A cereal aphid effector promotes barley susceptibility in a species-specific manner through suppression of defence gene expression

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Abstract

The bird cherry aphid *Rhopalosiphum padi* and the green peach aphid *Myzus persicae* are devastating insect pests on agriculturally important crops. While the host range of *R. padi* consists mainly of cereal plants, *M. persicae* can infest thousands of plant species from across different plant families. However, despite a broad host range, which includes *Nicotiana benthamiana*, *M. persicae* performs poorly on barley. To gain insights into the role of aphid effectors in determining aphid host range, we undertook a comparative functional characterization approach for 3 predicted orthologous effector pairs from *M. persicae* and *R. padi*. We compared subcellular localisation and protein stability of these effectors in *N. benthamiana* and determined their contribution to host susceptibility. While subcellular localization of members of each effector pair was similar, we observed differences in proteins stability, especially in the case of RpC002 (*R. padi* C002)/MpC002 (*M. persicae* C002). For example, RpC002 was less expressed and/or stable than MpC002 in *N. benthamiana* and, in contrast to MpC002, did not enhance susceptibility in this plant species. However, expression of RpC002, as well as *R. padi* effector Rp1, in transgenic barley lines enhanced plant susceptibility to *R. padi* but not *M. persicae*. Our data suggests that some aphid effectors specifically function when expressed in host species, and feature activities that benefit their corresponding aphid species. Characterization of Rp1 transgenic barley lines revealed reduced gene expression of plant hormone signalling genes relevant to plant-aphid interactions, suggesting this effector enhances susceptibility by suppressing plant defences in barley.

Introduction

Similar to plant pathogens, aphids form close associations with their hosts and secrete effector molecules to modulate host cell processes to their benefit. Over the past decade,

27 a combination of genomics- and proteomics-based approaches allowed the identification
28 of putative effectors from different aphid species, including economically important pests of
29 both monocot and dicot crops (Harmel et al., 2008; Carolan et al., 2011; Cooper et al.,
30 2010; Nicholson et al., 2012; Rao et al., 2013; Atamian et al., 2013; Vandermoten et al
31 2014; Thorpe et al., 2016; Zhang et al., 2017; Thorpe et al., 2018). Comparative analyses
32 of aphid effector repertoires across species has revealed core and diverse sets, provided
33 insight into effector diversity, and evidence for a shared transcriptional control mechanism
34 driving their expression (Thorpe et al., 2016); (Boulain et al., 2018); (Thorpe et al., 2018).
35 Moreover, functional characterization of aphid effectors increased our understanding of
36 how these proteins may function to enhance plant susceptibility during infestation (as
37 reviewed by Yates and Michel, 2018 and Nalam et al., 2019), and pointed to host-specific
38 effector activities (Pitino and Hogenhout, 2013; Elzinga et al., 2014; Rodriguez et al.,
39 2017).

40 The C002 salivary protein was first described as an effector in *Acyrthosiphon pisum*, and
41 promotes host susceptibility to aphids. However, while expression of MpC002 (*M. persicae*
42 C002) in host species *Arabidopsis* and *N. benthamiana* enhances susceptibility to *M.*
43 *persicae*, expression of ApC002 from *A. pisum* in these same plant species has no visible
44 impact on the host interaction with *M. persicae* (Pitino and Hogenhout, 2013). The
45 difference in effector activity was attributed to a motif sequence (NDQGEE) in the N-
46 terminal region of MpC002, which is lacking in ApC002 (Pitino and Hogenhout, 2013).
47 Whether the presence/absence of this motif affects protein stability rather than activity
48 across plant species remains to be determined. In addition, several effectors from the
49 broad host range pest *M. persicae* have been implicated in promoting host susceptibility,
50 including Mp1 and Mp58 (Pitino and Hogenhout, 2013; Elzinga et al., 2014; Rodriguez et
51 al., 2017). However, the underlying mechanisms by which these effectors impact
52 susceptibility remain largely unknown. We previously described that Mp1 associates with

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

68 *Rhopalosiphum padi* is an aphid species with a narrow host range, which includes grass
69 species, such as barley, oats and wheat (Blackman and Eastop, 2000). This aphid is an
70 important pest of cereal crops that causes feeding damage and transmits some of the
71 most destructive viral diseases of cereals, such as *Barley Yellow Dwarf Virus* (BYDV).
72 Whilst *R. padi* is highly specialized on cereals, other species, like *M. persicae,* feature an
73 exceptionally broad host range that includes more than 4000 different plant species,
74 including the model plants Arabidopsis or *N. benthamiana* (Blackman and Eastop, 2000).
75 Despite its broad host range, *M. persicae* is not a pest of barley and performs poorly on
76 this plant species (Escudero-Martinez et al., 2017). Recently, *M. persicae* and *R. padi*
77 effector repertoires were identified and compared, allowing the prediction of orthologue
78 effector pairs (Thorpe et al., 2016). To what extend the observed sequence variation

79 among these effector sets impacts host susceptibility and aphid host range is to be
80 determined and will require extensive functional characterization across different plant
81 species, including cereals.

82 Here, we characterized 3 predicted orthologous effector pairs from *M. persicae* and *R.*
83 *padi* with regards to their subcellular localization, protein stability, gene expression, and
84 contribution to susceptibility in barley and *N. benthamiana*. We demonstrate that
85 expression of the *R. padi* effectors Rp1 and RpC002 in transgenic barley lines enhances
86 plant susceptibility to *R. padi* (host interaction) but not to the same extent to *M. persicae*
87 (poor-host interaction), highlighting the importance of these effectors for barley
88 colonization in an aphid species-specific manner. In contrast, and unlike MpC002, RpC002
89 did not enhance *N. benthamiana* susceptibility to *M. persicae* (host interaction), which is
90 likely due to low protein expression and/or stability of RpC002 in this plant species. Further
91 characterization of Rp1 transgenic barley lines revealed reduced expression of several
92 markers of plant hormone signalling pathways relevant to plant-aphid interactions,
93 suggesting this effector may enhance susceptibility by suppressing plant defences.

94 **Results**

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

96 We predicted putative orthologs for 3 previously described *M. persicae* effectors, MpC002,
97 Mp1 and Mp58 from *R. padi* using reciprocal best blast hit analyses on available aphid
98 transcriptome datasets and aphid genome assemblies (threshold of 70 % identity and
99 50 % query coverage) (Thorpe et al., 2016; Thorpe et al., 2018). To confirm the sequences
100 of putative orthologous effector pairs we cloned and sequenced their coding sequences.
101 Amino acid and nucleotide sequence alignments show varying degrees of sequence
102 divergence across the selected effector pairs (Fig. 1, Supplemental Fig. S1). RpC002 is
103 smaller than MpC002, with 193 amino acids compared to 265, respectively, and these

104 effectors share 52.86% sequence identity. The difference in sequence length is partly due
105 to a lack of the NDNQGEE repeat in the N-terminal region of RpC002 (Fig. 1A,
106 Supplemental Fig. S1). Variation in the number of NDNQGEE repeats in MpC002 was
107 previously also detected within *M. persicae* (Thorpe et al., 2016), and in this study we
108 characterized the MpC002 version containing 5 repeats. MpC002 also has an extended C-
109 terminal domain compared to RpC002 (Fig. 1A). Rp1, which is similar to *M. persicae* Mp1,
110 is composed of 140 amino acids compared to 139 for Mp1, and these effectors share a
111 percentage sequence identity of 56.12% (Fig. 1B, Supplemental Fig. S1). Lastly, Mp58
112 and Rp58 contain 152 and 155 amino acids, respectively, share 64.94% sequence
113 identity, and are most divergent in the C-terminal region of the protein (Fig. 1C,
114 Supplemental Fig. S1).

