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1 # Abscisic acid is essential for rewiring of jasmonic acid-
2 # dependent defenses during herbivory

3

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19 **Abstract**

20 Jasmonic acid (JA) is an important plant hormone in the regulation of defenses
21 against chewing herbivores and necrotrophic pathogens. In *Arabidopsis thaliana*, the
22 JA response pathway consists of two antagonistic branches that are regulated by
23 MYC- and ERF-type transcription factors, respectively. The role of abscisic acid
24 (ABA) and ethylene (ET) in the molecular regulation of the MYC/ERF antagonism
25 during plant-insect interactions is still unclear. Here, we show that production of ABA
26 induced in response to leaf-chewing *Pieris rapae* caterpillars is required for both the
27 activation of the MYC-branch and the suppression of the ERF-branch during
28 herbivory. Exogenous application of ABA suppressed ectopic ERF-mediated *PDF1.2*
29 expression in *35S::ORA59* plants. Moreover, the GCC-box promoter motif, which is
30 required for JA/ET-induced activation of the ERF-branch genes *ORA59* and *PDF1.2*,
31 was targeted by ABA. Application of gaseous ET counteracted activation of the
32 MYC-branch and repression of the ERF-branch by *P. rapae*, but infection with the
33 ET-inducing necrotrophic pathogen *Botrytis cinerea* did not. Accordingly, *P. rapae*
34 performed equally well on *B. cinerea*-infected and control plants, whereas activation
35 of the MYC-branch resulted in reduced caterpillar performance. Together, these data
36 indicate that upon feeding by *P. rapae*, ABA is essential for activating the MYC-
37 branch and suppressing the ERF-branch of the JA pathway, which maximizes
38 defense against caterpillars.

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39 **Introduction**

40 In nature plants are a food source for over one million herbivorous insect species
41 (Howe and Jander, 2008). The evolutionary arms race between plants and their
42 herbivorous insect enemies has led to a highly sophisticated defense system in
43 plants that can recognize wounding and oral secretion of the insects and respond
44 with the production of nutritive value-diminishing enzymes, toxic compounds, or
45 predator-attracting volatiles (Kessler and Baldwin, 2002; Lawrence and Koundal,
46 2002; Wittstock et al., 2004; Chen et al., 2005; Mithöfer and Boland, 2012; Dicke,
47 2016). Conversely, insects can estimate the quality and suitability of the plant as a
48 food source by contact chemoreceptors on the insect mouthparts, antennae and tarsi
49 (Howe and Jander, 2008; Appel and Cocroft, 2014; Dicke, 2016). Because plant
50 defenses are costly, they are often only activated in case of insect or pathogen
51 attack and not constitutively expressed (Walters and Heil, 2007; Vos et al., 2013a).
52 The induced immune response is shaped by the induced production of diverse plant
53 hormones. The quantity, composition and timing of the hormonal blend tailors the
54 defense response specifically to the attacker at hand, thereby prioritizing effective
55 over ineffective defenses and minimizing fitness costs (De Vos et al., 2005; Pieterse
56 et al., 2012; Vos et al., 2013a; Vos et al., 2015).

57 Infestation with chewing herbivores or infection with necrotrophic pathogens
58 triggers the production of the plant hormone jasmonic acid (JA), and its bioactive
59 derivative JA-Ile (Creelman et al., 1992; Penninckx et al., 1996). Binding of JA-Ile to
60 the JA receptor complex consisting of the F-box protein COI1 and a JAZ repressor
61 protein (Xie et al., 1998; Yan et al., 2009; Sheard et al., 2010), leads to degradation
62 of JAZ proteins via the 26S proteasome pathway (Chini et al., 2007; Thines et al.,
63 2007). Without JA, JAZ proteins repress JA-responsive gene expression by binding
64 to transcriptional activators, such as MYC2, EIN3 and EIL1 (Pauwels and Goossens,
65 2011; Song et al., 2014b; Caarls et al., 2015). When JA accumulates the JAZ
66 proteins are degraded thereby releasing transcription factors that can activate JA-
67 regulated genes.

68 Within the JA pathway, two distinct, antagonistic branches of transcriptional
69 regulation are recognized; the MYC-branch and the ERF-branch. Feeding by
70 chewing herbivores activates the MYC-branch (Verhage et al., 2011; Vos et al.,
71 2013b). This branch is controlled by the basic helix-loop-helix leucine zipper
72 transcription factors MYC2, MYC3 and MYC4 leading to transcription of hundreds of

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73 JA-responsive MYC-branch regulated genes, including *VSP1* and *VSP2* (Anderson
74 et al., 2004; Lorenzo et al., 2004; Fernández-Calvo et al., 2011; Niu et al., 2011).
75 Furthermore, previous studies have indicated that ABA plays a co-regulating role in
76 the activation of the MYC-branch (Anderson et al., 2004; Bodenhausen and
77 Reymond, 2007; Sánchez-Vallet et al., 2012; Vos et al., 2013b). For example, in the
78 ABA-deficient mutant *aba2-1*, expression of the JA-responsive gene *VSP1* was
79 reduced upon feeding by caterpillars of *Pieris rapae* (small cabbage white) compared
80 to wild-type Col-0 plants (Vos et al., 2013b). In contrast to the herbivore-induced
81 MYC-branch, the ERF-branch is activated upon infection with necrotrophic
82 pathogens. The transcription factors EIN3 and EIL1 and the ERF transcription
83 factors ERF1 and ORA59 activate a large set of JA-responsive ERF-branch
84 regulated genes, including *PDF1.2* (Caarls et al., 2015). The expression of *ERF1*,
85 *ORA59* and *PDF1.2* is impaired in both JA- and ethylene (ET)-insensitive mutants,
86 indicating that joint activation of the JA and ET pathways is necessary for full
87 expression of the ERF-branch (Penninckx et al., 1998; Lorenzo et al., 2003; Pré et
88 al., 2008; Broekgaarden et al., 2015).

89 It has been shown that the ABA co-regulated MYC-branch and the ET co-
90 regulated ERF-branch of the JA pathway antagonize each other. For example, upon
91 infestation with *P. rapae* caterpillars, the MYC-branch is activated, while the ERF-
92 branch is suppressed (Verhage et al., 2011; Vos et al., 2013b). In *myc2* mutant
93 plants, *ORA59* and *PDF1.2* expression was highly upregulated after feeding by *P.*
94 *rapae*, indicating that in wild-type plants, MYC2 represses *ORA59* and *PDF1.2*
95 expression after feeding by *P. rapae* (Verhage et al., 2011; Vos et al., 2013b).
96 Additionally, exogenously applied ABA had a positive effect on expression of the
97 MYC-branch after feeding by *P. rapae* (Vos et al., 2013b) and caused suppression of
98 *PDF1.2* induction after exogenous application of JA (Anderson et al., 2004).
99 Recently, it was shown that the MYC-branch transcription factors MYC2, MYC3 and
100 MYC4 interact with the ERF-branch transcription factors EIN3 and EIL1 and that they
101 repress each other's transcriptional activity (Song et al., 2014a).

