosyringone 125
136 µM and $MgCl_2$ 10mM) to an optical density of OD_{600}= 0.1. *Agrobacterium* carrying the
137 GFP-effector constructs were then infiltrated in *N. benthamiana* leaves. RpC002 and
138 MpC002 were co-expressed with a plasma membrane molecular marker (Nelson *et al.*,
139 2007) and with the p19 silencing suppressor (OD_{600} = 0.1) for improving expression and
140 thereby detection under the confocal microscope. All other effector pairs were infiltrated
141 without p19. Three days after infiltration, plants were analysed under the confocal
142 microscope (Zeiss LSM 510 (Jena, Germany) using a Zeiss x20/x40 lens. GFP-fusion
143 proteins and free GFP were imaged with the GFP and chlorophyll filter (488 nm excitation),
144 and co-localization with the membrane marker was analysed with GFP-RFP filter (488-
145 561nm excitation). The experiment was repeated four times, and the resulting images
146 were processed using ImageJ (Schneider *et al.*, 2012).

147 **Western blotting to detect GFP-fusion proteins**

148 Effectors were cloned into the pB7WGF2 vector and constructs were transformed into
149 *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cells were treated as above
150 and infiltrated in *N. benthamiana* leaves to an optical density of OD_{600} = 0.3. After four
151 days, samples were harvested, and proteins were extracted with GTEN buffer (10%
152 Glycerol, 25mM Tris pH 7.5, 1mM EDTA, 150mM NaCl, 0.1% NP-40, 10mM DTT and 1x
153 protease inhibitor cocktail, Sigma). Western blots were incubated overnight with GFP-

154 antibody (Santa Cruz Biotechnology Inc, USA), for 1 hour with anti-rabbit-HRP (Santa
155 Cruz Biotechnology Inc, USA).

156

157 **Generation of transgenic barley lines expressing *R. padi* effectors**

158 Each of the effectors was cloned into the destination vector pBRACT214m containing the
159 ubiquitin promoter from maize for constitutive expression in all plant organs, and a
160 hygromycin marker gene for selection of transgenic lines. Constructs were transformed
161 into the *Agrobacterium* AGL1 strain, supplied with pSOUP, and delivered to the Functional
162 Genomics Facility (FUNGEN) at the James Hutton Institute for *Agrobacterium*-mediated
163 barley embryo transformation of the cultivar Golden Promise. After approximately four
164 months, we obtained different barley lines regenerated from independent calli. The T0
165 generation was tested for the expression of effector genes by PCR on cDNA from the
166 regenerated plants. RNA was extracted from T0 independent lines using the RNeasy Plant
167 Mini Kit (Qiagen). RNA plant samples were DNAse treated with Ambion® TURBO DNA-
168 free™. SuperScript® III Reverse Transcriptase (Invitrogen) and random primers were used
169 to prepare cDNA. The majority of these plants were positive in PCR tests using effector
170 gene-specific primers (Supplementary Table S1). T1 seeds were germinated on selective
171 media (AgarGel™- containing Hygromycin (100 µg/ml) to select for transformants. Lines
172 showing a 1:3 segregation, representing a single insertion (75 % survival rate on selective
173 media), were selected for further analyses. Universal Probe Library (UPL-Roche
174 Diagnostics ©) was used to quantify effector gene expression in T1 barley transgenic lines
175 ectopically expressing *R. padi* effectors. Barley cv Golden Promise, the background
176 genotype of the transgenic lines, was used as control. RNA from six different barley lines
177 per construct was reverse transcribed into cDNA. Probes and primers (Supplementary
178 Table S1) designed with the UPL System Assay Design (Roche) were tested for at least

179 95-105% efficiency. Internal controls were Actin-2 (MLOC_78511.2) and Pentatricopeptide
180 (AK373147/MLOC_80089.1) as described previously (Escudero-Martinez *et al.*, 2017).
181 Three technical replicates were included for each sample. Relative expression was
182 calculated with the method ΔCt (Delta Cycle threshold) with primer efficiency
183 consideration. One of each of the transgenic effector lines was used as a reference line to
184 calculate the fold-change in additional lines.

185 Lines positive for effector expression were then bulked into T2 and screened for
186 homozygosity based on complete resistance to hygromycin. Three independent
187 homozygous lines per effector were used to perform the aphid performance assays with *R.*
188 *padi* and *M. persicae*.

189 ***M. persicae* performance assays on *N. benthamiana***

190 Effectors were transiently expressed using vector pB7WG2 in *N. benthamiana* as
191 explained above. The empty vector pB7WG2 was used as a control. Twelve infiltration
192 sites were used per construct per biological replicate (n=12 per biological replicate). One
193 day after infiltration, the abaxial side of the infiltration sites was exposed to two *M. persicae*
194 adults enclosed in a clip cage. The following day, adult aphids were removed leaving three
195 1st instar nymphs at the underside of the leaves in a clip cage. Seven days later, *N.*
196 *benthamiana* plants were replaced by freshly infiltrated plants to ensure continued
197 expression of effectors in the plant tissue. After 14 days, the number of nymphs per adult
198 was counted and data were analysed using One way ANOVA (in R-studio) and the post-
199 hoc Fisher's protected Least Significant Differences (LSD) (cut-off $p \leq 0.05$). Three
200 biological replicates were performed with each replicate containing 12 infiltration sites per
201 construct.

202

203

204 **Aphid performance assays on barley transgenic lines**

205 Seven-day old transgenic barley plants expressing *R. padi* effectors were infested with two
206 1st instar age-synchronized nymphs (*M. persicae*) or with two 2-day old age-synchronized
207 nymphs (*R. padi*). Barley cv. Golden Promise wild-type plants were used as the control.
208 We used 6-8 plants per individual transgenic line for each biological replicate per aphid
209 species (n=6-8), and four biological replicates were performed. The number of nymphs per
210 adult was monitored at 11 days after infestation for *R. padi*, and after 14 days for *M.*
211 *persicae*. The resulting data was analysed One-way ANOVA (in R-studio) with post-hoc
212 Fisher's protected Least Significant Differences (LSD).

213 **Histochemical GUS staining**

214 To assess GUS expression driven by the maize ubiquitin promoter in transgenic barley
215 transformed with the pBRACT214m-GUS construct we collected different tissues and
216 stained these with 1mg/ml of X-gluc (5-bromo-4-chloro-3-indolyl-B-D-glucuronic acid,
217 Thermo Scientific, USA) in X-gluc buffer (100mM sodium phosphate buffer pH 7.0, 0.1%
218 Triton X-100, 2mM potassium ferricyanide and 2mM potassium ferrocyanide). Tissues
219 were vacuum-infiltrated and incubated in darkness at 37°C overnight. The next day,
220 chlorophyll was removed from the tissues with 1:3 acetic acid/ethanol. Pictures were taken
221 under the dissecting microscope with a Zeiss camera.

222 **Quantitative RT-PCR to assess defence gene expression in Rp1 transgenic lines**

223 Gene expression of different defence/hormone signalling pathways genes were analysed
224 by qRT-PCR, The transgenic barley Rp1 lines along with the control plants (cv. Golden
225 Promise) were pre-germinated in Petri dishes covered with wet filter paper for three days
226 in the dark at room temperature. Germinated seeds placed on soil and grown under
227 controlled conditions (8h light, 22°C, 70% humidity and 125 µmol photons/m2.s). For

228 basal gene expression the first leaf of the plants (n=6 per genotype) were collected and
229 flash frozen in liquid nitrogen. In addition, barley plants (n=6 plants per transgenic line or
230 wild type control) were exposed to either empty clip cages or clip cages containing 30
231 mixed-age *R. padi* aphids. Leaf tissues enclosed within the clip cages were collected after
232 24 h and 72 h. The experiment was performed in three biological replicates (n=6 plants per
233 transgenic or wildtype line per biological replicate) and samples were harvested at the
234 same time of the day: barley plants were treated and collected at 12 am for the 24 h
235 timepoint and at 3 pm for 72 h timepoint, avoiding any effects of the plant circadian cycle.

236 The local database *Morex genes* was used for retrieving the barely sequences and the
237 *Roche UPL assay design centre* for primer design (Supplementary Table S1). The
238 primers were tested for efficiency (85-115%) and relative gene expression was calculated
239 with the method ΔΔCt (Delta-delta Cycle threshold). Three technical replicates were
240 included for each sample. Cycle threshold values were normalized with two
241 reference genes, pentatricopeptide (AK373147/MLOC_80089) and ubiquitin (AK248472).
242 Expression of these two reference genes was unaffected in our previous microarray
243 experiment (Escudero-Martinez *et al.*, 2017). The Wilcoxon Rank Sum Test (cut-off p
244 ≤0.05) was used to assess differences in expression between plant genotypes and
245 treatments.

246 **Results**

247 **Effector sequence divergence between the aphid species *R. padi* and *M. persicae***

248 We predicted putative orthologs for 3 previously described *M. persicae* effectors, MpC002,
249 Mp1 and Mp58 from *R. padi* using reciprocal best blast hit analyses on available aphid
250 transcriptome datasets and aphid genome assemblies (threshold of 70 % identity and
251 50 % query coverage) (Thorpe *et al.*, 2016; Thorpe *et al.*, 2018). To confirm the sequences
252 of putative orthologous effector pairs we cloned and sequenced their coding sequences.