115 **Effector gene expression varies between aphid species but is consistent across**
116 **host and non-/poor-host interactions**

117 We were interested in assessing how gene expression of the three effector pairs was
118 affected in the two different aphid species, *M. persicae* and *R. padi* upon exposure to host,
119 non-/poor-host plants and artifical diet. We made use of previously generated aphid
120 RNAseq datasets (Thorpe et al., 2018) to investigate gene expression of our effectors of
121 interest by plotting their gene counts across different treatments (exposure to diet, host
122 and poor/nonhost plants) and timepoints (3h and 24h exposure). All 6 aphid effectors were
123 expressed with only limited variation in expression across the different aphid treatments
124 and timepoints (Fig. 2). Whilst the three selected effectors from *M. persicae* displayed
125 more similar gene expression levels compared to one another, ranging from 280 counts for
126 MpC002 to 904 counts for Mp1 (Fig. 2B and C), the different effectors from *R. padi*
127 showed a wider range of expression. For instance, gene counts varied from 271 for
128 RpC002 to 2112 for Rp1 over the various treatments and timepoints (Fig. 2A). .

129 **Similar subcellular localization of putative orthologous effectors from *M. persicae***
130 **and *R. padi* in *N. benthamiana***

131 The subcellular localization of effectors can provide important information on the cellular
132 compartment that is targeted by these proteins. We hypothesised that effector variants
133 from different aphid species may have different subcellular localization patterns when
134 expressed in a host or in a non-host plant due to divergence of potential host targets. We
135 transiently expressed GFP-effector fusion proteins (N-terminal GFP tag) for the 3 effector
136 pairs in leaves of *N. benthamiana*, which is a host for *M. persicae*, but a non-host for *R.*
137 *padi*. Western blotting showed that all GFP-fusion proteins were expressed, but that two of
138 the *R. padi* effectors, RpC002 and Rp58, were less expressed compared to MpC002 and
139 Mp58 from *M. persicae*, respectively (Supplemental Fig. S2), with RpC002 only detected
140 once in three biological replicates (Supplemental Fig. S2). In contrast, *M. persicae* effector
141 Mp1 was less expressed compared to its putative orthologue Rp1 from *R. padi*
142 (Supplemental Fig. S2). We detected GFP signal corresponding to the MpC002- and
143 RpC002-fusion proteins by confocal microscopy at the plasma membrane, and in some
144 cases, a weak signal was present in the nucleus or the cytoplasm (Fig. 3A and
145 Supplemental Fig. S3). It should be noted that expression of RpC002 was very low,
146 especially compared to MpC002 (Supplemental Fig. S2), and only a few transformed cells
147 were visible. We validated the plasma membrane localization of RpC002/MpC002
148 effectors upon co-expression with a plasma membrane marker (Nelson et al., 2007) (Fig.
149 3A). Both the Mp1/Rp1 and Mp58/Rp58 pair were detected in the cytoplasm and nucleus,
150 similar to the free GFP control (Fig. 3B and Fig. 3C). Similarly, we tried to express tagged
151 effectors in barley epidermal cells using particle bombardment, but due to low signal we
152 were unable to reliably localize effectors in this system. Overall, members of the predicted
153 orthologous effector pairs show similar subcellular localization patterns in *N. benthamiana*,
154 but show differences in protein expression and/or stability in this plant species.

155 **Specific versus conserved effects of putative orthologous aphid effectors on host**
156 **susceptibility to *M. persicae***

157 To assess whether the members of the 3 predicted orthologous effector pairs similarly
158 impacted host susceptibility to *M. persicae*, we performed aphid performance assays on *N.*
159 *benthamiana* leaves transiently expressing the different effectors under the control of a
160 35S promoter. In line with previous reports (Bos et al., 2010; Pitino and Hogenhout, 2013),
161 we found that ectopic expression of MpC002 significantly increased the number of *M.*
162 *persicae* nymphs produced per adult by 27% (One-way ANOVA post-hoc Fisher's
163 protected Least Significant Differences (LSD); p>0,05) (Fig. 4A). In contrast, RpC002 did
164 not alter *N. benthamiana* host susceptibility to *M. persicae*. Western blot analyses of GFP-
165 MpC002 and GFP-RpC002 showed that RpC002 protein is detected at a much lower level
166 than MpC002 (Fig. S2), which likely accounts for the observed difference in affecting plant
167 susceptibility. No significant differences in host susceptibility were noted upon expression
168 of Mp1 from *M. persicae* and Rp1 from *R. padi* (Fig. 4B), in line also with a previous report
169 that transient expression of Mp1 under the 35S promoter in *N. benthamiana* does not
170 affect susceptibility (Bos et al., 2010). The expression of Mp58, but not Rp58, resulted in
171 significantly lower *M. persicae* nymph production compared to the vector control, with 55%
172 and 27% less nymphs being produced per adult, respectively (One way ANOVA post-hoc
173 Fisher's protected Least Significant Differences (LSD); p>0,05) (Fig. 4C).

174

175 **Expression of RpC002 and Rp1 in transgenic barley enhances susceptibility to *R.***
176 ***padi***

177 Aphid effector characterization studies to date have focused on dicot plant species. With
178 *R. padi* being a major pest of cereals, we aimed to extend aphid effector characterization
179 studies to the monocot crop barley to explore the contribution of *R. padi* effectors to host

180 susceptibility. We generated barley transgenic lines in the cultivar Golden Promise to
181 ectopically express the three *R. padi* effectors Rp1, RpC002 and Rp58 using a modified
182 version of the pBRACT214 vector (Colas et al., 2019), containing the ubiquitin promoter
183 from maize to allow constitutive expression in all plant organs
184 (http://www.bract.org/constructs.htm#barley). To determine where candidate genes of
185 interest are potentially expressed when transformed into barley using this pBRACT214m
186 construct, we performed GUS-staining on different plant tissues, such as leaves, stems,
187 spikes and roots of a barley transgenic line generated by transformation with
188 pBRACT214m:GUS (Supplemental Fig. S4). We observed GUS expression in all tissues,
189 with particularly strong expression in leaf vascular tissues and roots (Supplemental Fig.
190 S4). After barley transformation, we obtained 13 independent lines for the RpC002
191 effector, 4 lines for the Rp1 effector, and 16 lines for Rp58. In the first generation, lines
192 with a single effector insertion were selected based on around 75% survival on
193 Hygromycin (hygromycin phosphotransferase is the pBRACT selection marker), yielding 8
194 independent transgenic lines for RpC002, 3 lines for Rp1, and 7 lines for Rp58. The
195 presence of effector coding sequences (lacking the signal peptide encoding sequence)
196 was confirmed in the T0 generation by semi quantitative RT-PCR (data not shown) and
197 was verified in the T1 generation by qRT-PCR (Fig. S5). We did not observe any visual
198 differences in plant growth and development for any of the transgenic lines selected (Fig.
199 S5). Three homozygous T3 lines per effector construct were selected for aphid
200 performance assays with *R. padi* and *M. persicae* to assess how barley host and poor-host
201 interactions with aphids were affected. Each plant was infested with two nymphs and
202 reproduction was assessed after 11 days for *R. padi* and after 14 days for *M. persicae.*

203 For *M. persicae* we did not find consistent significant differences in aphid performance on
204 the barley transgenic lines expressing the *R. padi* effectors compared to the wild-type
205 control (Fig. 5). For one of the Rp1 lines, Rp1_2A, however, we noted increased nymph

206 production (Fig. 5B). Line Rp1_2A was also the highest Rp1 expressing line we identified
207 (Supplemental Fig. S5), and additional lines with similar expression levels would need to
208 be identified to rule out the possibility that the insertion site in this line causes the
209 enhanced susceptibility phenotype.