102 These antagonistic effects between the MYC- and ERF-branch on gene
103 expression levels also have an effect on plant resistance. ABA-deficient mutants
104 have been reported to be more susceptible to herbivory (Thaler and Bostock, 2004;
105 Bodenhausen and Reymond, 2007; Dinh et al., 2013) and more resistant to
106 necrotrophic pathogens (Anderson et al., 2004; Sánchez-Vallet et al., 2012).

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107 Conversely, ET insensitive mutants are in general more susceptible to necrotrophic
108 pathogens and more resistant to herbivorous insects compared to wild-type plants
109 (Van Loon et al., 2006; Broekgaarden et al., 2015). Hence, the interplay between the
110 MYC- and the ERF-branch may allow the plant to activate a specific set of JA-
111 responsive genes that is required for an optimal defense against the attacker
112 encountered (Pieterse et al., 2012).
113 To study the role of ABA and ET in the molecular regulation of the MYC/ERF
114 balance in *Arabidopsis thaliana* (hereafter Arabidopsis) upon attack by *P. rapae*, we
115 analyzed hormone signaling mutants for their gene expression response, hormone
116 production and defense against *P. rapae*. We provide evidence that after *P. rapae*
117 infestation ABA accumulation plays an essential modulating role in the activation of
118 the MYC-branch, possibly by activating the MYC2, MYC3 and MYC4 transcription
119 factors. Concomitantly, ABA can suppress the ERF-branch independently of the
120 MYC transcription factors, by targeting the GCC-box, which is present in the
121 promoters of *ORA59* and *PDF1.2*. Furthermore, activation of the MYC-branch, either
122 by application of JA or ABA or by using the *ein2-1* mutant, resulted in a negative
123 effect on caterpillar performance, whereas activation of the ERF-branch by infection
124 with the necrotrophic pathogen *Botrytis cinerea* did not.
125
126 **Results**
127 ABA- and ET-dependency of JA-dependent defense gene expression upon *P. rapae*
128 feeding
129 The JA-dependent transcriptional response of Arabidopsis to *P. rapae* feeding is
130 predominantly regulated through activation of the MYC-branch of the JA pathway
131 and concomitant suppression of the ERF-branch (Verhage et al., 2011). Here, we
132 investigated whether ABA and ET have a role in the differential expression of the
133 MYC- and the ERF-branch during induction of JA-dependent defense signaling by *P.*
134 *rapae* feeding. Expression of the MYC-branch marker gene *VSP2* and the ERF-
135 branch marker gene *PDF1.2* was monitored in wild-type Col-0, *MYC2*-impaired
136 mutant *jin1-7* (hereafter called *myc2*), *MYC2*, *MYC3*, *MYC4* triple mutant *myc2,3,4*,
137 ABA biosynthesis mutant *aba2-1* and ET response mutant *ein2-1*. First-instar *P.*
138 *rapae* caterpillars were allowed to feed for 24 h on the different Arabidopsis
139 genotypes, after which they were removed. Comparable to Col-0, *ein2-1* plants
140 showed strong *P. rapae*-induced transcription of *VSP2* at 24 h and 30 h (Figure 1).

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141 *VSP2* transcript levels decreased to basal levels at 48 h in both Col-0 and *ein2-1*
142 plants, suggesting that stimulation of the MYC-branch lasted until at least 6 h after
143 removal of the caterpillars. At 24 h, the *ein2-1* plants showed a significantly
144 enhanced transcription level of *VSP2* compared with Col-0 (Figure 1), indicating a
145 primed responsiveness to the MYC-branch. *PDF1.2* transcript levels were very low in
146 both Col-0 and *ein2-1*. In *myc2* as well as in *aba2-1* mutants, the transcriptional
147 patterns of *VSP2* and *PDF1.2* were opposite to those observed in Col-0, showing
148 low *VSP2* expression and high *PDF1.2* expression up to 30 h. In *myc2,3,4* mutants,
149 expression of *VSP2* was almost zero. *PDF1.2* levels in *myc2,3,4* plants were similar
150 to Col-0 up to 30 h, after which they increased significantly at 48 h (Figure 1).
151 Together these results confirm that the MYC transcription factors function as a
152 switch between the two branches of the JA pathway, whereby *myc2,3,4* plants show
153 a delay in expression of the ERF-branch. Furthermore, ABA is essential for
154 activation of the MYC-branch and repression of the ERF-branch upon *P. rapae*
155 feeding, while the ET pathway has only a small, though significant, effect on the
156 MYC/ERF-balance during *P. rapae* feeding.
157

158 <u>Hormone accumulation upon *P. rapae* feeding</u>

159 To study whether the mutants used in this study are affected in herbivore-induced
160 levels of jasmonates (JAs; JA, the biologically highly active conjugate JA-Ile and the
161 JA-precursor OPDA) and ABA we monitored their accumulation in response to *P.*
162 *rapae* feeding. We also determined the production of ET in Col-0 wild-type plants.
163 For the measurement of JAs and ABA, first-instar caterpillars were allowed to feed
164 for 24 h after which they were removed from the leaves. Subsequently, hormone
165 levels were measured in caterpillar-damaged leaves at different time points after
166 caterpillar removal. Figure 2 shows that *P. rapae* feeding induced the accumulation
167 of JA, JA-Ile, OPDA and ABA in Col-0 wild-type plants, confirming previous findings
168 (Vos et al., 2013b). In *ein2-1* plants OPDA levels increased to a similar extent as in
169 Col-0, but in contrast, enhanced levels of JA-Ile, JA and ABA were detected. This
170 correlates with the observed enhanced *VSP2* expression in *ein2-1* plants upon *P.*
171 *rapae* feeding (Figure 1). In *myc2* and *aba2-1* plants, the levels of the JAs raised in
172 general to a similar extent as in Col-0 plants, only the JA and JA-Ile levels did not
173 drop to basal levels at 48 h in *aba2-1* (Figure 2). This indicates that the biosynthesis
174 of JAs is not significantly affected by the *myc2* mutation and only relatively late