253 Amino acid and nucleotide sequence alignments show varying degrees of sequence
254 divergence across the selected effector pairs (Fig. 1, Supplementary Fig. S1). RpC002 is
255 smaller than MpC002, with 193 amino acids compared to 265, respectively, and these
256 effectors share 52.86% sequence identity. The difference in sequence length is partly due
257 to a lack of the NDNQGEE repeat in the N-terminal region of RpC002 (Fig. 1A,
258 Supplementary Fig. S1). Variation in the number of NDNQGEE repeats in MpC002 was
259 previously also detected within *M. persicae* (Thorpe *et al.*, 2016), and in this study we
260 characterized the MpC002 version containing 5 repeats. MpC002 also has an extended C-
261 terminal domain compared to RpC002 (Fig. 1A). Rp1, which is similar to *M. persicae* Mp1,
262 is composed of 140 amino acids compared to 139 for Mp1, and these effectors share a
263 percentage sequence identity of 56.12% (Fig. 1B, Supplementary Fig. S1). Lastly, Mp58
264 and Rp58 contain 152 and 155 amino acids, respectively, share 64.94% sequence
265 identity, and are most divergent in the C-terminal region of the protein (Fig. 1C,
266 Supplementary Fig. S1).

267

268 **Effector gene expression is consistent across different feeding/plant environments,**
269 **but the range of expression varies between aphid species**

270 We were interested in assessing how gene expression of the three effector pairs was
271 affected in *R. padi* and *M. persicae* upon exposure to different feeding/plant environments.
272 We made use of previously generated aphid RNAseq datasets (Thorpe *et al.*, 2018) to
273 investigate gene expression of our effectors of interest by plotting their gene counts across
274 different treatments (exposure to diet, host and poor/nonhost plants) and timepoints (3h
275 and 24h exposure). All 6 aphid effectors were expressed with only limited variation in
276 expression across the different aphid treatments and timepoints (Fig. 2). Whilst the three
277 selected effectors from *M. persicae* displayed more similar gene expression levels
278 compared to one another, ranging from 280 counts for *MpC002* to 904 counts for *Mp1*

279 (Fig. 2B and C), the three effectors from *R. padi* showed a wider range of expression. For
280 instance, gene counts varied from 271 for *RpC002* to 2112 for *Rp1* over the various
281 treatments and timepoints (Fig. 2A).

282

283 ***R. padi* effectors show similar subcellular localisation as their putative *M. persicae***
284 **orthologs *in N. benthamiana***

285 The subcellular localization of effectors can provide important information on the cellular
286 compartment that is targeted by these proteins. We used confocal microscopy of GFP-
287 tagged *R. padi* effectors alongside their *M. persicae* putative orthologs to compare
288 subcellular localisation *in planta*. The GFP-effector fusion proteins (N-terminal GFP tag)
289 were transiently expressed in leaves of *N. benthamiana*, which is a host for *M. persicae*,
290 but a non-host for *R. padi*. Western blotting showed that all GFP-fusion proteins were
291 expressed, but that two of the *R. padi* effectors, RpC002 and Rp58, showed lower protein
292 levels than their putative *M. persicae* orthologs (Supplementary Fig. S2), with RpC002 only
293 detected once in three biological replicates (Supplementary Fig. S2). In contrast, Rp1 from
294 *R. padi* was detected more strongly than its putative *M. persicae* ortholog Mp1
295 (Supplementary Fig. S2). We detected GFP signal corresponding to the RpC002- and
296 MpC002-fusion proteins by confocal microscopy at the plasma membrane, and in some
297 cases, a weak signal was present in the nucleus or the cytoplasm (Fig. 3A and
298 Supplementary Fig. S3). It should be noted that expression of RpC002 was very low,
299 especially compared to MpC002 (Supplementary Fig. S2), and only a few transformed
300 cells were visible. We validated the plasma membrane localization of RpC002/MpC002
301 effectors upon co-expression with a plasma membrane marker (Nelson *et al.*, 2007) (Fig.
302 3A). Both the Rp1 and Rp58 were detected in the cytoplasm and nucleus, similar to their
303 putative *M. persicae* orthologs and the free GFP control (Fig. 3B and Fig. 3C). Similarly,

304 we tried to express tagged effectors in barley epidermal cells using particle bombardment,
305 but due to low signal we were unable to reliably localize effectors in this system. Overall,
306 the three selected *R. padi* effector showed similar subcellular localization patterns in *N.*
307 *benthamiana* as their putative *M. persicae* orthologs.

308

309 **Expression of Rp58 in *Nicotiana benthamiana* reduces host susceptibility to *M.***
310 ***persiae***

311 To assess whether the three selected *R. padi* effectors can impact host susceptibility to *M.*
312 *persicae* when expressed in a *R. padi* nonhost plant species, we performed aphid
313 performance assays on *N. benthamiana* leaves transiently expressing the different
314 effectors under the control of a 35S promoter. In line with previous reports (Bos *et al.*,
315 2010) (Pitino and Hogenhout, 2013), we found that ectopic expression of MpC002
316 significantly increased the number of *M. persicae* nymphs produced per adult by 27%
317 (One-way ANOVA post-hoc Fisher's protected Least Significant Differences (LSD);
318 p>0,05) (Fig. 4A). In contrast, RpC002 did not alter *N. benthamiana* host susceptibility to
319 *M. persicae*. Western blot analyses of GFP-MpC002 and GFP-RpC002 showed that
320 RpC002 protein is detected at a much lower level than MpC002 (Supplementary Fig. S2),
321 and therefore it is possible that also the untagged MpC002 and RpC002 proteins
322 expressed in the aphid performance assays have different levels of abundance which
323 affects the phenotypic observations. No significant differences in host susceptibility were
324 noted upon expression of Mp1 from *M. persicae* and Rp1 from *R. padi* (Fig. 4B), in line
325 also with a previous report that transient expression of Mp1 under the 35S promoter in *N.*
326 *benthamiana* does not affect susceptibility (Bos *et al.*, 2010). The expression of Mp58, but
327 not Rp58, resulted in significantly lower *M. persicae* nymph production compared to the
328 vector control, with 55% and 27% less nymphs being produced per adult, respectively

329 (One way ANOVA post-hoc Fisher's protected Least Significant Differences (LSD);
330 p>0,05) (Fig. 4C).

331

332 **Expression of RpC002 and Rp1 in transgenic barley enhances susceptibility to *R.***
333 ***padi***

334 Aphid effector characterization studies to date have focused on dicot plant species. With
335 *R. padi* being a major pest of cereals, we aimed to extend aphid effector characterization
336 studies to the monocot crop barley to explore the contribution of *R. padi* effectors to host
337 susceptibility. We generated barley transgenic lines in the cultivar Golden Promise to
338 ectopically express the three *R. padi* effectors Rp1, RpC002 and Rp58 using a modified
339 version of the pBRACT214 vector (Colas *et al.*, 2019), containing the ubiquitin promoter
340 from maize to allow constitutive expression in all plant organs
341 (http://www.bract.org/constructs.htm#barley). To determine where candidate genes of
342 interest are potentially expressed when transformed into barley using this pBRACT214m
343 construct, we performed GUS-staining on different plant tissues, such as leaves, stems,
344 spikes and roots of a barley transgenic line generated by transformation with
345 pBRACT214m:GUS (Supplementary Fig. S4). We observed GUS expression in all tissues,
346 with particularly strong expression in leaf vascular tissues and roots (Supplementary Fig.
347 S4). After barley transformation, we obtained 13 independent lines for the RpC002
348 effector, 4 lines for the Rp1 effector, and 16 lines for Rp58. In the first generation, lines
349 with a single effector insertion were selected based on around 75% survival on
350 Hygromycin (hygromycin phosphotransferase is the pBRACT selection marker), yielding 8
351 independent transgenic lines for RpC002, 3 lines for Rp1, and 7 lines for Rp58. The
352 presence of effector coding sequences (lacking the signal peptide encoding sequence)
353 was confirmed in the T0 generation by semi quantitative RT-PCR (data not shown) and
354 was verified in the T1 generation by qRT-PCR (Supplementary Fig. S5). We did not

355 observe any visual differences in plant growth and development for any of the transgenic
356 lines selected (Supplementary Fig. S5). Three homozygous T3 lines per effector construct
357 were selected for aphid performance assays with *R. padi* and *M. persicae* to assess how
358 barley host and poor-host interactions with aphids were affected. Each plant was infested
359 with two nymphs and reproduction was assessed after 11 days for *R. padi* and after 14
360 days for *M. persicae.*

361 For *M. persicae* we did not find consistent significant differences in aphid performance on
362 the barley transgenic lines expressing the *R. padi* effectors compared to the wild-type
363 control (Fig. 5). For one of the Rp1 lines, Rp1_2A, however, we noted increased nymph
364 production (Fig. 5B). Line Rp1_2A was also the highest Rp1 expressing line we identified
365 (Supplementary Fig. S5), and additional lines with similar expression levels would need to
366 be identified to rule out the possibility that the insertion site in this line causes the
367 enhanced susceptibility phenotype.

368 Ectopic expression of both RpC002 and Rp1 in transgenic barley lines enhanced
369 susceptibility to *R. padi* (Fig. 6A and B). Specifically, two out of three independent RpC002
370 barley lines, RpC002_1A and RpC002_2A, showed 16% and 12% increased nymph
371 production compared to the wild-type control, respectively (One way ANOVA post-hoc
372 Fisher's protected Least Significant Differences (LSD); p>0,05) (Fig. 6A). The transgenic
373 line with the strongest susceptibility phenotype (RpC002_1A) also showed the highest
374 *RpC002* expression level (Fig. 6A; Supplementary Fig. S5). In addition, all three
375 independent Rp1 barley lines showed enhanced susceptibility to *R. padi* with an increased
376 nymph production of 11-22% across lines compared to the wild-type control (One way
377 ANOVA post-hoc Fisher's protected Least Significant Differences (LSD); p>0,05) (Fig. 6B).
378 Also, we observed a correlation between the level of effector gene expression and impact
379 on host susceptibility to aphids, with the lines showing the most pronounced susceptibility

380 phenotype towards *R. padi* (Rp1_2A and Rp1_3B) also showing the higher *Rp1* transcript
381 levels (Fig. 6B; Supplementary Fig. S5).