210 Ectopic expression of both RpC002 and Rp1 in transgenic barley lines enhanced
211 susceptibility to *R. padi* (Fig. 6A and B). Specifically, two out of three independent RpC002
212 barley lines, RpC002_1A and RpC002_2A, showed 16% and 12% increased nymph
213 production compared to the wild-type control, respectively (One way ANOVA post-hoc
214 Fisher's protected Least Significant Differences (LSD); p>0,05) (Fig. 6A). The transgenic
215 line with the strongest susceptibility phenotype (RpC002_1A) also showed the highest
216 *RpC002* expression level (Fig. 6A; Supplemental Fig. S5). In addition, all three
217 independent Rp1 barley lines showed enhanced susceptibility to *R. padi* with an increased
218 nymph production of 11-22% across lines compared to the wild-type control (One way
219 ANOVA post-hoc Fisher's protected Least Significant Differences (LSD); p>0,05) (Figure
220 6B). Also, we observed a correlation between the level of effector gene expression and
221 impact on host susceptibility to aphids, with the lines showing the most pronounced
222 susceptibility phenotype towards *R. padi* (Rp1_2A and Rp1_3B) also showing the higher
223 *Rp1* transcript levels (Fig. 6B; Supplemental Fig. 4).

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

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

227 To gain further insight into how Rp1 may enhance barley host susceptibility to aphids, we
228 investigated the basal and induced expression levels of defence-related genes in the three
229 independent transgenic lines we generated (lines 2A, 3B and 4E), compared to the wild-
230 type cultivar Golden Promise. Based on our previous work (Escudero-Martinez et al.,

231 2017) we selected barley genes strongly induced upon *R. padi* infestation: antimicrobial
232 peptide beta-thionin (AK252675), cysteine proteinase SAG12-like (MLOC_74627.1), a
233 jasmonate ZIM domain gene 3 (JAZ3, MLOC_9995), lipoxygenase 2 (LOX2,
234 MLOC_AK357253) and the jasmonate-induced gene (JI, MLOC_15761). We further
235 expand our selected genes set based on markers of different hormone signalling
236 pathways, with focus on the jasmonate pathway, which is strongly activated upon aphid
237 infestation (Escudero-Martinez et al., 2017): lipoxygenase 5 (LOX5, MLOC_71948), the
238 WRKY transcription factor 50 (WRKY50, MLOC_66204), the allene cyclase oxydase
239 (AOC, MLOC_68361) and jasmonate-induced gene 2 (JI2, MLOC_56924) markers for
240 jasmonate; but also the salicylic acid marker non-expressor of pathogenesis-related 1-like
241 (NPR1, AM050559.1), the ethylene-response factor 1 (EFR1, MLOC_38561) and abscisic
242 acid-inducible late embryogenesis abundant (A1, MLOC_72442). We analysed basal gene
243 expression levels as well as expression levels upon 24h and 72h exposure to clip cages
244 with or without aphids. It should be noted that the use of clip-cages, even when empty,
245 triggers changes in gene expression due to mechanical damage. Therefore, we compared
246 basal gene expression levels across plant lines not infested with aphids and without being
247 exposed to a clip-cage (Fig. 7A).

248 We found that expression of a gene encoding a SAG-12 like cysteine protease
249 (MLOC_74627.1) was most strongly reduced at basal levels in Rp1 lines compared to the
250 wild-type control, but differences were only significant for lines Rp1-2A and Rp1-4E,
251 possibly due to sample variation for line Rp1-3B (Fig. 7A). SAG12-like expression was
252 also reduced in the transgenic lines upon exposure to either empty clip cages (Fig. 7B and
253 D) or clip cages containing aphids (Fig. 7C and E), compared to wild-type plants, but not
254 consistently to statistically significant levels. The basal level of EFR1 expression was
255 slightly but significantly higher in Rp1 transgenic lines compared to the wild-type control
256 (Fig. 7A).

257

258 In addition, four genes (WRKY50, AOC, beta-thionin, NPR1) were significantly less
259 expressed across all transgenic lines compared to the wild-type control when leaves were
260 exposed for 24h to empty clip-cages (Fig. 7B). LOX2, JI, and JI2 showed a trend towards
261 reduced expression in transgenic lines, but differences were not consistently significant
262 across all lines (Fig. 7B). In response to clip-cages with aphids for 24h, only LOX2 showed
263 a significant reduction in expression in all transgenic lines, whereas JI2 reduced
264 expression was noticeable not consistently significant (Fig. 7C). For the 72h timepoint, 3
265 marker genes (beta-thionin, NPR1, EFR1) showed a significant reduction in expression in
266 all lines compared to wild-type plants when exposed to clip-cages containing aphids, and
267 similar trends were observed for WRKY50, JI, JI2 and SAG12-like (Fig. 7E). Overall, we
268 observed a reduction of several marker genes of defence/hormone signalling pathways
269 relevant to plant-aphid interactions in the Rp1 transgenic barley lines, which may translate
270 into their enhanced susceptibility to aphids.

271

272 **Discussion**

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

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

298 All selected aphid effectors were expressed regardless whether aphids were exposed to a
299 host, non-host plant or artificial diet. It is possible that, unlike the case for plant pathogens
300 where effector gene expression varies across different infections stages (O'Connell et al.,
301 2012; Jupe et al., 2013; Hacquard et al., 2013; Cotton et al., 2014), aphid effectors are
302 constitutively expressed to ensure aphids are generally ready to infest a plant. This
303 hypothesis is in line with other reports where no significant overall effector gene
304 expression variation was reported when aphids were adapted to different plant
305 environments (Lu et al., 2016; Mathers et al., 2017; Thorpe et al., 2018). The Rp1/Mp1
306 and Rp58/Mp58 pair was more similarly expressed in the two aphid species relative to the
307 RpC002/MpC002 effector pair. Interestingly, the Mp1- and Mp58-like effectors are co-
308 located in a non-syntenic region across the genomes of 5 different aphid species, and their

309 expression is tightly co-regulated with a large set of aphid genes, including many
310 (predicted) effectors such as MpC002 (Thorpe et al., 2018). Whether and how these
311 effectors work together to enable aphid infestation remains to be explored.

312 The selected putative orthologous effectors localized in the same cellular compartments,
313 with 2 of the pairs localizing to the cytoplasm and one pair, RpC002/MpC002, localizing to
314 the plasma membrane indicating these could be their sites of activity. Both the nucleus
315 and plant plasma membrane play key roles in activating plant defences against plant
316 pathogenic microbes (reviewed by Boutrot and Zipel, 2017; Motion et al., 2015). The
317 plasma membrane is the site of many immune receptors, such as receptor-like kinases,
318 required for pathogen recognition and initiation of an immune response (Boutrot and Zipel,
319 2017). The plasma membrane localization of MpC002/RpC002 might reflect a role in
320 interfering with immune receptors or any other cell membrane associated defences.
321 Moreover, it should be noted that effector localization using highly expressed effectors
322 (35S-based) may be affected by (endogenous) expression levels of their host targets. For
323 example, in the case where the host target is in low abundance in the epidermal cells
324 analysed by confocal microscopy, the highly abundant effector may not visibly localize to
325 the site of the endogenous target. This is the case for Mp1, which only co-localizes to
326 vesicles in the presence of over-expressed VPS52 (interacting host protein), with
327 endogenous levels of VPS52 being low in leaf tissues (Rodriguez et al., 2017).

328 Despite a similar subcellular localization of MpC002 and RpC002 in *N. benthamiana*, only
329 MpC002 enhanced plant susceptibility in this plant species to *M. persicae*. Species-
330 specific activity within the aphid C002 family was previously reported and linked the
331 presence/absence of the NDNQGEE repeat motif (Pitino and Hogenhout, 2013). Here we
332 show that this difference could be due to different protein stability of these effectors in *N.*
333 *benthamiana*, which may be affected by the NDNQGEE motif. In contrast, RpC002

334 increases barley susceptibility to *R. padi*, indicating the effector is functional when
335 expressed in an appropriate host plant. It is possible that some aphid effectors are less
336 stable in non-host plants due to the absence of interacting host proteins that do not only
337 facilitate effector activity but also stability.