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175 affected by the *aba2-1* mutation. In *myc2,3,4* plants, OPDA levels were significantly
176 reduced at all time points after caterpillar feeding compared with Col-0. JA levels
177 were also reduced in *myc2,3,4*, but only at 24 h. On the contrary, JA-Ile levels were
178 significantly enhanced in *myc2,3,4* plants at 30 h and 48 h compared with Col-0. This
179 suggests that the JA biosynthesis pathway is perturbed in the *myc2,3,4* plants,
180 resulting in low levels of OPDA, but enhanced production of JA-Ile. ABA levels were
181 highly induced by *P. rapae* feeding in Col-0 at 24 h, but not in *myc2* and *myc2,3,4*
182 plants. At later time points the ABA levels dropped in Col-0 and the differences
183 between the *myc* mutants and Col-0 were no longer significant. These data suggest
184 that herbivore-induced ABA biosynthesis is regulated via MYC transcription factors.
185 To monitor the emission of ET during *P. rapae* feeding, caterpillar-infested Col-0
186 plants were placed in 2-l air-tight cuvettes, which allows for continuous ET
187 measurements under climate chamber growth conditions. The positive control,
188 infection with the necrotrophic fungus *B. cinerea,* showed strongly enhanced ET
189 production (Figure 3A), whereas *P. rapae* infestation did not lead to changes in ET
190 production over a 72-h feeding period compared to non-treated control plants (Figure
191 3B). This indicates that *P. rapae* feeding does not influence ET production.
192
193 <u>The role of ABA in regulation of MYC/ERF antagonism</u>
194 To further investigate the role of ABA in the regulation of the MYC/ERF antagonism
195 upon feeding by *P. rapae*, we determined the effect of exogenously applied ABA on
196 the *P. rapae*-induced expression levels of *VSP2* and *PDF1.2*. Application of 100 µM
197 ABA alone did not significantly activate or repress the expression of *VSP2* or *PDF1.2*
198 in any of the tested lines at any of the tested time points (Figure 4 shows the 30 h
199 time point). Interestingly, caterpillar-induced transcription levels of *VSP2* were
200 significantly enhanced in Col-0, *aba2-1* and *ein2-1* plants at 30 h when ABA was
201 applied to the plants 24 h prior to the start of *P. rapae* infestation (Figure 4). This
202 ABA-mediated enhancement of *P. rapae*-induced *VSP2* expression was not
203 observed in *myc2* and *myc2,3,4* plants. Furthermore, the induction of *MYC2* gene
204 expression by *P. rapae* feeding was blocked in *aba2-1* plants and restored by ABA
205 treatment (Supplemental Figure 1A). This indicates that ABA acts positively on the *P.*
206 *rapae*-induced MYC-branch, possibly by inducing expression and activity of MYC2.
207 On the other hand, ABA application diminished the high *P. rapae*-induced *PDF1.2*
208 transcript levels in *myc2* and *aba2-1* plants at 30 h. In *myc2,3,4* plants, *PDF1.2*

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209 levels were significantly reduced by ABA at 48 h (Supplemental Figure 1B), but not
210 yet significantly at 30 h (Figure 4). This indicates that ABA antagonizes the activation
211 of the ERF-branch independently of the MYC2, MYC3 and MYC4 transcription
212 factors.

213 ORA59 is a crucial transcription factor for activation of the ERF-branch marker
214 gene *PDF1.2* (Pré et al., 2008). To test if ABA can interfere with *PDF1.2* activation
215 downstream of the ORA59 protein, we used a *35S::ORA59* overexpression line in
216 which *PDF1.2* is constitutively expressed. The *VSP2* expression pattern in the
217 *35S::ORA59* line was similar to that in Col-0 after feeding by *P. rapae* and
218 application of ABA (Figure 5A). *ORA59* levels were constitutively high in the
219 *35S::ORA59* plants and were not significantly influenced by *P. rapae* or ABA
220 treatment. As expected, *PDF1.2* was expressed constitutively in untreated
221 *35S::ORA59* plants and was increased further upon feeding by *P. rapae*, which likely
222 can be ascribed to the elevated JA content in response to herbivory. Application of
223 ABA significantly repressed the *PDF1.2* levels in *P. rapae*-infested *35S::ORA59*
224 plants and a similar trend was found in the non-infested plants (Figure 5A). These
225 results suggest that ABA antagonizes *PDF1.2* expression downstream of ORA59.

226 The GCC-box motif that is present in the promoter region of the *PDF1.2* gene
227 and is the binding site for ERF transcription factors has previously been shown to be
228 sufficient for transcriptional activation by JA and suppression thereof by salicylic acid
229 (SA; Brown et al., 2003; Spoel et al., 2003; Van der Does et al., 2013). Therefore, we
230 tested if the GCC-box is also targeted for suppression by ABA. We used a
231 transgenic *GCC::GUS* line containing 4 copies of the GCC-box fused to a minimal
232 *35S* promoter and the *GUS* reporter gene (Zarei et al., 2011). We treated the plants
233 with 100 µM MeJA, 100 µM ABA or a combination of MeJA and ABA and determined
234 GUS activity after 24 h. Both the histochemical staining (Figure 5B) and the
235 quantification of the GUS activity (Figure 5C) showed that MeJA induced GUS
236 activity, confirming that MeJA activates the GCC-box. Treatment with ABA alone
237 significantly repressed the background GUS activity. Moreover, the combination
238 treatment resulted in a significant suppression of the MeJA-induced activation of the
239 GCC-box by ABA. Also the promoter region of the *ORA59* gene contains a GCC-box
240 and in accordance with the *PDF1.2* expression pattern (Figure 4), the high *P. rapae*-
241 induced expression level of *ORA59* in *myc2* plants could be suppressed by prior
242 treatment with ABA (Supplemental Figure 2). Together, these results indicate that, in

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243 line with the reported antagonism of JA-dependent gene transcription by SA, the
244 GCC-box is similarly targeted by ABA, which likely contributes to suppression of the
245 ERF-branch of the JA pathway during herbivory.
246
247 The role of ET in regulation of MYC/ERF antagonism
248 Although the impact of ET signaling on the expression of the MYC- and the ERF-
249 branch upon *P. rapae* feeding is not merely as great as that of ABA, we did observe
250 that in *ein2-1* plants *VSP2* transcription was significantly enhanced at 24 h compared
251 to Col-0 (Figure 1). Furthermore, the production of JA, JA-Ile and ABA was
252 significantly enhanced in *ein2-1* plants at 30 h compared to Col-0 (Figure 2). To
253 investigate whether activation of ET signaling could influence the balance between
254 the MYC- and ERF-branch of the JA pathway during *P. rapae* feeding, we
255 exogenously applied gaseous ET before and during infestation of Col-0 and *ein2-1*
256 plants with *P. rapae* caterpillars. Treatment with 1 ppm of gaseous ET alone induced
257 the expression of *PDF1.2* in Col-0, which was further enhanced by the combination
258 with *P. rapae* feeding (Figure 6). This is likely due to synergistic action between ET
259 and *P. rapae*-induced JAs on *PDF1.2* expression (Penninckx et al., 1998).
260 Additionally, ET treatment strongly reduced the level of both basal and *P. rapae*-
261 induced expression of *VSP1/2*, indicating that induced ET signaling can antagonize
262 the MYC-branch. Both the stimulating effect of ET on *PDF1.2* and the suppressive
263 effect of ET on *VSP1/2* were absent in *P. rapae*-infested *ein2-1* plants, indicating that
264 both ET-mediated processes are dependent on EIN2 and thus regulated via the ET
265 signaling pathway.
266 Infection with *B. cinerea* induced ET production (Figure 3B) and we tested if *B.*
267 *cinerea* infection can also suppress the *P. rapae*-induced activation of the MYC-
268 branch, thereby influencing the MYC/ERF antagonism. Per Col-0 plant, six leaves
269 were inoculated with droplets of *B. cinerea* spores and 24 h later one first-instar
270 caterpillar was placed on the plant. Caterpillars were allowed to feed for 24 h, after
271 which they were removed. *B. cinerea* infection strongly induced the expression of
272 *ORA59* and *PDF1.2* (Figure 7), indicating that the ERF-branch was activated. *P.*
273 *rapae* infestation activated the MYC-branch as evidenced by enhanced transcription
274 of *VSP2* (Figure 7). Surprisingly, infection with *B. cinerea* prior to *P. rapae* infestation
275 did not antagonize the *P. rapae*-induced activation of *VSP2*. In contrast, *P. rapae*
276 infestation subsequent to *B. cinerea* infection suppressed the *B. cinerea*-induced