382 No differences were observed for the Rp58 transgenic lines, which showed susceptibility
383 levels similar to the wild-type control.

384 **Rp1 suppresses defence signalling in transgenic barley lines**

385 To gain further insight into how Rp1 may enhance barley host susceptibility to aphids, we
386 investigated the basal and induced expression levels of defence-related genes in the three
387 independent transgenic lines we generated (lines 2A, 3B and 4E), compared to the wild-
388 type cultivar Golden Promise. Based on our previous work (Escudero-Martinez *et al.*,
389 2017) we selected barley genes strongly induced upon *R. padi* infestation: *beta-thionin*
390 (AK252675), *SAG12-like* (MLOC_74627.1), a *jasmonate ZIM domain gene 3* (*JAZ3*,
391 MLOC_9995), *lipoxygenase 2* (*LOX2*, MLOC_AK357253) and the *jasmonate-induced*
392 *gene* (*JI*, MLOC_15761). We further expand our selected genes set based on markers of
393 different hormone signalling pathways, with focus on the jasmonate pathway, which is
394 strongly activated upon aphid infestation (Escudero-Martinez *et al.*, 2017): *lipoxygenase 5*
395 (*LOX5*, MLOC_71948), the *WRKY transcription factor 50* (*WRKY50*, MLOC_66204), the
396 *allene cyclase oxydase* (*AOC*, MLOC_68361) and *jasmonate-induced gene 2* (*JI2*,
397 MLOC_56924) markers for jasmonate; but also the salicylic acid marker *non-expressor of*
398 *pathogenesis-related 1-like* (*NPR1*, AM050559.1), *the ethylene-response factor 1* (*EFR1*,
399 MLOC_38561) and *abscisic acid-inducible late embryogenesis abundant 1* (*A1*,
400 MLOC_72442). We analysed basal gene expression levels as well as expression levels
401 upon 24h and 72h exposure to clip cages with or without aphids. It should be noted that
402 the use of clip-cages, even when empty, triggers changes in gene expression due to
403 mechanical damage, and that all selected genes were induced by aphid challenge
404 (Supplementary Fig. S6 and S7).

405 First, we compared basal gene expression levels across plant lines not infested with
406 aphids and without being exposed to a clip-cage (Fig. 7A). We found that expression of a
407 gene encoding a SAG-12 like cysteine protease (MLOC_74627.1) was most strongly
408 reduced at basal levels in Rp1 lines compared to the wild-type control, but differences
409 were only significant for lines Rp1-2A and Rp1-4E, possibly due to sample variation for line
410 Rp1-3B (Fig. 7A). *SAG12-like* expression was also reduced in the transgenic lines upon
411 exposure to either empty clip cages (Supplementary Fig. S6A and B) or clip cages
412 containing aphids (Fig. 7B and C), compared to wild-type plants, but not consistently to
413 statistically significant levels. The basal level of *EFR1* expression was slightly but
414 significantly higher in Rp1 transgenic lines compared to the wild-type control (Fig. 7A).
415 In addition, four genes (*WRKY50*, *AOC*, *beta-thionin*, *NPR1*) were significantly less
416 expressed across all transgenic lines compared to the wild-type control when leaves were
417 exposed for 24h to empty clip-cages (Supplementary Fig. S6A and B). *LOX2*, *JI*, and *JI2*
418 showed a trend towards reduced expression in transgenic lines, but differences were not
419 consistently significant across all lines (Supplementary Fig. S6A and B). In response to
420 clip-cages with aphids for 24h, only *LOX2* showed a significant reduction in expression in
421 all transgenic lines, whereas *JI2* reduced expression was noticeable not consistently
422 significant (Fig. 7B). For the 72h timepoint, 3 marker genes (*beta-thionin*, *NPR1*, *EFR1*)
423 showed a significant reduction in expression in all lines compared to wild-type plants when
424 exposed to clip-cages containing aphids, and similar trends were observed for *WRKY50*,
425 *JI*, *JI2* and *SAG12-like* (Fig. 7C). Overall, we observed a reduction of several marker
426 genes of defence/hormone signalling pathways relevant to plant-aphid interactions in the
427 Rp1 transgenic barley lines, which may translate into their enhanced susceptibility to
428 aphids.

429

430

431 **Discussion**

432 Aphids are damaging pests on cereals, including barley. Aphid effector characterization
433 efforts to date have focused on dicot plant species including Arabidopsis, tomato, and
434 *Nicotiana benthamiana* (Mutti *et al.*, 2008) (Bos *et al.*, 2010) (Pitino *et al.*, 2011) (Atamian
435 *et al.*, 2013) (Rodriguez *et al.*, 2014) (Elzinga *et al.*, 2014; Pitino and Hogenhout, 2013;
436 Rodriguez *et al.*, 2017a) (Chaudhary *et al.*, 2019), and have not yet been described for
437 monocot crops. It is crucial to understand the mechanisms employed by aphids and other
438 insects to infest cereals, as well as to gain insight into how aphid effector function may
439 have diverged across different plant-aphid species interactions. Although challenging,
440 functional characterization of aphid effectors not only in dicot (model) plants, but also in
441 monocot crops, promises to reveal novel insight into effector function and evolution.

442 Effector diversity across different aphid species might reflect the adaptation to different
443 host plants (Schulze-Lefert and Panstruga, 2011). Amino acid alignments of the putative
444 orthologous effectors we selected showed different levels of sequence divergence which
445 might reflect the different lifestyles of the two aphid species *R. padi* (cereal specialist) and
446 *M. persicae* (broad host range pest). In general, the signal peptide sequences of these
447 effectors tend to be more conserved than their C-terminal regions, indicating divergence
448 mainly occurred within the functional effector domains. The NDNQGEE repeat motif, which
449 is absent in RpC002, was previously shown to be linked to virulence in *M. persicae*, since
450 MpC002 transgenic Arabidopsis lines, but not lines expressing a deletion mutant missing
451 the repeat motifs, showed enhanced susceptibility to aphids (Pitino and Hogenhout, 2013).
452 We noticed that the RpC002 protein, which lacks the NDNQGEE repeats, is less
453 expressed/stable in *N. benthamiana*, which could explain the limited impact on plant
454 susceptibility in this plant species. Noteworthy, within *M. persicae*, different MpC002
455 variants have been reported with different numbers of the NDNQGEE repeat (Thorpe *et*
456 *al.*, 2016). The biological significance of this repeat variation remains to be elucidated.

457 All selected aphid effectors were expressed regardless whether aphids were exposed to a
458 host, non-host plant or artificial diet. It is possible that, unlike the case for plant pathogens
459 where effector gene expression varies across different infections stages (Cotton *et al.*,
460 2014; Hacquard *et al.*, 2013; Jupe *et al.*, 2013; O'Connell *et al.*, 2012), aphid effectors are
461 constitutively expressed to ensure aphids are generally ready to infest a plant. This
462 hypothesis is in line with other reports where no significant overall effector gene
463 expression variation was reported when aphids were adapted to different plant
464 environments (Lu *et al.*, 2016; Mathers *et al.*, 2017; Thorpe *et al.*, 2018). The Rp1/Mp1
465 and Rp58/Mp58 pair was more similarly expressed in the two aphid species relative to the
466 RpC002/MpC002 effector pair. Interestingly, the Mp1- and Mp58-like effectors are co-
467 located in a non-syntenic region across the genomes of 5 different aphid species, and their
468 expression is tightly co-regulated with a large set of aphid genes, including many
469 (predicted) effectors such as MpC002 (Thorpe *et al.*, 2018). Whether and how these
470 effectors work together to enable aphid infestation remains to be explored.

471 Both RpC002 from *R. padi* and MpC002 from *M. persicae* showed similar localisation to
472 the plasma membrane in *N. benthamiana* indicating this could be their sites of activity.
473 Both the nucleus and plant plasma membrane play key roles in activating plant defences
474 against plant pathogenic microbes (reviewed by (Boutrot and Zipfel, 2017; Motion *et al.*,
475 2015)). The plasma membrane is the site of many immune receptors, such as receptor-like
476 kinases, required for pathogen recognition and initiation of an immune response (Boutrot
477 and Zipfel, 2017). The plasma membrane localization of these aphid effectors might reflect
478 a role in interfering with immune receptors or any other cell membrane associated
479 defences. It should be noted that effector localization using highly expressed effectors
480 (35S-based) may be affected by (endogenous) expression levels of their host targets. For
481 example, in the case where the host target is in low abundance in the epidermal cells
482 analysed by confocal microscopy, the highly abundant effector may not visibly localize to

483 the site of the endogenous target. This is the case for Mp1, which only co-localizes to
484 vesicles in the presence of over-expressed VPS52 (interacting host protein), with
485 endogenous levels of VPS52 being low in leaf tissues (Rodriguez *et al.*, 2017a).

486 Despite a similar subcellular localization of MpC002 and RpC002 in *N. benthamiana*, only
487 MpC002 enhanced plant susceptibility in this plant species to *M. persicae*. Species-
488 specific activity within the aphid C002 family was previously reported and linked the
489 presence/absence of the NDNQGEE repeat motif (Pitino and Hogenhout, 2013). In
490 contrast, RpC002 increases barley susceptibility to *R. padi*, indicating the effector is
491 functional when expressed in an appropriate host plant. Whether the NDNQEE motif is
492 associated with reduced protein expression and/or stability in certain plant species
493 remains to be investigated.