338 Both Mp58 and Rp58 similarly reduced *N. benthamiana* susceptibility to *M. persicae*
339 pointing to a potentially conserved function of this effector pair. The reduction in
340 susceptibility mediated by Mp58 is in line with a report by Elzinga et al., (Elzinga et al.,
341 2014). Potentially, the artificially high levels of Mp58/Rp58 expression leads to an
342 exaggerated host targeting response and subsequent activation of defences. Alternatively,
343 Mp58/Rp58 was not expressed in tissues where these effectors are usually delivered and
344 active, or these proteins may only function in combination with additional effectors in
345 enhancing plant susceptibility. In contrast to our observations, Atamian et al., (Atamian et
346 al., 2013) reported that the putative orthologue of Mp58/Rp58 in *Macrosiphum euphorbiae*
347 (Me10) increased tomato and *N. benthamiana* susceptibility to the potato aphid. Perhaps
348 these effectors function in a different way across plant-aphid interactions.

349 The lack of an impact of Mp1/Rp1 on *N. benthamiana* susceptibility to *M. persicae* was not
350 surprising as it was previously shown that Mp1, when expressed under the 35S promoter,
351 does not alter plant susceptibility (Bos et al., 2010; Elzinga et al., 2014). However, when
352 expressed under a phloem-specific promoter, Mp1, but not Rp1, enhances *N.*
353 *benthamiana* susceptibility to *M. persicae* (Pitino and Hogenhout, 2013; (Rodriguez et al.,
354 2017). Interestingly, Rp1 expression in barley, driven by a ubiquitin-promoter, enhanced
355 barley susceptibility to *R. p*adi but not to the same extent to *M. persicae*, suggesting that
356 not only Mp1, but also Rp1, functions in a species-specific manner but in a different host.
357 In contrast to the 35S promoter in *N. benthamiana*, the maize ubiquitin promoter in
358 transgenic barley lines is expressed quite strongly in the plant vasculature, which likely is

359 the plant tissue where many aphid effectors function. Barley resistance to *M. persicae* is
360 likely phloem-based and barley transcriptional responses to this aphid species include a
361 strong activation of a specific set of defence-related genes (Escudero-Martinez et al.,
362 2017). It is possible that effectors from the cereal specialist *R. padi* do not affect barley
363 resistance mechanisms against *M. persicae* and as a result susceptibility remains
364 comparable to wild-type plants.

365 The effect of Rp1 on barley susceptibility to *R. padi* is likely linked to the suppression of
366 several defence genes we observed in transgenic lines expressing this effector. SAG12-
367 like encodes a cysteine protease involved in hypersenescence and has been implicated in
368 Arabidopsis PAD4-mediated defence against aphids (Pegadaraju et al., 2005). Barley
369 genes encoding beta-thionins contribute to defence against aphids (Escudero-Martinez et
370 al., 2017), as well as encoding for components of the JA signaling pathway (reviewed by
371 Zust and Agrawal, 2016). For example, LOX2 overexpression in barley increased
372 resistance towards *R. padi* and *M. persicae*, possibly by activating a group of JA-related
373 genes . In line with this, knock-down of lox2 in barley resulted in enhanced susceptibility to
374 these same aphid species (Losvik et al., 2017). The WRKY50 transcription factor also is
375 implicated in JA signalling, but negatively regulates JA responses while promoting SA-
376 induced expression of PR1 (Gao et al., 2011; Hussain et al., 2018). The mechanical
377 damage caused by the clip cages may have led to activation of WRKY50, at reduced
378 levels in Rp1 transgenic lines compared to the control. The consistent reduction of both SA
379 and ethylene signalling markers (NPR1 and EFR1) 72h after aphid exposure in the Rp1
380 transgenic, despite higher basal levels in most of the lines, suggests that defence
381 pathways are suppressed upon expression of the Rp1 effector. Our work represents an
382 important step towards understanding the function of aphid effectors promoting
383 susceptibility in a monocot crop. The future identification of barley host targets of effectors

384 like Rp1, will help us further link the observed suppression of defence gene expression to
385 host susceptibility and reveal the underlying mechanisms.
386

387 **Material & methods**

388 **Aphid cultures**

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

393 **Identification of orthologues effector pairs and plasmid construction**

394 Effector annotation and identification of orthologs was performed as described by Thorpe
395 et al. 2016. Similarity searches were performed by reciprocal best BLAST hit analysis
396 between *R. padi* and *M. persicae* transcriptomes with the minimum thresholds of 70 %
397 identity and 50 % query coverage. Pair-wise sequence analysis was performed in Jalview
398 2.10.4 (Waterhouse et al., 2009) with T-coffee and default parameters. Signal peptide
399 sequences were predicted with SignalP 4.1 (Petersen et al., 2011). Coding sequences
400 were amplified from *R. padi* and *M. persicae* cDNAs, without the region encoding for the
401 signal peptide, and verified by sequencing (Primers in Supplementary Table S1). The
402 resulting amplicons were cloned by Gateway technology into pDONR201, pDONR207 or
403 pENTR_D-TOPO (Gateway®, Invitrogen). Sequence verified inserts were cloned into
404 different destination vectors by LR reaction. Destination vectors pB7WGF2 (35S promoter,
405 N-terminal GFP) and pB7WG2 (35S promoter, no tag) (Karimi, et al. 2002) were used for
406 transient overexpression in *N. benthamiana*, and pBRACT214m (maize ubiquitin promoter,
407 no tag), kindly provided by Abdellah Barakate (JHI) (Colas et al., 2019), was used for
408 generating transgenic barley lines.

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

411 The experimental set-up for determining aphid effector expression in aphids exposed to
412 the different feeding environments is explained in detail in Thorpe et al., (Thorpe et al.,
413 2018). Briefly, aphids were exposed to an artificial diet, host, poor/nonhost plant for 3 and
414 24h and collected for RNA samples preparation and their transcriptome was sequenced by
415 RNAseq. More specifically, *R. padi* was exposed to barley (host) and Arabidopsis (non-
416 host), and *M. persicae* was exposed to Arabidopsis (host) and barley (poor-host). Both
417 aphids were also exposed to artificial diet for 3 and 24h. A total of five independent
418 replicates were used for this experiment and differential expression (DE) analyses was
419 performed as described (Thorpe et al., 2018). For each selected effector (Mp1/Rp1,
420 MpC002/RpC002 and Mp58/Rp58), we performed BLAST searches against the RNAseq
421 datasets described in Thorpe et al., 2018 to identify their corresponding gene models.
422 Transcripts were normalized by the fragments per kilo-base of exon per million reads
423 mapped (TMM-FPKM) method, which normalized the gene counts to the gene length and
424 the library size (Conesa et al, 2016).

425 **Effector localization**

426 Effectors were cloned into pB7WGF2 and the constructs were transformed into
427 *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cells were harvested by
428 centrifugation (8min, 6000rpm) and resuspended in infiltration buffer (acetosyringone 125
429 μM and $MgCl_2$ 10mM) to an optical density of OD_{600}= 0.1. *Agrobacterium* carrying the
430 GFP-effector constructs were then infiltrated in *N. benthamiana* leaves. RpC002 and
431 MpC002 were co-expressed with a plasma membrane molecular marker (Nelson et al.,
432 2007) and with the p19 silencing suppressor (OD_{600} = 0.1) for improving expression and
433 thereby detection under the confocal microscope. All other effector pairs were infiltrated

434 without p19. Three days after infiltration, plants were analysed under the confocal
435 microscope (Zeiss LSM 510 (Jena, Germany) using a Zeiss x20/x40 lens. GFP-fusion
436 proteins and free GFP were imaged with the GFP and chlorophyll filter (488 nm excitation),
437 and co-localization with the membrane marker was analysed with GFP-RFP filter (488-
438 561nm excitation). The experiment was repeated four times, and the resulting images
439 were processed using ImageJ (Schneider et al., 2012).