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277 activation of *ORA59* and *PDF1.2* to basal expression levels (Figure 7). Together,
278 these results suggest that in this set-up Arabidopsis plants prioritize their MYC-
279 branch controlled defenses to combat *P. rapae* infestation, even when the plants
280 were first conditioned to express the ERF-branch defenses against *B. cinerea*
281 infection.
282
283 The effect of ABA and ET on preference and performance of *P. rapae* caterpillars
284 Previously, Verhage et al. (2011) showed that *P. rapae* caterpillars prefer to feed
285 from plants that express the ERF-branch over plants that express the MYC-branch.
286 Here, we determined the effect of ABA and ET signaling on the preference of *P.*
287 *rapae* by conducting two-choice assays, in which two plants of each of two
288 genotypes were placed together in a two-choice arena. Leaves were in physical
289 contact with each other, which allowed the caterpillars to freely move from plant to
290 plant. Two first-instar *P. rapae* caterpillars were placed on each plant at the start of
291 the assay (eight caterpillars per arena) and after 4 days the number of caterpillars
292 per plant genotype was determined in 20-30 independent two-choice arenas. As
293 demonstrated previously (Verhage et al., 2011), significantly more caterpillars were
294 detected on *myc2* than on Col-0 plants (Figure 8A). Similarly, *aba2-1* plants
295 contained more caterpillars than Col-0 when tested in a choice assay (Figure 8A).
296 This finding is in accordance with a preference of *P. rapae* caterpillars for plants that
297 express the ERF-branch, as shown for *myc2* and *aba2-1* upon infestation with *P.*
298 *rapae* (Figure 1). Mutant *ein2-1* plants that, like Col-0, expressed the MYC-branch
299 and not the ERF-branch (Figure 1) accommodated a similar amount of caterpillars as
300 Col-0 plants in a two-choice set-up. These results suggest that MYC2- and ABA-
301 dependent suppression of the ERF-branch in wild-type Col-0 plants during feeding
302 by *P. rapae* reduces the preference of the caterpillars, whereas ET signaling does
303 not influence caterpillar preference.
304 To investigate whether the preference of *P. rapae* caterpillars for the ERF-
305 branch-expressing *myc2* and *aba2-1* mutant plants coincides with increased
306 performance of the caterpillars on these genotypes, we assessed their growth in no-
307 choice assays with Col-0, *myc2*, *myc2,3,4*, *aba2-1*, *ein2-1*, and JA-nonresponsive
308 *coi1-1* plants. One first-instar *P. rapae* caterpillar was placed on each plant and
309 allowed to feed for 7 days, after which the caterpillar was weighed. Figure 8B shows
310 that there was no significant difference between the growth of caterpillars that fed

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311 from Col-0, *myc2* or *aba2-1*. In contrast, on *ein2-1* mutants, caterpillar growth was
312 significantly inhibited. The growth of caterpillars on *myc2,3,4* mutants was increased
313 to the same extent as on *coi1-1* mutants. Next, we tested whether pretreatment of
314 Col-0 plants with solutions of 100 µM MeJA, 100 µM ABA or 1 µM of the ET
315 precursor 1-aminocyclopropane-1-carboxylic acid (ACC) had an effect on caterpillar
316 performance. MeJA or ABA pretreatment significantly reduced the weight of the
317 caterpillars, whereas pretreatment with ACC did not have an effect (Figure 8C).
318 Finally, the effect of prior infection with ET-inducing *B. cinerea* on *P. rapae*
319 performance was tested. Figure 8D shows that *P. rapae* performance was not
320 altered on *B. cinerea*-infected plants compared to control plants.
321 These results indicate that, although caterpillars have a preference for the ERF-
322 branch-expressing *myc2* and *aba2-1* plants, there is no direct positive effect on their
323 performance by these plants. In accordance, the ERF-branch-activating ACC and *B.*
324 *cinerea* pretreatments had no effect on caterpillar performance. On the other hand,
325 enhanced activation of the MYC-branch as is evident in *ein2-1* plants upon caterpillar
326 feeding (Figure 1) correlates with reduced performance of the caterpillars on these
327 plants. Moreover, also the MYC-branch-activating/priming MeJA and ABA
328 pretreatments significantly reduced caterpillar performance (Figure 8C). In
329 conclusion, enhancement of the MYC-branch, by activating the JA or ABA pathway
330 or by suppressing the ET pathway reduced caterpillar performance. The preference
331 for plants expressing the ERF-branch, as is demonstrated in the two-choice assays,
332 might be a strategy to avoid plants with an effective defense.
333
334 **Discussion**

335 The complex plant immune regulatory network that is activated upon recognition of
336 attackers is largely controlled by plant hormones (Pieterse et al., 2012). JA has a
337 decisive regulatory role in the defense responses against herbivorous insects and
338 necrotrophic pathogens (Howe and Jander, 2008; Pieterse et al., 2012). Several
339 studies indicated that ABA co-regulates the JA-induced activation of the MYC-
340 branch, while ET co-regulates activation of the ERF-branch (Penninckx et al., 1998;
341 Lorenzo et al., 2003; Anderson et al., 2004; Lorenzo et al., 2004; Pré et al., 2008;
342 Vos et al., 2013b). Previously, Verhage et al. (2011) showed that feeding of *P. rapae*
343 caterpillars on Arabidopsis leads to activation of the MYC-branch while the
344 herbivore-preferred ERF-branch is strongly suppressed. However, the role of ABA

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345 and ET in the antagonistic interaction between the MYC- and the ERF-branch during
346 herbivory was not clear. Here, we show that ABA and ET are important regulators of
347 the balance between the MYC- and the ERF-branch in herbivore-infested plants,
348 thereby activating the appropriate defense response and suppressing costly
349 unnecessary defenses.