494 Both Rp58 and Mp58 similarly reduced *N. benthamiana* susceptibility to *M. persicae*
495 pointing to a potentially conserved function of these effectors. The reduction in
496 susceptibility mediated by Mp58 is in line with a report by Elzinga et al., (Elzinga *et al.*,
497 2014). Potentially, the artificially high levels of Rp58/Mp58 expression leads to an
498 exaggerated host targeting response and subsequent activation of defences. Alternatively,
499 Rp58/Mp58 was not expressed in tissues where these effectors are usually delivered and
500 active, or these proteins may only function in combination with additional effectors in
501 enhancing plant susceptibility. In contrast to our observations, Atamian et al., (Atamian *et*
502 *al.*, 2013) reported that the putative ortholog of Rp58/Mp58 in *Macrosiphum euphorbiae*
503 (Me10) increased tomato and *N. benthamiana* susceptibility to the potato aphid. Perhaps
504 these effectors function in a different way across plant-aphid interactions.

505 The lack of an impact of Rp1 and Mp1 on *N. benthamiana* susceptibility to *M. persicae*
506 was not surprising as it was previously shown that Mp1, when expressed under the 35S
507 promoter, does not alter plant susceptibility (Bos *et al.*, 2010) (Elzinga *et al.*, 2014).

508 However, when expressed under a phloem-specific promoter, Mp1, but not Rp1, enhances
509 *N. benthamiana* susceptibility to *M. persicae* (Pitino and Hogenhout, 2013) (Rodriguez *et*
510 *al.*, 2017a). Interestingly, Rp1 expression in barley, driven by a ubiquitin-promoter,
511 enhanced barley susceptibility to *R. p*adi but not to the same extent to *M. persicae*,
512 suggesting that not only Mp1, but also Rp1, promotes aphid susceptibility in a specific
513 plant-aphid system. In contrast to the 35S promoter in *N. benthamiana*, the maize ubiquitin
514 promoter in transgenic barley lines is expressed quite strongly in the plant vasculature,
515 which likely is the plant tissue where many aphid effectors function. Barley resistance to *M.*
516 *persicae* is likely phloem-based (Escudero-Martinez *et al.*, 2019) and barley transcriptional
517 responses to this aphid species include a strong activation of a specific set of defence-
518 related genes (Escudero-Martinez *et al.*, 2017). It is possible that effectors from the cereal
519 specialist *R. padi* do not affect barley resistance mechanisms against *M. persicae* and as a
520 result susceptibility remains comparable to wild-type plants.

521 The effect of Rp1 on barley susceptibility to *R. padi* is likely associated with the
522 suppression of several defence genes we observed in transgenic lines expressing this
523 effector. *SAG12-like* encodes a cysteine protease involved in hypersenescence and has
524 been implicated in Arabidopsis PAD4-mediated defence against aphids (Pegadaraju *et al.*,
525 2007). Barley genes encoding beta-thionins contribute to defence against aphids
526 (Escudero-Martinez *et al.*, 2017), as well as encoding for components of the JA signaling
527 pathway (reviewed by (Züst and Agrawal, 2016). For example, *LOX2* overexpression in
528 barley increased resistance towards *R. padi* and *M. persicae*, possibly by activating a
529 group of JA-related genes. In line with this, knock-down of *LOX2* in barley resulted in
530 enhanced susceptibility to these same aphid species (Losvik *et al.*, 2017). The WRKY50
531 transcription factor also is implicated in JA signalling, but negatively regulates JA
532 responses while promoting SA-induced expression of PR1 (Gao *et al.*, 2011; Hussain *et*
533 *al.*, 2018). The mechanical damage caused by the clip cages may have led to activation of

534 WRKY50, at reduced levels in Rp1 transgenic lines compared to the control. The
535 consistent reduction of both SA and ethylene signalling markers (*NPR1* and *EFR1*) 72h
536 after aphid exposure in the Rp1 transgenic, despite higher basal levels in most of the lines,
537 suggests that defence pathways are suppressed upon expression of the Rp1 effector. Our
538 work represents an important step towards understanding the function of aphid effectors
539 promoting susceptibility in a monocot crop. The future identification of barley host targets
540 of effectors like Rp1, will help us further link the observed suppression of defence gene
541 expression to host susceptibility and reveal the underlying mechanisms.

542

543 **Supplementary data**

544 Fig. S1. Pair-wise nucleotide sequence alignments of putative orthologous effectors from
545 *Rhopalosiphum padi* and *Myzus persicae*.

546 Fig. S2. Western blots showing the expression of GFP and the GFP-effector fusion
547 proteins in *Nicotiana benthamiana*.

548 Fig. S3. Localization of *Rhopalosiphum padi* effector RpC002 in *Nicotiana benthamiana*.

549 Fig. S4. Expression of GUS (β-glucuronidase) under control of the maize ubiquitin
550 promoter in different tissue of the barley transgenic line generated using
551 pBRACT214:GUS.

552 Fig. S5. Effector transcript levels in transgenic barley lines expressing *Rhopalosiphum*
553 *padi* effectors and plant phenotypes.

554 Fig. S6. Defence-related gene expression in barley Rp1 lines after exposure to empty clip
555 cages without *Rhopalosiphum padi*.

556

557

558

559

560 **Acknowledgements**

561 We thank Abdellah Barakate (JHI, UK) for help and advice on selection of barley
562 transgenic lines and for providing the pBRACT214m construct, Peter Thorpe for help and
563 advice on analysing gene expression of effectors across RNAseq datasets.

564

565 This work was supported by the Biotechnology and Biological Sciences Research Council
566 (grant no. BB/J005258/1 to J.I.B.B.), the European Research Council (grant no.
567 APHIDHOST-310190 to J.I.B.B.

568

569

570 **Author contributions**

571

572 J.I.B.B. conceived and directed the project; C.E-M and J.I.B.B designed the experiments;
573 C.E-M, P.A.R. and P.A.S performed the experiments; C.E-M, P.A.R. and J.I.B.B. analysed
574 the data; J.S. generated the barley transgenic lines; C.E-M and J.I.B.B. wrote the
575 manuscript; all authors approved the final manuscript.

References

Atamian HS, Chaudhary R, Cin VD, Bao E, Girke T, Kaloshian I. 2013. In planta expression or delivery of potato aphid Macrosiphum euphorbiae effectors Me10 and Me23 enhances aphid fecundity. Mol Plant Microbe Interact **26**, 67-74.

Blackman R, Eastop V. 2000. Aphids on the World's Crops: An Identification and Information Guide. Wiley-Blackwell.

Bos JI, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA. 2010. A functional genomics approach identifies candidate effectors from the aphid species Myzus persicae (green peach aphid). PLoS Genet **6**, e1001216.

Boulain H, Legeai F, Guy E, Morlière S, Douglas NE, Oh J, Murugan M, Smith M, Jaquiéry J, Peccoud J, White FF, Carolan JC, Simon JC, Sugio A. 2018. Fast Evolution and Lineage-Specific Gene Family Expansions of Aphid Salivary Effectors Driven by Interactions with Host-Plants. Genome Biol Evol **10**, 1554-1572.

Boutrot F, Zipfel C. 2017. Function, Discovery, and Exploitation of Plant Pattern Recognition Receptors for Broad-Spectrum Disease Resistance. Annu Rev Phytopathol **55**, 257-286.

Chaudhary R, Peng HC, He J, MacWilliams J, Teixeira M, Tsuchiya T, Chesnais Q, Mudgett MB, Kaloshian I. 2019. Aphid effector Me10 interacts with tomato TFT7, a 14-3-3 isoform involved in aphid resistance. New Phytol **221**, 1518-1528.

Colas I, Barakate A, Macaulay M, Schreiber M, Stephens J, Vivera S, Halpin C, Waugh R, Ramsay L. 2019. desynaptic5 carries a spontaneous semi-dominant mutation affecting Disrupted Meiotic cDNA 1 in barley. J Exp Bot **70**, 2683-2698.

Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, Szcześniak MW, Gaffney DJ, Elo LL, Zhang X, Mortazavi A. 2016. A survey of best practices for RNA-seq data analysis. Genome Biol **17**, 13.

Cooper WR, Dillwith JW, Puterka GJ. 2011. Comparisons of salivary proteins from five aphid (Hemiptera: Aphididae) species. Environ Entomol **40**, 151-156.

Cotton JA, Lilley CJ, Jones LM, Kikuchi T, Reid AJ, Thorpe P, Tsai IJ, Beasley H, Blok V, Cock PJ, Eves-van den Akker S, Holroyd N, Hunt M, Mantelin S, Naghra H, Pain A, Palomares-Rius JE, Zarowiecki M, Berriman M, Jones JT, Urwin PE. 2014. The genome and life-stage specific transcriptomes of Globodera pallida elucidate key aspects of plant parasitism by a cyst nematode. Genome Biol **15**, R43.

Elzinga DA, De Vos M, Jander G. 2014. Suppression of plant defenses by a Myzus persicae (green peach aphid) salivary effector protein. Mol Plant Microbe Interact **27**, 747-756.

Escudero-Martinez C, Leybourne DJ, Bos JIB. 2019. Plant resistance in different cell layers affects aphid probing and feeding behaviour during poor- and non-host interactions. bioRxiv **doi:** https://doi.org/10.1101/372839.

Escudero-Martinez CM, Morris JA, Hedley PE, Bos JIB. 2017. Barley transcriptome analyses upon interaction with different aphid species identify thionins contributing to resistance. Plant Cell Environ **40**, 2628-2643.