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

441 Effectors were cloned into the pB7WG2 vector and constructs were transformed into
442 *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* cells were treated as above
443 and infiltrated in *N. benthamiana* leaves to an optical density of OD_{600} = 0.3. After four
444 days, samples were harvested, and proteins were extracted with GTEN buffer (10%
445 Glycerol, 25mM Tris pH 7.5, 1mM EDTA, 150mM NaCl, 0.1% NP-40, 10mM DTT and 1x
446 protease inhibitor cocktail, Sigma). Western blots were incubated overnight with GFP-
447 antibody (Santa Cruz Biotechnology Inc, USA), for 1 hour with anti-rabbit-HRP (Santa
448 Cruz Biotechnology Inc, USA).

449

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

451 Each of the effectors was cloned into the destination vector pBRACT214m containing the
452 ubiquitin promoter from maize for constitutive expression in all plant organs, and a
453 hygromycin marker gene for selection of transgenic lines. Constructs were transformed
454 into the *Agrobacterium* AGL1 strain, supplied with pSOUP, and delivered to the Functional
455 Genomics Facility (FUNGEN) at the James Hutton Institute for *Agrobacterium*-mediated
456 barley embryo transformation of the cultivar Golden Promise. After approximately four
457 months, we obtained different barley lines regenerated from independent calli. The T0
458 generation was tested for the expression of effector genes by PCR on cDNA from the

459 regenerated plants. RNA was extracted from T0 independent lines using the RNeasy Plant
460 Mini Kit (Qiagen). RNA plant samples were DNAse treated with Ambion® TURBO DNA-
461 free™. SuperScript® III Reverse Transcriptase (Invitrogen) and random primers were used
462 to prepare cDNA. The majority of these plants were positive in PCR tests using effector
463 gene-specific primers (Supplementary Table S1). T1 seeds were germinated on selective
464 media (AgarGel™- containing Hygromycin (100 µg/ml) to select for transformants. Lines
465 showing a 1:3 segregation, representing a single insertion (75 % survival rate on selective
466 media), were selected for further analyses. Universal Probe Library (UPL-Roche
467 Diagnostics ©) was used to quantify effector gene expression in T1 barley transgenic lines
468 ectopically expressing *R. padi* effectors. Barley cv Golden Promise, the background
469 genotype of the transgenic lines, was used as control. RNA from six different barley lines
470 per construct was reverse transcribed into cDNA. Probes and primers (Supplementary
471 Table S1) designed with the UPL System Assay Design (Roche) were tested for at least
472 95-105% efficiency. Internal controls were Actin-2 (MLOC_78511.2) and Pentatricopeptide
473 (AK373147/MLOC_80089.1) as described previously (Escudero-Martinez et al., 2017).
474 Three technical replicates were included for each sample. Relative expression was
475 calculated with the method ΔCt (Delta Cycle threshold) with primer efficiency
476 consideration. One of each of the transgenic effector lines was used as a reference line to
477 calculate the fold-change in additional lines.

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

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

483 Effectors were transiently expressed using vector pB7WG2 in *N. benthamiana* as
484 explained above. The empty vector pB7WG2 was used as a control. Two infiltration sites
485 per plant (n=12) were used per construct. One day after infiltration, the abaxial side of the
486 infiltration sites was exposed to two *M. persicae* adults enclosed in a clip cage. The
487 following day, adult aphids were removed leaving three 1st instar nymphs at the underside
488 of the leaves in a clip cage. Seven days later, *N. benthamiana* plants were replaced by
489 freshly infiltrated plants to ensure continued expression of effectors in the plant tissue.
490 After 14 days, the number of nymphs per adult was counted and data were analysed by
491 One way ANOVA and the post-hoc Fisher's protected Least Significant Differences (LSD)
492 (cut-off $p \leq 0.05$).

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

494 Seven-day old transgenic barley plants expressing *R. padi* effectors were infested with two
495 1st instar age-synchronized nymphs (*M. persicae*) or with two 2-day old age-synchronized
496 nymphs (*R. padi*). Barley cv. Golden Promise wild-type plants were used as the control.
497 We used n=6-8 plants per individual transgenic line for each biological replicate (per aphid
498 species), with four biological replicates. The number of nymphs per adult was monitored at
499 11 days after infestation for *R. padi*, and after 14 days for *M. persicae*. The resulting data
500 was analysed by One-way ANOVA with post-hoc Fisher's protected Least Significant
501 Differences (LSD).

502 **Histochemical GUS staining**

503 To assess GUS expression driven by the maize ubiquitin promoter in transgenic barley
504 transformed with the pBRACT214m-GUS construct we collected different tissues and
505 stained these with 1mg/ml of X-gluc (5-bromo-4-chloro-3-indolyl-B-D-glucuronic acid,
506 Thermo Scientific, USA) in X-gluc buffer (100mM sodium phosphate buffer pH 7.0, 0.1%
507 Triton X-100, 2mM potassium ferricyanide and 2mM potassium ferrocyanide). Tissues

508 were vacuum-infiltrated and incubated in darkness at 37°C overnight. The next day,
509 chlorophyll was removed from the tissues with 1:3 acetic acid/ethanol. Pictures were taken
510 under the dissecting microscope with a Zeiss camera.

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

512 Gene expression of different defence/hormone signalling pathways genes were analysed
513 by qRT-PCR, The transgenic barley Rp1 lines along with the control plants (cv. Golden
514 Promise) were pre-germinated in Petri dishes covered with wet filter paper for three days
515 in the dark at room temperature. Germinated seeds placed on soil and grown under
516 controlled conditions (8h light, 22°C, 70% humidity and 125 µmol photons/m2.s). For
517 basal gene expression the first leaf of the plants (n=6 per genotype) were collected and
518 flash frozen in liquid nitrogen. In addition, barley plants (n=6 per genotype) were exposed
519 to either empty clip cages or clip cages containing 30 mixed-age *R. padi* aphids. Leaf
520 tissues enclosed within the clip cages were collected after 24 h and 72 h. The experiment
521 was performed in three biological replicates and samples were harvested at the same time
522 of the day: barley plants were treated and collected at 12 am for the 24 h timepoint and at
523 3 pm for 72 h timepoint, avoiding any effects of the plant circadian cycle.

524 The local database *Morex genes* was used for retrieving the barely sequences and the
525 *Roche UPL assay design centre* for primer design (STable_1). The primers were tested
526 for efficiency (85-115%) and relative gene expression was calculated with the method
527 ΔΔCt (Delta-delta Cycle threshold). Three technical replicates were included for each
528 sample. Cycle threshold values were normalized with two reference genes,
529 pentatricopeptide (AK373147/MLOC_80089) and ubiquitin (AK248472). Expression of
530 these two reference genes was unaffected in our previous microarray experiment
531 (Escudero-Martinez et al., 2017). The Wilcoxon Rank Sum Test (cut-off $p \leq 0.05$) was used
532 to assess differences in expression between plant genotypes and treatments.

533 **Acknowledgements**

534 We thank Abdellah Barakate (JHI, UK) for help and advice on selection of barley
535 transgenic lines and for providing the pBRACT214m construct, Peter Thorpe for help and
536 advice on analysing gene expression of effectors across RNAseq datasets.
537
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542
543
544 **Author contributions**
545 J.I.B.B. conceived and directed the project; C.E-M and J.I.B.B designed the experiments;
546 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
547 the data; J.S. generated the barley transgenic lines; C.E-M and J.I.B.B. wrote the
548 manuscript; all authors approved the final manuscript.