350

351 ABA is required for *P. rapae*-induced activation of the MYC-branch and repression of
352 the ERF-branch

353 There is ample evidence for the production of JA upon feeding by chewing
354 herbivores (Wasternack and Hause, 2013), but the production of ABA is not often
355 taken along. We demonstrated that in wild-type Col-0 plants, *P. rapae* feeding
356 enhanced the production of JAs, as well as that of ABA (Figure 2 & Figure 9; Vos et
357 al., 2013b). Also in maize and rice plants, an increased production of JAs and ABA
358 has been demonstrated upon root herbivory (Erb et al., 2009; Lu et al., 2015). We
359 show that *aba2-1* plants fail to activate the MYC-branch in response to *P. rapae*
360 feeding, evidenced by the reduced activation of *MYC2* and the MYC-branch marker
361 *VSP2* (Figure 1 and Supplemental Figure 1A). Importantly, *aba2-1* plants are also
362 deficient in suppression of the ERF-branch in response to herbivory, apparent from
363 enhanced activation of *PDF1.2* after *P. rapae* feeding (Figure 1). Since *aba2-1* plants
364 differ from Col-0 in the herbivory-induced production of ABA and only minimally in
365 the production of JAs (Figure 2), it seems plausible that in wild-type Arabidopsis ABA
366 is essential for shifting the MYC/ERF balance towards the MYC-branch upon
367 herbivory by *P. rapae*. This was confirmed by experiments in which ABA was applied
368 exogenously 24 h prior to infestation with *P. rapae*. The ABA treatment stimulated
369 the herbivore-induced MYC-branch in Col-0 plants, while in *myc2* and *myc2,3,4*
370 plants ABA treatment strongly inhibited the enhanced expression of the ERF-branch
371 (Figure 4 & Supplemental Figure 1B). In line with this, induction of the ERF-branch
372 by *B. cinerea* infection was strongly suppressed by subsequent *P. rapae* feeding
373 (Figure 7), likely due to enhanced ABA levels in response to *P. rapae* feeding (Vos et
374 al., 2015). Treatment with exogenous ABA in the absence of herbivory did not alter
375 the expression of the marker genes *VSP2* and *PDF1.2* (Figure 4), indicating that
376 ABA alone is not sufficient for influencing the expression levels of these marker
377 genes, but requires additional activation of the JA pathway. Likewise, we previously
378 demonstrated that systemic induction of *MYC2* in non-damaged leaves of *P. rapae*-

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379 infested plants only led to downstream activation of *VSP2* if ABA levels increased as
380 well (Vos et al., 2013b).
381 Interestingly, the suppression of the ERF-branch by exogenous application of
382 ABA to *P. rapae*-infested plants occurred in the *myc2* and *myc2,3,4* plants (Figure 4
383 & Supplemental Figure 1), showing that this response is independent of the
384 previously reported MYC2/MYC3/MYC4-EIN3/EIL1 protein-protein interactions
385 (Song et al., 2014a). However, the ABA biosynthesis that was induced upon *P.*
386 *rapae* feeding was largely dependent on MYC transcription factors, as indicated by
387 basal ABA levels in *P. rapae*-infested *myc2* and *myc2,3,4* plants compared with Col-
388 0 at 24 h (Figure 2). Also in Arabidopsis roots JA-dependent signaling was reported
389 to be necessary for the production of ABA (De Ollas et al., 2015). Hence, despite the
390 fact that exogenous ABA can suppress ERF-branch-induced responses
391 independently of the MYC transcription factors, there seems to be a positive
392 feedback loop between the ABA and JA response pathways that are induced by
393 herbivory, in which ABA signaling enhances MYC transcription factor activity, which
394 in turn is important for the production of ABA (Figure 9).
395
396 ABA antagonizes the ERF-branch downstream of ORA59 at the GCC-box
397 Analysis of the *35S::ORA59* transgenic line showed that ABA is able to suppress
398 *PDF1.2* even when ectopic *ORA59* expression levels are constitutively high (Figure
399 5A). Previously, Van der Does et al. (2013) investigated the suppressive effect of SA
400 on JA-induced *PDF1.2* expression. They also found that SA could suppress
401 activation of *PDF1.2* in the *35S::ORA59* line. Moreover, they reported that the GCC-
402 box, which is present in the promoter of *PDF1.2*, and required for the JA-responsive
403 expression, is essential and sufficient for transcriptional suppression by SA.
404 Similarly, we show here that ABA strongly inhibits activation of the GCC-box in
405 mock- or MeJA-treated plants (Figure 5B & C). In addition to *PDF1.2* also *ORA59*
406 harbors a GCC-box in its promoter, and activated expression of *ORA59* is shown to
407 be suppressed by (*P. rapae*-induced) ABA (Figure 4, Figure 7 & Supplemental
408 Figure 2), as well as by SA (Zander et al.,2014). Together, these data point towards
409 a similar mechanism for SA-dependent and ABA-dependent suppression of the
410 expression levels of *ORA59* and *PDF1.2* at the level of transcriptional regulation at
411 the GCC-box.
412

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413 Strong activation of the ET pathway is necessary for suppression of the MYC-branch
414 Continuous monitoring of the production of ET in *P. rapae*-infested Arabidopsis
415 plants revealed no changes in the emission of ET in this set-up (Figure 3 & Figure 9).
416 However, ET-nonresponsive *ein2-1* plants showed enhanced activation of the MYC-
417 branch, as evidenced by an increase in *VSP2* transcription at 24 h after introduction
418 of *P. rapae* (Figure 1). The production of JA-Ile, JA and especially ABA was
419 enhanced in the *ein2-1* plants compared with Col-0 upon *P. rapae* feeding (Figure 2),
420 suggesting that in wild-type plants basal activity of the ET pathway can inhibit
421 herbivory-induced production of JA and ABA, which tempers the activation of the
422 MYC-branch.
423 Strong evidence for a role for ET in shifting the MYC/ERF balance was provided
424 by the experiment in which we applied gaseous ET to the plants. This ET treatment
425 led to activation of the ERF-branch during *P. rapae* feeding, while the MYC-branch
426 was suppressed (Figure 6). Both effects were absent in the ET-insensitive mutant
427 *ein2-1,* indicating that the modulating effect of ET was mediated via the ET pathway.
428 Infection with the necrotrophic pathogen *B. cinerea* induced ET emission (Figure 3),
429 but in contrast to exogenously applied gaseous ET, *B. cinerea* infection was not able
430 to suppress activation of the MYC-branch in response to *P. rapae* feeding (Figure 7).
431 In a previous study, where *P. rapae* feeding preceded *B. cinerea* infection, the MYC-
432 branch was not suppressed either (Vos et al., 2015). A likely explanation for the
433 discrepancy between the continuous application of gaseous ET and infection with *B.*
434 *cinerea* is that only the gaseous ET treatment activates the ET pathway to a great
435 enough extent to suppress the *P. rapae*-induced MYC-branch.
436 ET has been described to play a role in resistance to herbivores in many plant
437 species (Von Dahl and Baldwin, 2007), for example as a volatile signal
438 (Broekgaarden et al., 2015). However, the specific effect of ET on plant defense
439 varies per plant species and attacking insect (Nguyen et al., 2016). Although ET has
440 the potential to modulate the balance between the MYC- and the ERF-branch in
441 Arabidopsis, ET levels do not change upon feeding by *P. rapae* only excessive
442 amounts of ET seem to be able to suppress the MYC-branch upon *P. rapae* feeding.
443 Therefore, the induction of the ET pathway is unlikely to play a major role in the
444 defense response of Arabidopsis to *P. rapae* feeding (Figure 9).
445