Gao QM, Venugopal S, Navarre D, Kachroo A. 2011. Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins. Plant Physiol **155**, 464-476.

Hacquard S, Kracher B, Maekawa T, Vernaldi S, Schulze-Lefert P, Ver Loren van Themaat E. 2013. Mosaic genome structure of the barley powdery mildew pathogen and conservation of transcriptional programs in divergent hosts. Proc Natl Acad Sci U S A **110**, E2219-2228.

Hussain RMF, Sheikh AH, Haider I, Quareshy M, Linthorst HJM. 2018. Arabidopsis WRKY50 and TGA Transcription Factors Synergistically Activate Expression of. Front Plant Sci **9**, 930.

Jupe J, Stam R, Howden AJ, Morris JA, Zhang R, Hedley PE, Huitema E. 2013. Phytophthora capsici-tomato interaction features dramatic shifts in gene expression associated with a hemi-biotrophic lifestyle. Genome Biol **14**, R63.

Karimi M, Inzé D, Depicker A. 2002. GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7**, 193-195.

Losvik A, Beste L, Glinwood R, Ivarson E, Stephens J, Zhu LH, Jonsson L. 2017. Overexpression and Down-Regulation of Barley Lipoxygenase LOX2.2 Affects Jasmonate-Regulated Genes and Aphid Fecundity. Int J Mol Sci **18**.
Lu H, Yang P, Xu Y, Luo L, Zhu J, Cui N, Kang L, Cui F. 2016. Performances of survival, feeding behavior, and gene expression in aphids reveal their different fitness to host alteration. Sci Rep **6**, 19344.
Mathers TC, Chen Y, Kaithakottil G, Legeai F, Mugford ST, Baa-Puyoulet P, Bretaudeau A, Clavijo B, Colella S, Collin O, Dalmay T, Derrien T, Feng H, Gabaldón T, Jordan A, Julca I, Kettles GJ, Kowitwanich K, Lavenier D, Lenzi P, Lopez-Gomollon S, Loska D, Mapleson D, Maumus F, Moxon S, Price DR, Sugio A, van Munster M, Uzest M, Waite D, Jander G, Tagu D, Wilson AC, van Oosterhout C, Swarbreck D, Hogenhout SA. 2017. Rapid transcriptional plasticity of duplicated gene clusters enables a clonally reproducing aphid to colonise diverse plant species. Genome Biol **18**, 27.
Motion GB, Amaro TM, Kulagina N, Huitema E. 2015. Nuclear processes associated with plant immunity and pathogen susceptibility. Brief Funct Genomics **14**, 243-252.
Mutti NS, Louis J, Pappan LK, Pappan K, Begum K, Chen MS, Park Y, Dittmer N, Marshall J, Reese JC, Reeck GR. 2008. A protein from the salivary glands of the pea aphid, Acyrthosiphon pisum, is essential in feeding on a host plant. Proc Natl Acad Sci U S A **105**, 9965-9969.
Nalam V, Louis J, Shah J. 2019. Plant defense against aphids, the pest extraordinaire. Plant Sci **279**, 96-107.
Nelson BK, Cai X, Nebenführ A. 2007. A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J **51**, 1126-1136.
Nicholson SJ, Hartson SD, Puterka GJ. 2012. Proteomic analysis of secreted saliva from Russian wheat aphid (Diuraphis noxia Kurd.) biotypes that differ in virulence to wheat. J Proteomics **75**, 2252-2268.
O'Connell RJ, Thon MR, Hacquard S, Amyotte SG, Kleemann J, Torres MF, Damm U, Buiate EA, Epstein L, Alkan N, Altmüller J, Alvarado-Balderrama L, Bauser CA, Becker C, Birren BW, Chen Z, Choi J, Crouch JA, Duvick JP, Farman MA, Gan P, Heiman D, Henrissat B, Howard RJ, Kabbage M, Koch C, Kracher B, Kubo Y, Law AD, Lebrun MH, Lee YH, Miyara I, Moore N, Neumann U, Nordström K, Panaccione DG, Panstruga R, Place M, Proctor RH, Prusky D, Rech G, Reinhardt R, Rollins JA, Rounsley S, Schardl CL, Schwartz DC, Shenoy N, Shirasu K, Sikhakolli UR, Stüber K, Sukno SA, Sweigard JA, Takano Y, Takahara H, Trail F, van der Does HC, Voll LM, Will I, Young S, Zeng Q, Zhang J, Zhou S, Dickman MB, Schulze-Lefert P, Ver Loren van Themaat E, Ma LJ, Vaillancourt LJ. 2012. Lifestyle transitions in plant pathogenic Colletotrichum fungi deciphered by genome and transcriptome analyses. Nat Genet **44**, 1060-1065.
Pegadaraju V, Louis J, Singh V, Reese JC, Bautor J, Feys BJ, Cook G, Parker JE, Shah J. 2007. Phloem-based resistance to green peach aphid is controlled by Arabidopsis PHYTOALEXIN DEFICIENT4 without its signaling partner ENHANCED DISEASE SUSCEPTIBILITY1. Plant Journal **52**, 332-341.
Petersen TN, Brunak S, von Heijne G, Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods **8**, 785-786.
Pitino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA. 2011. Silencing of aphid genes by dsRNA feeding from plants. PLoS One **6**, e25709.
Pitino M, Hogenhout SA. 2013. Aphid protein effectors promote aphid colonization in a plant species-specific manner. Mol Plant Microbe Interact **26**, 130-139.
Rao SA, Carolan JC, Wilkinson TL. 2013. Proteomic profiling of cereal aphid saliva reveals both ubiquitous and adaptive secreted proteins. PLoS One **8**, e57413.
Rodriguez PA, Escudero-Martinez C, Bos JI. 2017a. An Aphid Effector Targets Trafficking Protein VPS52 in a Host-Specific Manner to Promote Virulence. Plant Physiol **173**, 1892-1903.
Rodriguez PA, Escudero-Martinez C, Bos JIB. 2017b. An Aphid Effector Targets Trafficking Protein VPS52 in a Host-Specific Manner to Promote Virulence. Plant Physiology **173**, 1892--1903.
Rodriguez PA, Stam R, Warbroek T, Bos JIB. 2014. Mp10 and Mp42 from the Aphid Species Myzus persicae Trigger Plant Defenses in Nicotiana benthamiana Through Different Activities. Molecular Plant-Microbe Interactions **27**, 30-39.
Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods **9**, 671-675.

Schulze-Lefert P, Panstruga R. 2011. A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. Trends Plant Sci **16**, 117-125.
Thorpe P, Cock PJA, Bos J. 2016. Comparative transcriptomics and proteomics of three different aphid species identifies core and diverse effector sets. Bmc Genomics **17**.
Thorpe P, Escudero-Martinez CM, Cock PJA, Eves-van den Akker S, Bos JIB. 2018. Shared transcriptional control and disparate gain and loss of aphid parasitism genes. Genome biology and evolution.
Vandermoten S, Harmel N, Mazzucchelli G, De Pauw E, Haubruge E, Francis F. 2014. Comparative analyses of salivary proteins from three aphid species. Insect Mol Biol **23**, 67-77.
Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. 2009. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics **25**, 1189-1191.
Yates AD, Michel A. 2018. Mechanisms of aphid adaptation to host plant resistance. Curr Opin Insect Sci **26**, 41-49.
Zhang Y, Fan J, Sun J, Francis F, Chen J. 2017. Transcriptome analysis of the salivary glands of the grain aphid, Sitobion avenae. Sci Rep **7**, 15911.
Züst T, Agrawal AA. 2016. Mechanisms and evolution of plant resistance to aphids. Nat Plants **2**, 15206.