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Figures

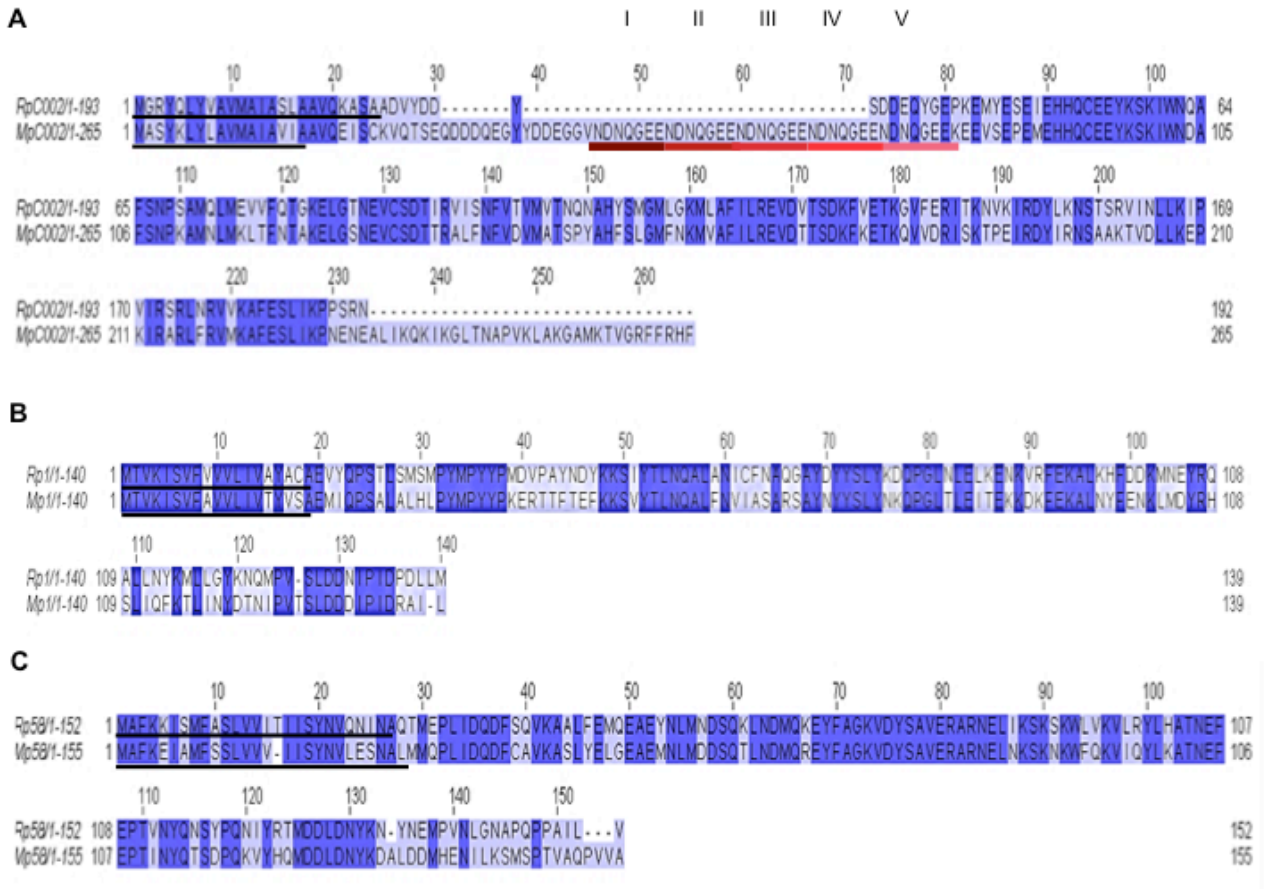


Figure 1. Pair-wise amino acid sequence alignments of effectors from *Rhopalosiphum padi* and *Myzus persicae*.

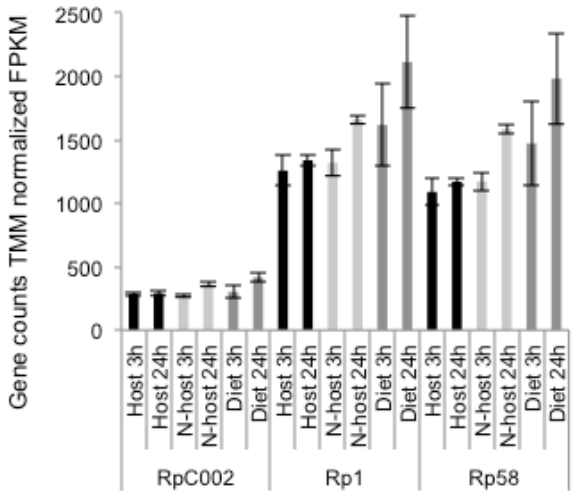
Alignments were generated using Jalview 2.10.4. The level of sequence conservation is indicated by dark (high conservation) to light purple colour (low conservation). Predicted signal peptide (Signal P4.1) sequences are underlined in black.

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.

A



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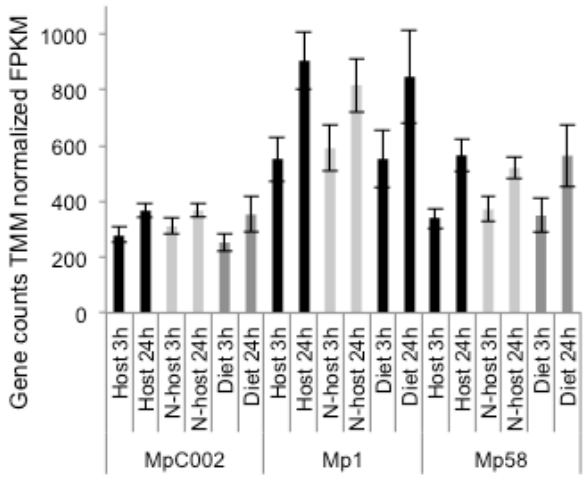

C

	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]

Figure 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 ($p<0.05$ *; $p<0.01$ ***).