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446 The differential role of the MYC- and the ERF-branch on preference and
447 performance of *P. rapae* caterpillars

448 The importance of ABA in defense against insect herbivory became further apparent
449 from bioassays in which preference and performance of *P. rapae* caterpillars was
450 determined. In two-choice assays *P. rapae* caterpillars were found to prefer to feed
451 from the *aba2-1* and *myc2* plants over wild-type Col-0 plants (Figure 8A). These
452 findings confirm previous results that *P. rapae* caterpillars prefer to feed from plants
453 expressing the ERF-branch (Verhage et al., 2011). There was no preference for
454 either *ein2-1* or Col-0 plants when caterpillars were given a choice between those
455 two genotypes in a two-choice assay (Figure 8A). Comparably, the specialist
456 caterpillar *P. xylostella* did not move away faster from leaves in which the MYC-
457 branch was activated compared to control leaves (Perkins et al., 2013). The feeding
458 preference of *P. rapae* caterpillars for *aba2-1* and *myc2* plants was not obviously
459 correlated with enhanced performance (weight gain) on these mutants in no-choice
460 assays (Figure 8B), which corresponds with the observation that the ERF-branch
461 activating *B. cinerea* infection or ACC pretreatment did not affect caterpillar
462 performance (Figure 5C & 5D; Davila Olivas et al.,2016). Performance of both
463 specialist *P. rapae* caterpillars (Figure 8B) and generalist *S. littoralis* caterpillars
464 (Bodenhausen and Reymond, 2007) was highly reduced on *ein2-1* plants, which
465 corresponds with the observation that the MYC-branch-activating/priming MeJA or
466 ABA treatment significantly reduced caterpillar performance (Figure 8C). Moreover,
467 caterpillar performance on the *myc2,3,4* and *coi1-1* mutants was greatly enhanced
468 (Figure 8B). This indicates that full activation of the MYC-branch is needed to
469 effectively reduce growth of the caterpillars, while activation of the ERF-branch
470 determines preference of specialist caterpillars, which may enable them to choose
471 plants on which their performance is not negatively affected.

472 Altogether, this study highlights the interplay between JA on the one hand, and
473 ABA and ET on the other hand, in shaping the outcome of the defense response that
474 is triggered upon caterpillar feeding. We show that induced ABA can activate the
475 MYC-branch upon *P. rapae* feeding and suppress the ERF-branch by targeting the
476 GCC-box. Although ET is also capable of suppressing the MYC-branch, ET
477 production is not induced by *P. rapae* feeding and is not likely to play a role in the
478 defense response upon feeding. By prioritizing the MYC-branch over the ERF-

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479 branch during insect herbivory, Arabidopsis is capable of prioritizing its JA-induced
480 response to defenses that contribute to maximizing the chance of survival.
481

482 Material and Methods

483 Plant material and cultivation

484 Seeds of *Arabidopsis thaliana* accession Col-0 and mutants *jin1-7* (*myc2*), *myc2,3,4*,
485 *aba2-1*, *ein2-1* and *coi1-1* (Koornneef et al., 1982; Feys et al., 1994; Alonso et al.,
486 1999; Lorenzo et al., 2004; Fernández-Calvo et al., 2011) and the transgenic lines
487 *35S::ORA59* and *GCC::GUS* (Pré et al., 2008; Zarei et al., 2011) were sown on river
488 sand. Two weeks later, seedlings were transplanted into 60-ml pots containing a
489 sand-potting soil mixture (5:12 v/v) that had been autoclaved twice for 20 min with a
490 24 h interval. Plants were cultivated in a growth chamber with a 10-h day and 14-h
491 night cycle at 70% relative humidity and 21°C. Plants were watered every other day
492 and received 10 ml of half-strength Hoagland solution (Hoagland and Arnon, 1938)
493 containing 10 µM sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.
494

495 *Pieris rapae* assays

496 *Pieris rapae* (small cabbage white) was reared on white cabbage plants (*Brassica*
497 *oleracea*) as described (Van Wees et al., 2013). First-instar caterpillars were used in
498 all experiments. For gene expression analysis, two caterpillars were placed on fully
499 expanded leaves of 5-week-old Arabidopsis plants using a fine paintbrush.
500 Caterpillars were removed 24 h later and leaves were harvested at different time
501 points after infestation. For the ET production measurement, caterpillars remained
502 on the leaves for the entire assay.

503 For the two-choice assays, two or three *aba2-1* and *ein2-1* mutant plants
504 (instead of one plant), were grown in one pot to compensate for their smaller size.
505 Biomass and leaf area were measured from a representable subset of 6-week-old
506 plants before the start of the assay to verify that the amount of leaf tissue was equal
507 among the different genotypes tested. Two pots containing Col-0 wild-type plants
508 and two pots a mutant were placed together in an arena, such that there was
509 physical contact between the plants. Two first-instar caterpillars were released on
510 the plants in each pot in the arena (n=20-30), so that there were eight caterpillars per
511 arena that could freely move through the arena. After 4 days, the number of

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512 caterpillars present on each genotype was monitored and the frequency distribution
513 of the caterpillars over the different genotypes was calculated.
514 To examine caterpillar performance, a single first-instar caterpillar was placed on
515 a 5-week-old plant inside a plastic cup covered with an insect-proof mesh to contain
516 the caterpillars. After 7 days of feeding, caterpillars were weighed to the nearest 0.1
517 mg on a microbalance.
518
519 *Botrytis cinerea* inoculation
520 *Botrytis cinerea* inoculations were performed with strain B05.10 (Van Kan et al.,
521 1997) as described previously (Van Wees et al., 2013). *B. cinerea* solution was
522 made into a final density of $1{\cdot}10^5$ spores/ml and 5 µL droplets of the spores were
523 applied to six leaves of 5-week-old plants. Plants were used immediately for
524 measurement of ethylene production or were placed under a lid for 24 h to increase
525 relative humidity and stimulate the infection, after which the lids were removed and
526 *P. rapae* caterpillars were placed on the plants.
527
528 Chemical treatments
529 For gene expression analysis, plants were treated with MeJA (Serva, Brunschwig
530 Chemie, Amsterdam, the Netherlands) or ABA (Sigma, Steinheim, Germany) by
531 dipping the rosettes in a solution containing either 100 µM MeJA, 100 µM ABA or a
532 combination of both chemicals and 0.015% (v/v) Silwet L77 (Van Meeuwen
533 Chemicals BV, Weesp, the Netherlands) 24 h before caterpillar feeding. MeJA and
534 ABA solutions were diluted from a 1000-fold concentrated stock in 96% ethanol. The
535 mock solution contained 0.015% Silwet L77 and 0.1% ethanol.
536 For analysis of caterpillar performance, plants were treated with 100 µM MeJA,
537 100 µM ABA or 1 µM ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC;
538 Sigma, Steinheim, Germany) by applying 20 ml of the solutions to the plants as a
539 root drench, 5 and 2 days before introduction of the *P. rapae* caterpillars. MeJA and
540 ABA solutions were diluted from a 1000-fold concentrated stock in 96% ethanol. The
541 mock solution contained 0.1% ethanol.
542 Treatment with gaseous ET was performed as described previously (Millenaar et
543 al., 2005). In short, gaseous ET (100 µl/l; Hoek Loos, Amsterdam, the Netherlands)
544 and air (70% relative humidity) were mixed using flow meters (Brooks Instruments,
545 Veenendaal, the Netherlands) to generate an output concentration of 1 µl/l ET, which