Fig. 1

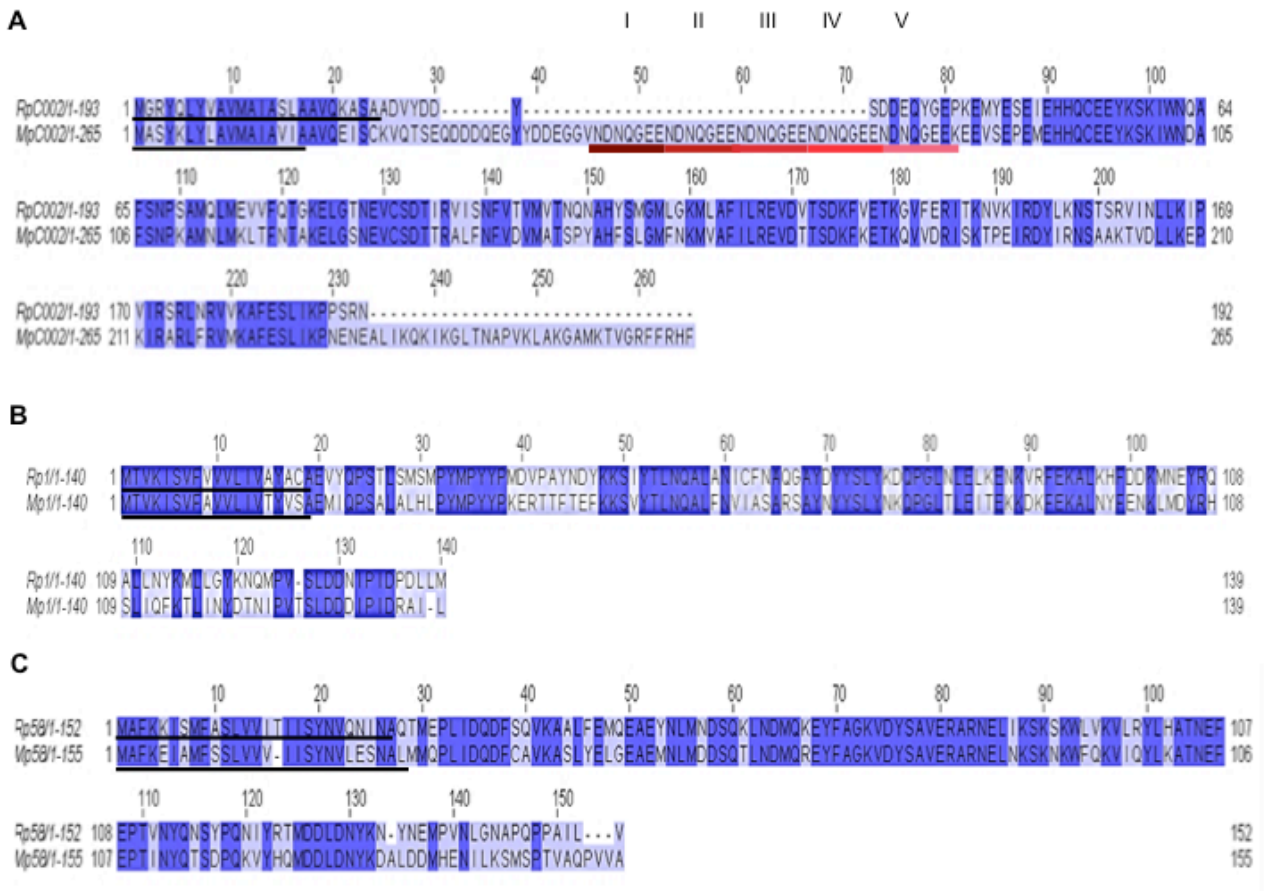


Figure Legends

Fig. 1. Pair-wise amino acid sequence alignments of three selected effectors from *Rhopalosiphum padi* and *Myzus persicae*.

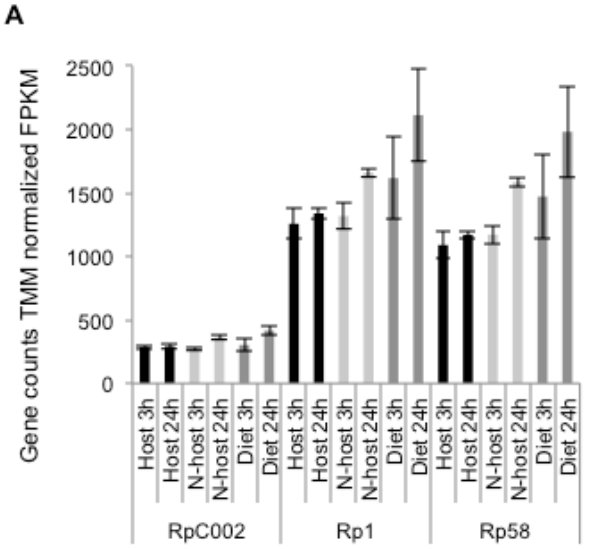
Alignments were generated using Jalview 2.10.4. The level of sequence conservation is indicated by dark (high identity) to light purple colour (low identity). Predicted signal peptide (Signal P4.1) sequences are underlined in black.

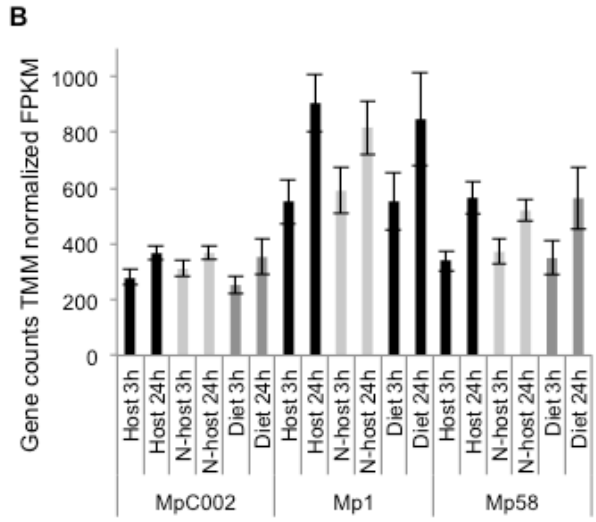
A) RpC002/MpC002 alignment. The 5x repeat motif (NDNQGEE) in MpC002 is underlined with different shades of red to pink.

B) Rp1/Mp1 alignment.

C) Rp58/Mp58 alignment.

Fig. 2





C

	RpC002	Rp1	Rp58	MpC002	Mp1	Mp58
Host 3 h	287[ab]	1257[a]	1089[a]	279[a]	551[a]	338[a]
Host 24 h	292 [ab]	1339[a]	1169[ab]	368[a]	904[a]	564[b]
N-host 3 h	271[a]	1317[a]	1171[ab]	310[a]	590[a]	371[a]
N-host 24 h	364[bc]	1660[a]	1585[bc]	366[a]	817[a]	519[ab]
Diet 3 h	302[ab]	1617[a]	1471[abc]	252[a]	550[a]	349[a]
Diet 24 h	416[c]	2113[a]	1981[c]	353[a]	846[a]	564[b]

Fig. 2. Effector gene expression in *Rhopalosiphum padi* and *Myzus persicae* upon exposure to different feeding environments.

A) Expression of *R. padi* effectors *RpC002*, *Rp1* and *Rp58* upon aphid exposure to barley (host), Arabidopsis (non-host) or artificial diet for 3h or 24h. The expression of transcripts was normalized by the TMM-FPKM method (fragments per kilo-base of exon per million reads mapped). Bars indicate standard error.

B) Expression of *M. persicae* effectors *MpC002*, *Mp1* and *Mp58* upon aphid exposure to Arabidopsis (host), barley (poor-host) or artificial diet. The expression of transcripts was normalized by the TMM-FPKM method (fragments per kilo-base of exon per million reads mapped). Bars indicate standard error.

C) Table displaying expression values for each effector. Letters indicate significant differences as determined by one-way ANOVA and post-hoc protected Least Significant Differences ($p<0.05$ *; $p<0.01$ ***).

Fig. 3

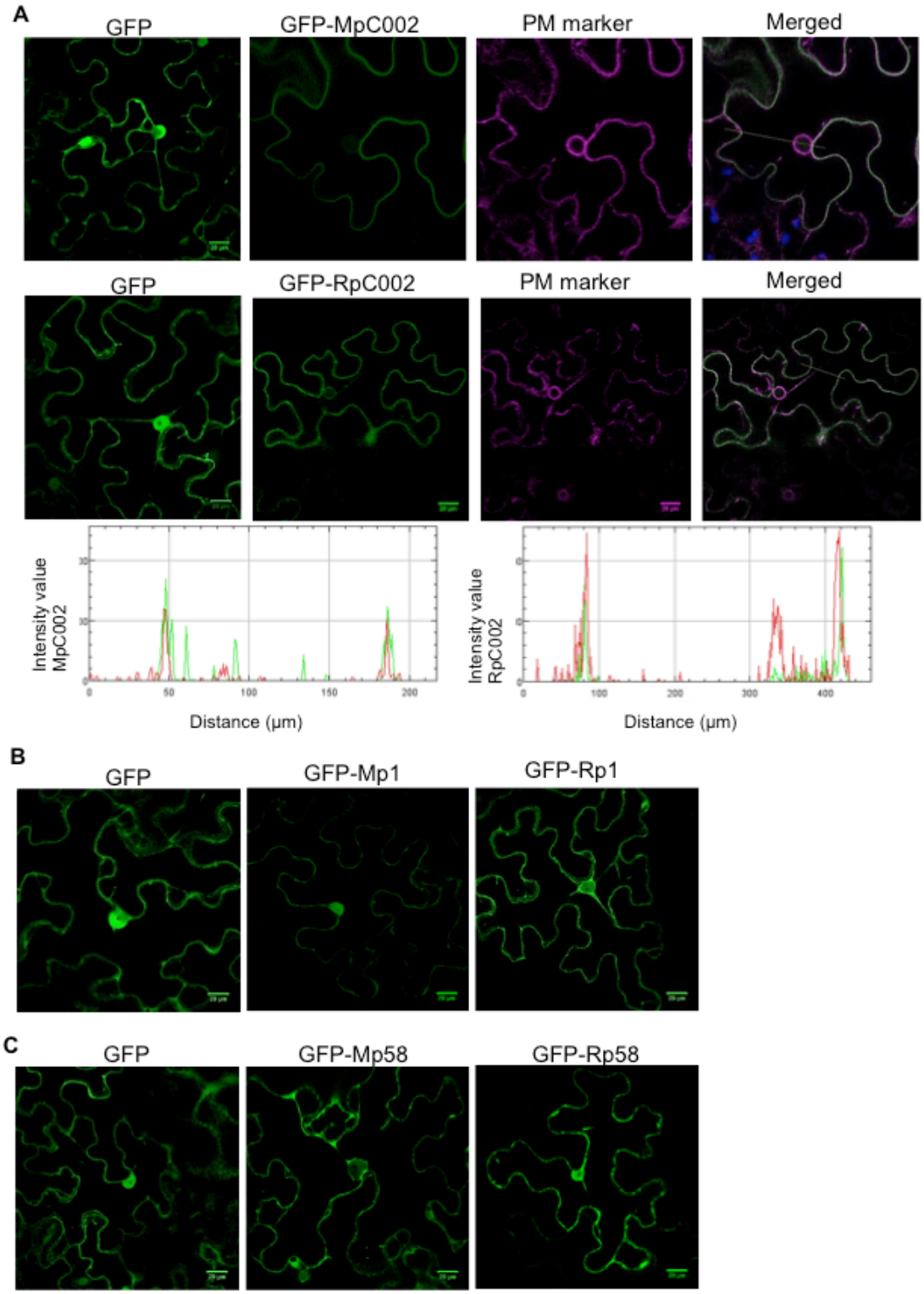


Fig. 3. Localization of aphid effectors in *Nicotiana benthamiana*

A) Confocal microscopy images of free GFP (empty vector, pB7WG2F), and effectors GFP-MpC002 and GFP-RpC002 (middle section) transiently overexpressed in *N.*

benthamiana. Both effectors were co-expressed with a plasma membrane marker (PM marker; Nelson et al., 2007). Merged pictures represent the overlay image of the GFP and RFP channels. Graphs represent overlay between the fluorescence intensity of the GFP (green series) and RFP (red series) channels of a particular selected region of interest (ROI) shown as a line in the merged picture. The images were taken 3 days after agroinfiltration. The co-localization was analysed by Fiji software and the plugin RGB profiler.