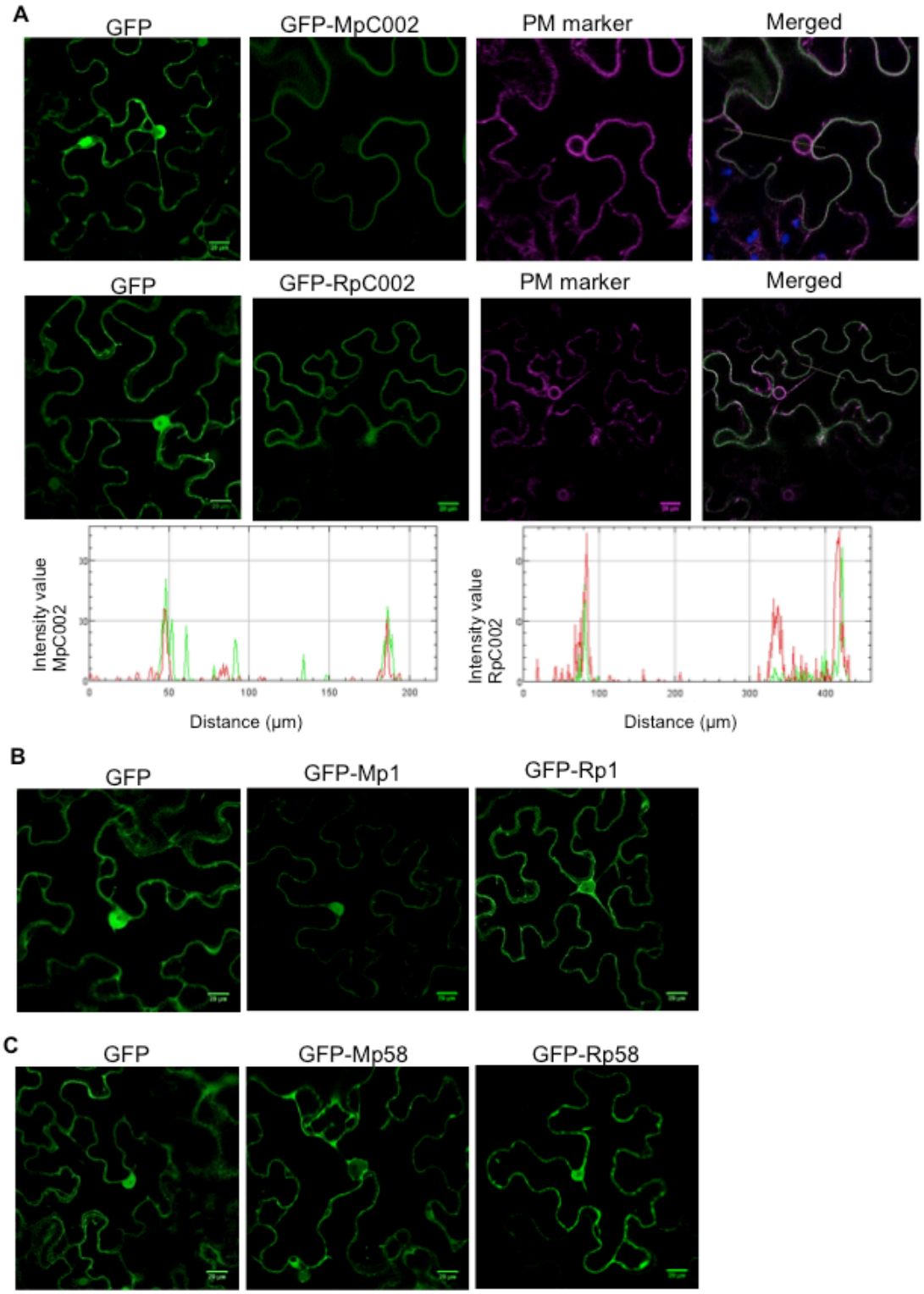

Figure 3. Localization of aphid effectors in *Nicotiana benthamiana*

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.

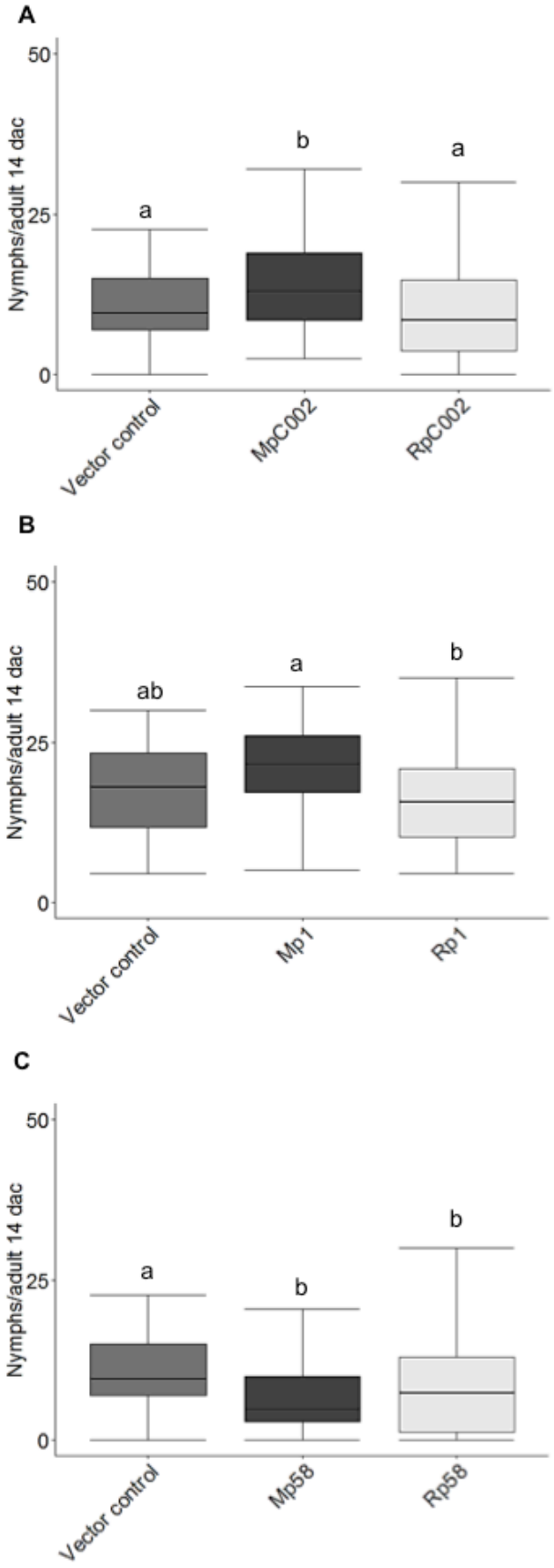

Figure 4. Ectopic expression of aphid effectors in *Nicotiana benthamiana* alters susceptibility to *Myzus persicae*.

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.

Bar graphs show the average number of nymphs per adult 14 days after challenge (dac) from three independent replicates (n=12). Error bars represent the standard error and 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 ($p>0.05$).

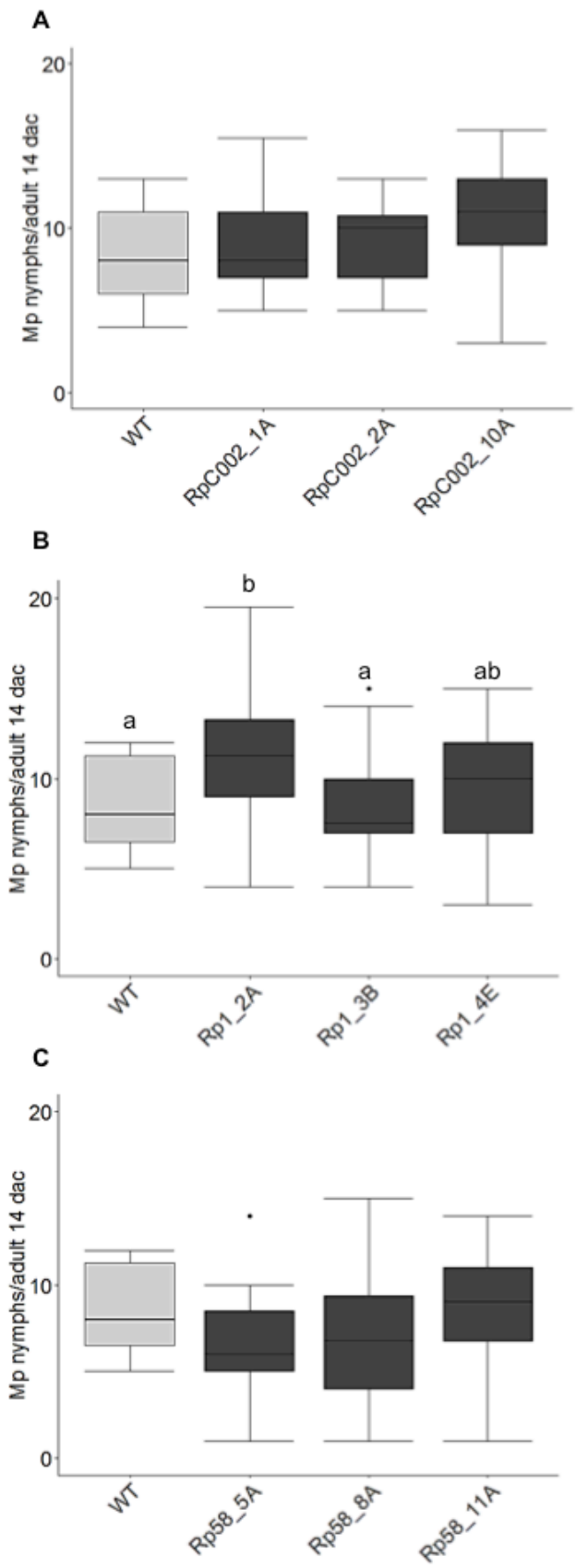

Figure 5. *Myzus persicae* performance on barley plants ectopically expressing different *Rhopalosiphum padi* effectors.

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.

Bar graphs show the average number of nymphs per adult 14 days after challenge (dac) from at least three independent replicates (n=6-8). Error bars represent the standard error and different letters indicate significant differences as determined with one-way ANOVA post-hoc Fisher's protected least significant difference test ($p>0.05$).