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546 was flushed continuously through glass cuvettes (13.5 x 16.0 x 29.0 cm) at a flow
547 rate of 75 l/h and then vented to the outside of the building. The concentration of ET
548 in the airflow was verified using gas chromatography. Five-week-old plants were
549 placed separately in the cuvettes and remained there for the duration of the
550 experiment. Control plants were placed in cuvettes which were flushed with air (70%
551 relative humidity) at the same flow rate. ET and air treatments started 1 day prior to
552 introduction of *P. rapae* to the plants in the cuvettes and continued for the duration of
553 the experiment. Light and temperature conditions were the same as described
554 above.
555

556 RNA extraction, RT-qPCR and northern blot analysis

557 Total RNA was isolated as described (Oñate-Sánchez and Vicente-Carbajosa,
558 2008). RevertAid H minus Reverse Transcriptase (Fermentas) was used to convert
559 DNA-free total RNA into cDNA. PCR reactions were performed in optical 384-well
560 plates (Applied Biosystems) with an ABI PRISM® 7900 HT sequence detection
561 system using SYBR ® Green to monitor the synthesis of double-stranded DNA. A
562 standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of
563 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after
564 cycle 40 by heating from 60 to 95°C with a ramp speed of 1.0°C/min. Transcript
565 levels were calculated relative to the reference gene At1g13320 (Czechowski et al.,
566 2005) using the $2^{-\Delta\Delta CT}$ method described previously (Livak and Schmittgen, 2001;
567 Schmittgen and Livak, 2008).

568 For northern blot analysis, 15 µg of RNA was denatured using glyoxal and
569 dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on 1.5%
570 agarose gel, and blotted onto Hybond-N^+ membranes (Amersham, 's-
571 Hertogenbosch, the Netherlands) by capillary transfer. The electrophoresis and
572 blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively.
573 Equal loading was confirmed by staining rRNA bands with ethidium bromide.
574 Northern blots were hybridized with gene-specific probes for *PDF1.2* and *VSP1/2*
575 (Leon-Reyes et al., 2010). After hybridization with α-^{32}P-dCTP-labeled probes, blots
576 were exposed for autoradiography.

577 The AGI numbers of the studied genes are At1g32640 (*MYC2*), At5g24780
578 (*VSP1*), At5g24770 (*VSP2*), At1g06160 (*ORA59*) and At5g44420 (*PDF1.2*).
579

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580 Jasmonates and ABA analysis

581 For JA, JA-Ile, OPDA and ABA concentration analysis, 50-100 mg of *P. rapae*-
582 infested damaged leaves as well as undamaged leaves from non-infested control
583 plants were grinded. The extraction and hormone analysis was performed as
584 previously described (López-Ráez et al., 2010). At the start of the extraction 1 ml of
585 cold ethylacetate containing D_6-SA (25 ng/ml) and D_5-JA (25 ng/ml) was added to
586 the samples as an internal standard in order to calculate the recovery of the
587 hormones measured. Hormone levels were analyzed by LC-MS on a Varian 320
588 Triple Quad LC/MS/MS. Ten µl of each sample was injected onto a Pursuit column
589 (C18; 5 µm, 50 x 2.0 mm; Varian) that was connected to a precolumn (Pursuit
590 Metaguard C18; 5 µm; 2.0 mm). Multiple reaction monitoring was performed for
591 parent-ions and selected daughter-ions after negative ionization: JA 209/59
592 (fragmented under 12V collision energy), JA-Ile 322/130 (fragmented under 19V
593 collision energy), OPDA 291/165 (fragmented under 18V collision energy) and ABA
594 263/153 (fragmented under 9V collision energy). The mobile phase comprised
595 solvent A (0.05% formic acid) and solvent B (0.05% formic acid in MeOH) with
596 settings as described (Diezel *et al.*, 2009). The retention time of each compound was
597 confirmed with pure compounds (ChemIm Ltd, Olomouc, Czech Republic). The
598 surface area for each daughter-ion peak was recorded for the detected analytes.
599 Analytes were quantified using standard curves made for each individual compound.
600

601 Ethylene measurements

602 ET production was measured in a laser-driven photoacoustic detection system (ETD-
603 300, Sensor Sense, Nijmegen, the Netherlands) connected to a 6-channel valve
604 control box in line with a flow-through system (Voesenek et al., 1990). Five-week-old
605 plants were placed in 2-l air-tight cuvettes (four plants per cuvette), which were
606 incubated under growth chamber conditions. After an acclimation time of 2 h, the
607 cuvettes were continuously flushed with air (flow rate: 0.9 l/h), directing the flow-
608 through air from the cuvettes into a photoacoustic cell for ET measurements. ET
609 levels were measured over consecutive 0.5 h time intervals, after which the machine
610 switched to the next cuvette (n=6).
611

612 GUS assays

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613 For the histochemical GUS assay, GUS activity was assessed by transferring plants
614 to a GUS staining solution (1 mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10 mM EDTA
615 and 0.1% [v/v] Triton X-100). After vacuum infiltration and overnight incubation at
616 37°C, the plants were destained by repeated washes in 96% ethanol (Spoel et al.,
617 2003). For the quantitative GUS assay, protein was isolated from frozen plant
618 material and GUS activity was quantified using a microplate reader (BioTek
619 Instruments, Inc., Winooski, United States of America) as described (Pré et al.,
620 2008).
621
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632
633 **Author contributions**

634 I.A.V., A.V., C.M.J.P. and S.C.M.V.W. designed the research. I.A.V., A.V., L.G.W.
635 and I.V. performed the research. I.A.V., A.V., L.G.W., I.V., R.C.S., C.M.J.P. and
636 S.C.M.W. analyzed the data. I.A.V., C.M.J.P. and S.C.M.W. wrote the paper.
637
638 **Figure 1: Expression of JA-responsive MYC- and ERF-branch marker genes in**
639 **response to *P. rapae* feeding in Arabidopsis mutants.**
640 RT-qPCR analysis of *VSP2* and *PDF1.2* gene expression in *P. rapae*-infested leaves
641 of Col-0, *myc2*, *myc2,3,4*, *aba2-1* and *ein2-1* plants. Indicated are expression values
642 relative to non-infested Col-0 plants at 24 h after infestation. First-instar *P. rapae*
643 caterpillars were allowed to feed for 24 h after which they were removed. Infested
644 leaves were harvested at the indicated time points after introduction of the
645 caterpillars. Crosses indicate a statistically significant difference with the
646 corresponding non-infested control (expression data of non-infested controls are not