B) Confocal microscopy images of free GFP alone (pB7WG2F), and effectors GFP-Mp1 / GFP-Rp1.

C) Confocal microscopy images of free GFP alone (pB7WG2F), and effector GFP-Mp58 / Rp58.

Fig. 4

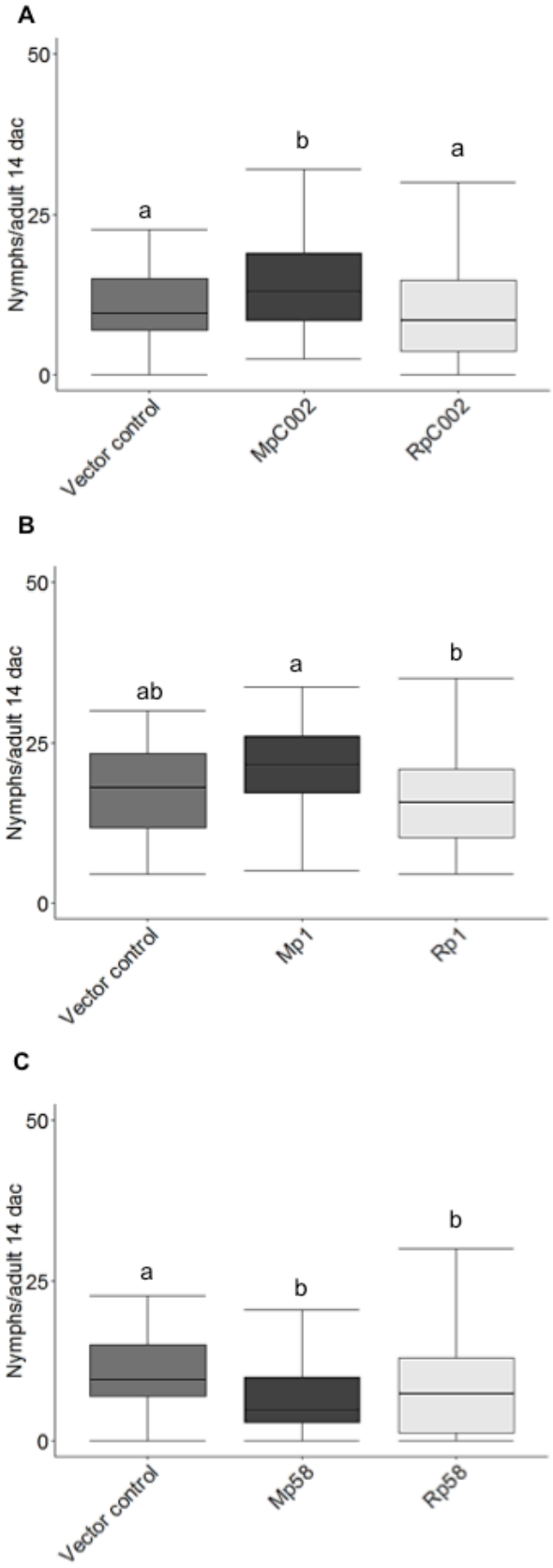


Fig. 4. *Myzus persicae* performance on *Nicotiana benthamiana* plants expressing aphid effectors

Leaves of *N. benthamiana* were agroinfiltrated with different effector constructs (35S-promoter) and infiltration sites were challenged with 3 *M. persicae* nymphs, which were allowed to develop and reproduce. Nymph production per aphid was monitored over a 14-day period, with the aphids being moved to freshly infiltrated leaves every 7 days. Empty vector was used as a control.

A) Number of nymphs produced per adult on *N. benthamiana* leaves expressing the vector control, MpC002 or RpC002.

B) Number of nymphs produced per adult on *N. benthamiana* leaves expressing the vector control, Mp1 or Rp1.

C) Number of nymphs produced per adult on *N. benthamiana* leaves expressing the vector control, Mp58 or Rp58.

Box plots show the average number of nymphs per adult 14 days after challenge (dac) from three independent biological replicates (number of plants per effector or control used on each replicate = 12). Different letters indicates significant differences at $p>0.05$. Statistical analyses were performed using one-way ANOVA post-hoc Fisher's protected Least Significant Differences ($p>0.05$).

Fig. 5

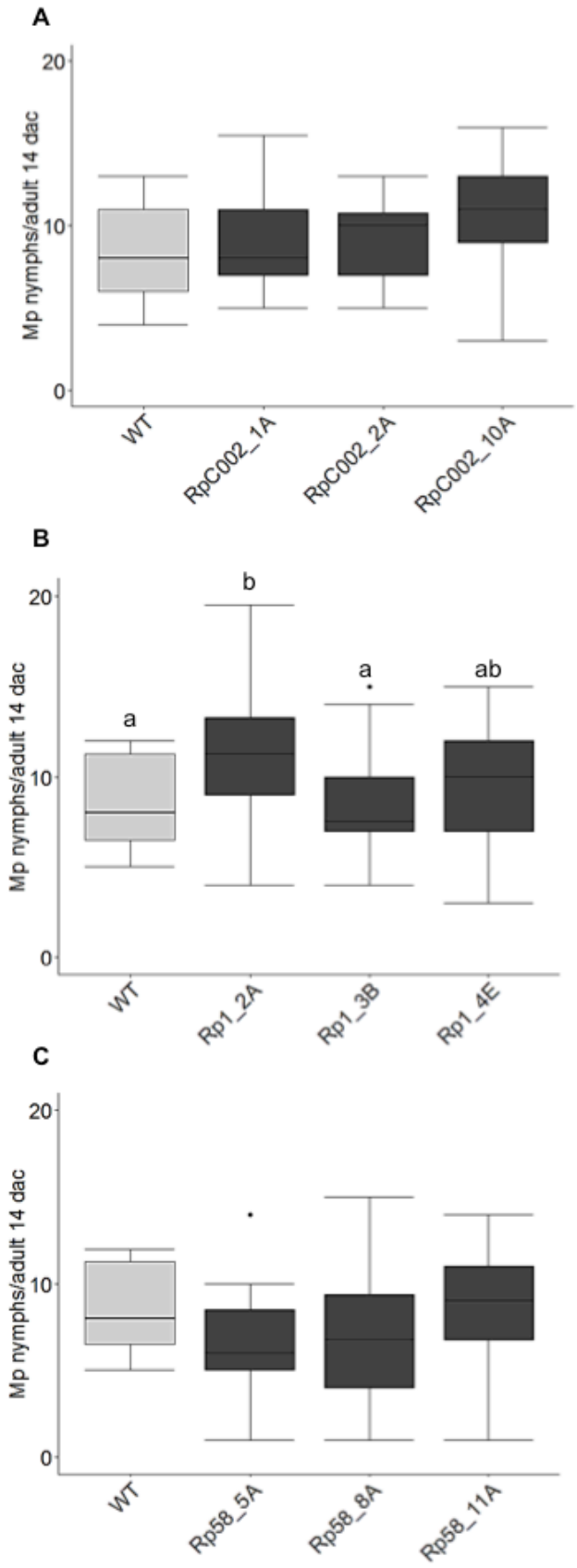


Fig. 5. *Myzus persicae* performance on barley plants ectopically expressing different *Rhopalosiphum padi* effectors.

Transgenic barley lines were challenged with aphids alongside wild-type plants cv Golden Promise (WT). Nymph production was monitored for 14 days.

A) Nymph production per adult on transgenic barley lines expressing effector RpC002. Three independent transgenic lines were assessed: RpC002_1A, RpC002_2A and RpC002_10A.

B) Nymph production per adult on transgenic barley lines expressing effector Rp1. Three independent transgenic lines were assessed: Rp1_2A, Rp1_3B and Rp1_4E.

C) Nymph production per adult on transgenic barley lines expressing effector Rp58. Three independent transgenic lines were assessed: Rp58_5A, Rp58_8A and Rp58_11A.

Box plots show the average number of nymphs per adult 14 days after challenge (dac) from at least three independent biological replicates (number of plants per effector or control used on each replicate = 6-8). Different letters indicate significant differences as determined with one-way ANOVA post-hoc Fisher's protected least significant difference test ($p>0.05$).