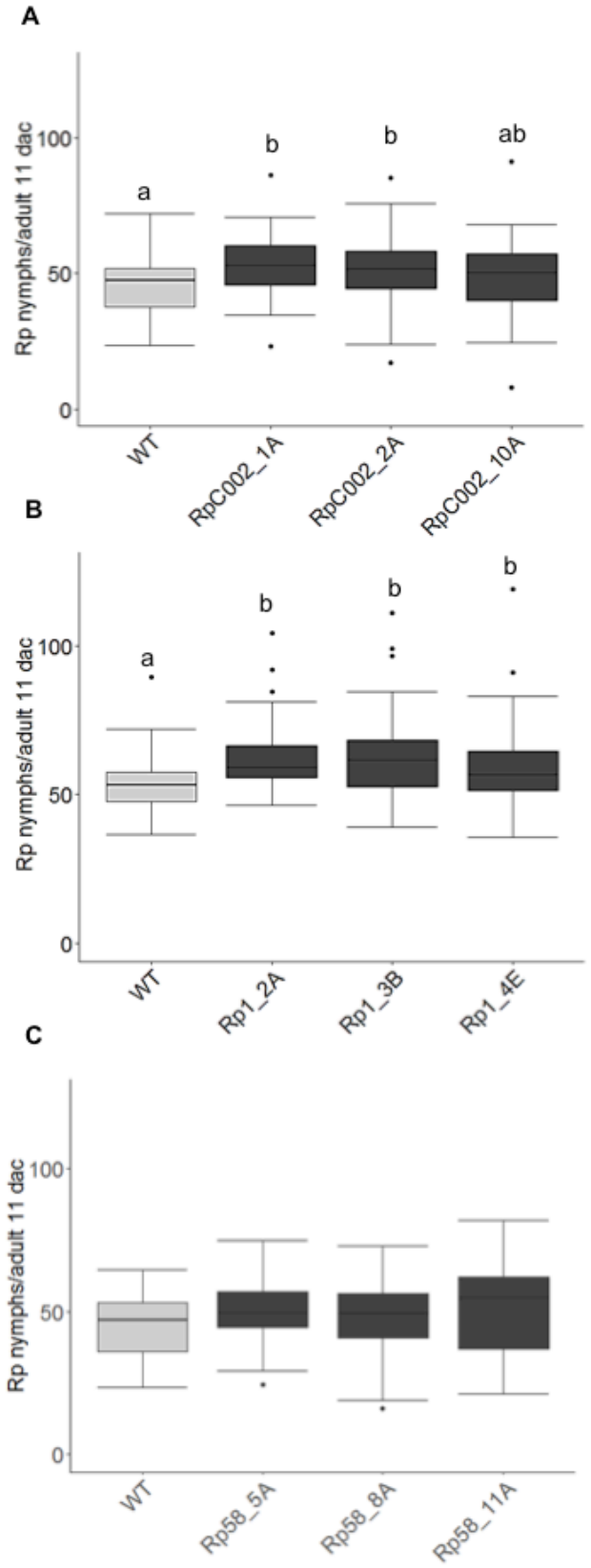

Figure 6. *Rhopalosiphum padi* performance on barley plants ectopically expressing different *R. padi* effectors. Transgenic barley lines were challenged with aphids alongside wild-type plants cv Golden Promise (WT). Nymph production was monitored for 11 days.

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.

Bar graphs show the average number of nymphs per adult 11 days after challenge (dac) from at least three independent replicates (n=5-10). Error bars represent the standard error and different letters indicate significant differences as determined with one-way ANOVA post-hoc Fisher's protected least significant difference test ($p>0.05$).

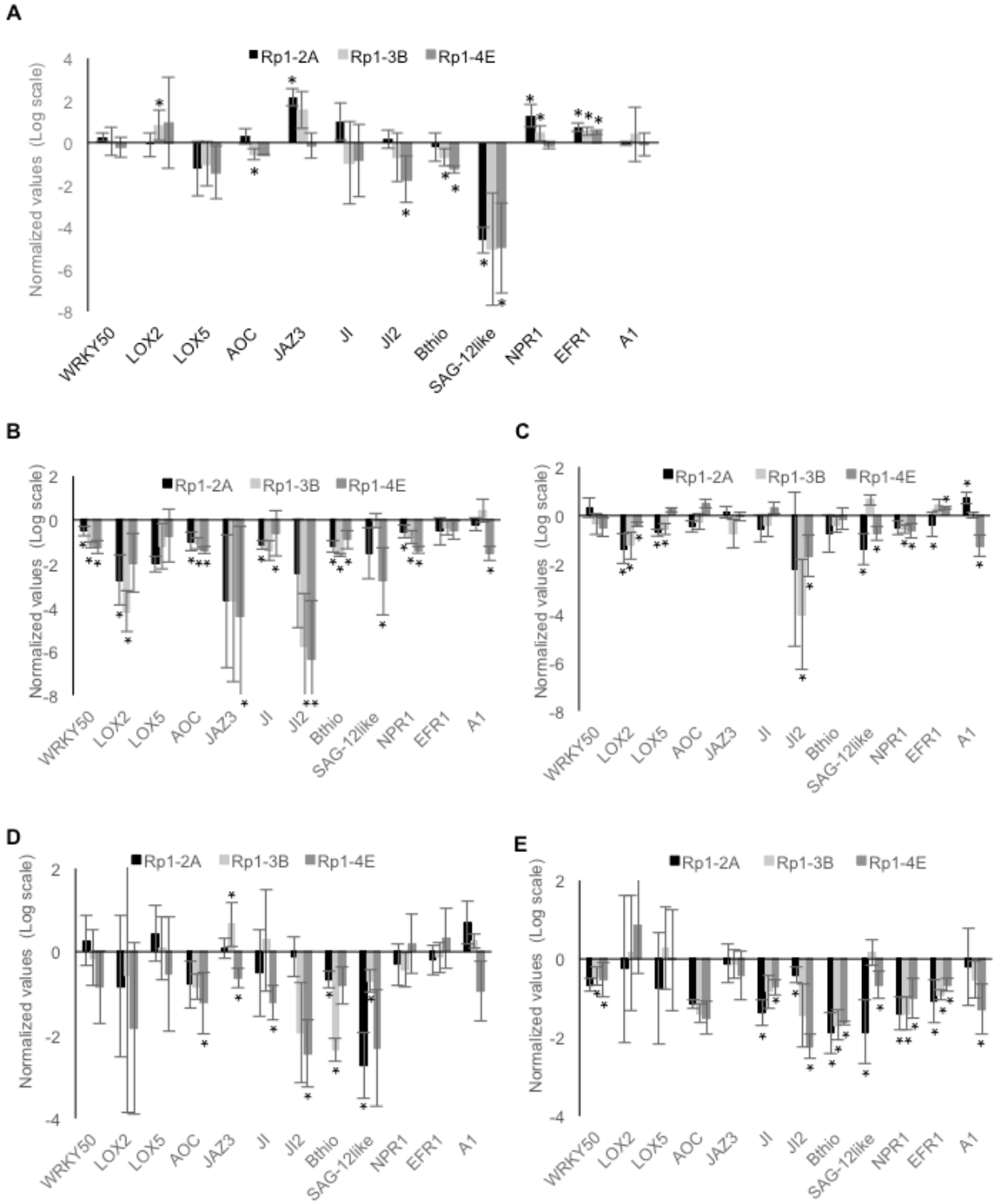

Figure 7. Barley Rp1 lines defence-related gene expression in basal and after infestation with *Rhopalosiphum padi*.

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 the three independent Rp1 barley lines relative to control lines (WT=0).

B) Log-fold changes of barley gene expression upon 24h exposure to empty clip cages (no aphids) in the three independent Rp1 barley lines relative to control lines (WT=0).

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

D) Log-fold changes of barley gene expression upon 72h exposure to empty clip cages (no aphids) in the three independent Rp1 barley lines relative to control lines (WT=0).

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

All gene expression analyses were based on three 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), cysteine proteinase 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 (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, $p \leq 0.05$).

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