Fig. 6

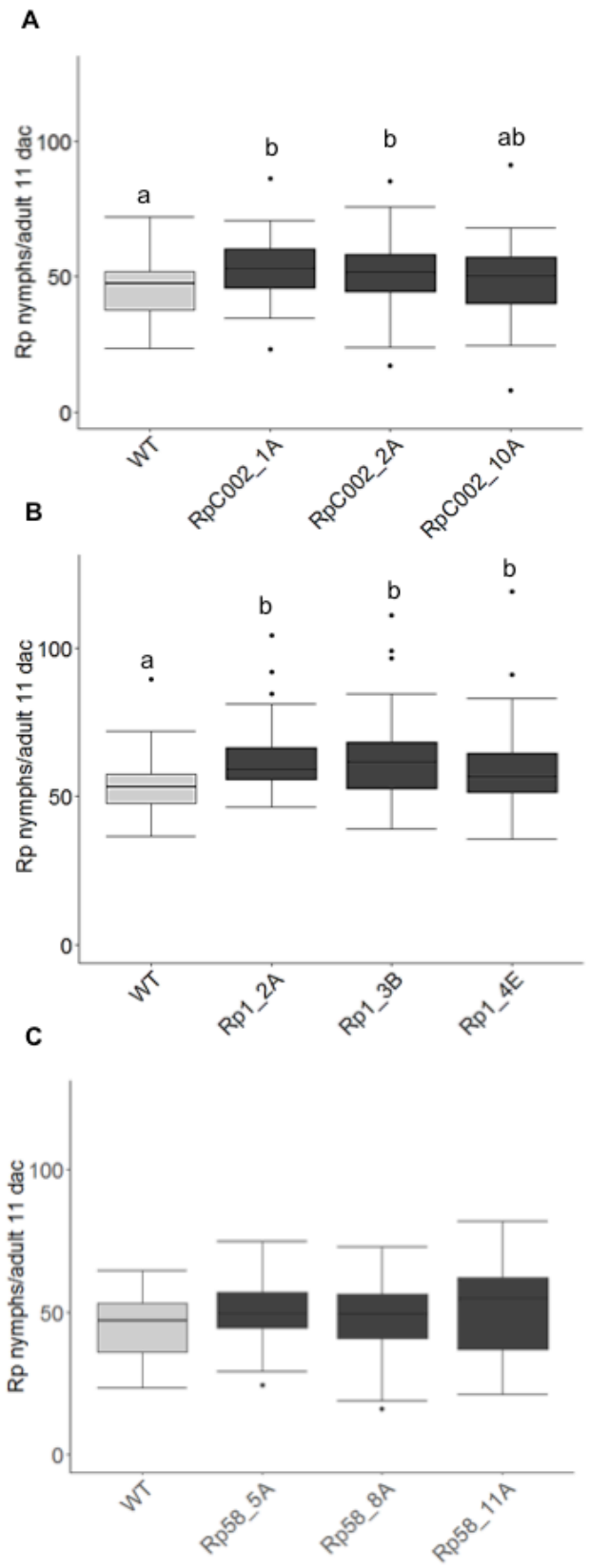


Fig. 6. *Rhopalosiphum padi* performance on barley plants ectopically expressing different *R. padi* effectors. Transgenic barley lines were challenged with aphids

alongside wild-type plants cv Golden Promise (WT). Nymph production was monitored for 11 days.

A) Nymph production per adult on transgenic barley lines expressing effector RpC002. Three independent transgenic lines were assessed: RpC002_1A, RpC002_2A and RpC002_10A.

B) Nymph production per adult on transgenic barley lines expressing effector Rp1. Three independent transgenic lines were assessed: Rp1_2A, Rp1_3B and Rp1_4E.

C) Nymph production per adult on transgenic barley lines expressing effector Rp58. Three independent transgenic lines were assessed: Rp58_5A, Rp58_8A and Rp58_11A.

Box plots show the average number of nymphs per adult 11 days after challenge (dac) from at least three independent biological replicates (number of plants per effector or control used on each replicate = 5-10). Different letters indicate significant differences as determined with one-way ANOVA post-hoc Fisher's protected least significant difference test ($p>0.05$).

Fig. 7

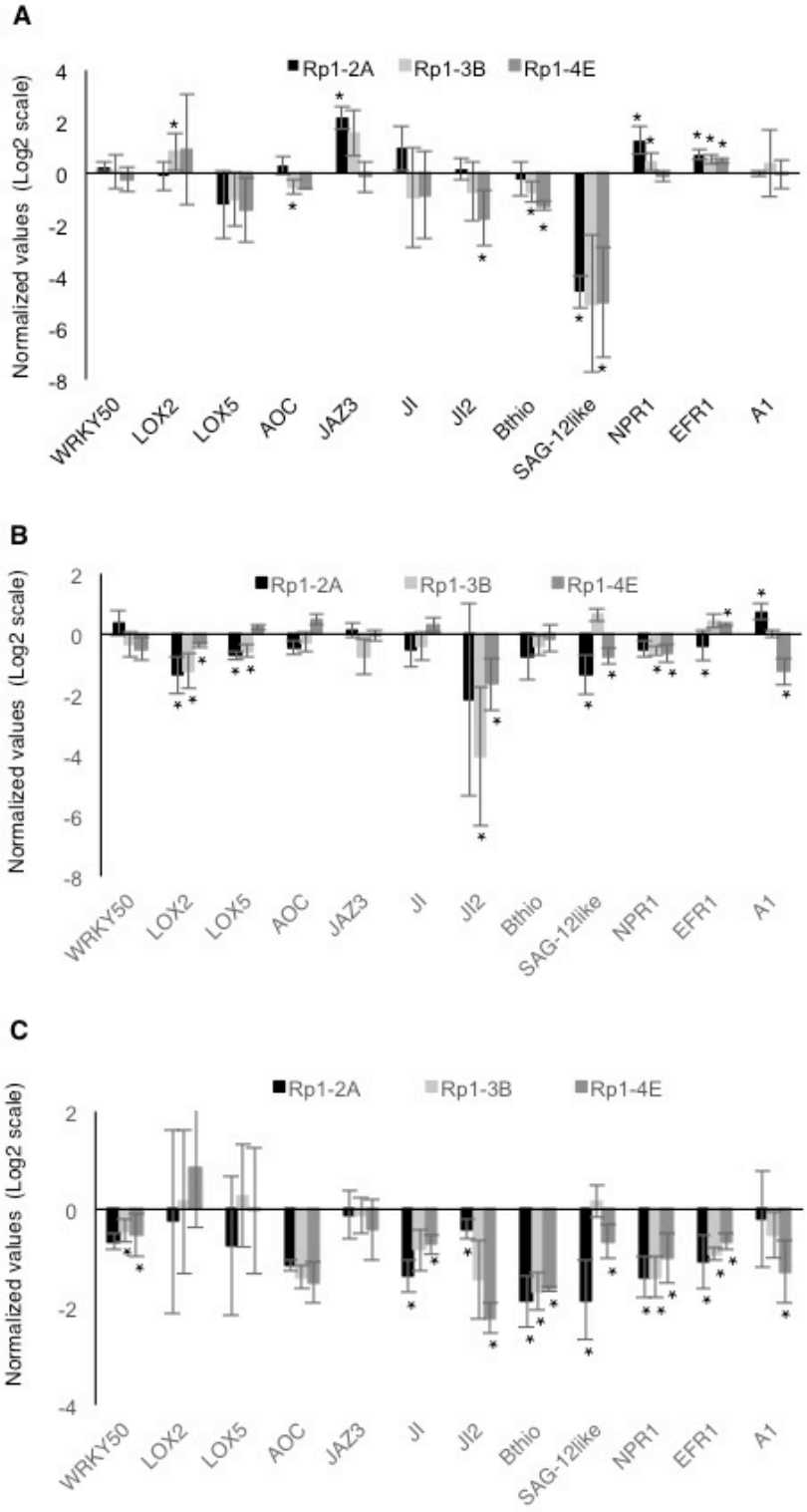


Fig. 7. Basal and aphid-induced defence gene expression in barley Rp1 lines.

Relative gene expression of defence-related/hormone-signalling genes was measured by qRT-PCR in control barley plants (cv. Golden Promise) and three independent barley lines expressing the *R. padi* effector Rp1.

A) Log-fold changes of barley basal gene expression (no aphids, no clip cage) in three transgenic Rp1 barley lines relative to control lines (WT=0).

B) Log-fold changes of barley gene expression upon 24h exposure to clip cages with *R. padi* aphids in three transgenic Rp1 barley lines relative to control plants (WT=0).

C) Log-fold changes of barley gene expression upon 72h exposure to clip cages with *R. padi* aphids in three transgenic Rp1 barley lines relative to control lines (WT=0).

All gene expression analyses were based on three independent biological replicates, and graphs represent mean expression. Genes are represented in the graphs are: *WRKY transcription factor 50* (*WRKY50*, MLOC_66204), *lipoxygenase 2* (*LOX2*, MLOC_AK357253), *lipoxygenase 5* (*LOX5*, MLOC_71948), *allene cyclase oxydase* (*AOC*, MLOC_68361), *jasmonate ZIM domain gene 3* (*JAZ3*, MLOC_9995), *jasmonate-induced gene* (*JI*, MLOC_15761), *jasmonate-induced gene 2* (*JI2*, MLOC_56924), *beta-thionin* (AK252675), *SAG12-like* (MLOC_74627.1), *non-expressor of pathogenesis-related 1-like* (*NPR1*, AM050559.1), the *ethylene-response factor 1* (*EFR1*, MLOC_38561) and *abscisic acid-inducible late embryogenesis abundant 1* (*A1*, MLOC_72442). Black bars represent gene expression levels in Rp1-2A lines, light grey bars represent gene expression levels in Rp1-3B lines and grey bars gene expression levels in Rp1-4E lines. Asterisks indicate significant differences between control plants (WT) and Rp1 transgenic lines (Wilcoxon Rank Sum Test, $p \leq 0.05$).