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647 shown; two-way ANOVA (treatment x time point), LSD test for multiple comparisons;
648 *P*<0.05). Asterisks indicate a statistically significant difference with Col-0 at the same
649 time point (two-way ANOVA (time point x genotype), LSD test for multiple
650 comparisons; *P*<0.05). Error bars represent SE, *n*=3 plants.
651
652 **Figure 2: Differential production of OPDA, JA, JA-Ile and ABA in *P. rapae*-**
653 **infested Arabidopsis mutants.**
654 Absolute values (ng/ml/mg FW) of OPDA, JA, JA-Ile and ABA levels that were
655 measured by Triple Quad LC/MS/MS in Col-0, *myc2*, *myc2,3,4*, *aba2-1* and *ein2-1*
656 plants. First-instar *P. rapae* caterpillars were allowed to feed for 24 h after which
657 hormone levels were determined in leaves of non-infested control plants and
658 caterpillar-damaged leaves. Arrows and horizontal dashed lines indicate the average
659 values of non-infested control plants. Crosses indicate a statistically significant
660 difference with the non-infested control of the same line (per genotype two-way
661 ANOVA (treatment x time point), LSD test for multiple comparisons; *P*<0.05).
662 Asterisks indicate a statistically significant difference with Col-0 at the same time
663 point (two-way ANOVA (time point x genotype), LSD test for multiple comparisons;
664 *P*<0.05). Error bars represent SE, *n*=4 plants.
665
666 **Figure 3: ET production of Col-0 plants during herbivory by *P. rapae* and**
667 **infection with *B. cinerea*.**
668 ET production was monitored in consecutive 3-h time intervals. Col-0 plants were
669 infected with *B. cinerea* (A) or infested with first-instar *P. rapae* caterpillars (B;
670 caterpillars fed on the leaves for the duration of the experiment) in 2-l air-tight
671 cuvettes that were connected to a photoacoustic detection system, which allowed
672 continuous detection of ET levels in the flush-through airflow. Error bars represent
673 SE, *n*=6 plants. White areas indicate the light period, shaded areas indicate the dark
674 period.
675
676 **Figure 4: Effect of ABA treatment on *P. rapae*-induced *VSP2* and *PDF1.2***
677 **expression.**
678 RT-qPCR analysis of *VSP2* and *PDF1.2* gene expression at 30 h in leaves of Col-0,
679 *myc2*, *myc2,3,4*, *aba2-1* and *ein2-1* plants that were treated with a mock solution or
680 with 100 µM ABA 24 h prior to infestation with *P. rapae*. For experimental detail on

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681 timing of *P. rapae* treatment, see legend Figure 1. Indicated are expression levels
682 relative to non-infested Col-0 plants at 24 h. Different letters indicate statistically
683 significant differences between treatments of one line. Indications above the
684 brackets specify whether there is an overall statistically significant difference
685 between the mutant line and Col-0 (two-way ANOVA (treatment x genotype), LSD
686 test for multiple comparisons; *** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$; NS = not
687 significant). Error bars represent SE, $n=3$ plants.
688

689 **Figure 5: Suppression of *P. rapae*-induced *PDF1.2* expression and MeJA-**
690 **induced *GCC::GUS* activity by ABA.**
691 A) RT-qPCR analysis of *VSP2*, *ORA59* and *PDF1.2* gene expression at 30 h in
692 leaves of Col-0 and *35S::ORA59* plants that were treated with a mock solution or
693 with 100 µM ABA 24 h prior to infestation with *P. rapae*. For experimental detail on
694 timing of *P. rapae* treatment, see legend Figure 1. Indicated are expression levels
695 relative to untreated Col-0 plants at 30 h. Different letters indicate a statistically
696 significant difference between treatments of one line. Indications above the brackets
697 specify whether there is an overall statistically significant difference between
698 *35S::ORA59* and Col-0 (two-way ANOVA (treatment x genotype), LSD test for
699 multiple comparisons; *** = $P<0.001$; NS = not significant). Error bars represent SE,
700 $n=3$ plants.
701 B, C) GUS activity of the *GCC::GUS* line. Plants were dipped in a solution containing
702 100 µM MeJA, 100 µM ABA, a combination of both chemicals or a mock solution and
703 harvested after 24 h. B) Rosettes were stained for GUS activity or C) GUS activity in
704 the leaves was quantified for 48 h using a microplate reader. Different letters indicate
705 statistically significant differences between treatments (regression analysis; $P<0.05$).
706 Error bars represent SE, $n=4$ plants.
707

708 **Figure 6: Effect of gaseous ET treatment on *P. rapae*-induced *VSP1/2* and**
709 ***PDF1.2* expression.**
710 Northern blot analysis of *VSP1/2* and *PDF1.2* gene expression in leaves of Col-0
711 and *ein2-1* plants that were infested with *P. rapae* and treated with a continuous flow
712 of gaseous ET (1 ppm) or ambient air (starting 24 h prior to infestation and
713 continuing until tissue was harvested). First-instar caterpillars of *P. rapae* were

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714 allowed to feed for 24 h after which they were removed. Infested leaves were
715 harvested at the indicated time points after *P. rapae* was introduced.
716
717 **Figure 7: Effect of *B. cinerea* infection on *P. rapae*-induced gene expression.**
718 A) RT-qPCR analysis of *VSP2,* ORA59 and *PDF1.2* gene expression in leaves of
719 Col-0 control plants and leaves infected with *B. cinerea* 24 h prior to infestation with
720 *P. rapae*. Indicated are expression levels relative to untreated Col-0 plants at 0 h.
721 Different letters indicate statistically significant differences between the treatments at
722 the indicated time point (ANOVA, Tukey post-hoc tests; *P*<0.05; NS = not
723 significant). Error bars represent SE, *n*=3 plants.
724
725 **Figure 8: Effect of ABA and ET signaling on the preference and performance of**
726 ***P. rapae.***
727 A) Caterpillar preference for Col-0 vs *myc2*, Col-0 vs *aba2-1* and Col-0 vs *ein2-1*
728 plants. Two-choice arenas (*n*=20-30) consisted of two pots per genotype. In each
729 two-choice arena, two first-instar *P. rapae* caterpillars were placed on the plants in
730 each pot (total eight caterpillars per arena). After 4 days the number of caterpillars
731 on each genotype was determined. The right panel displays which branch of the JA
732 pathway is predominantly activated in the corresponding genotypes that are
733 displayed in the left panel. Displayed are the average percentages (±SE) of the
734 distribution of the *P. rapae* caterpillars over the two genotypes (x-axis). *P*-values
735 indicate a statistically significant difference from the 50% percentile (Student's *t*-test).
736 In cases of statistically significant differences (*P*<0.05), the preferred branch of the
737 JA pathway is marked with a circle. Experiments were repeated with similar results.
738 B, C, D) Caterpillar performance on Col-0, *myc2*, *myc2,3,4*, *aba2-1*, *ein2-1* and *coi1-*
739 *1* plants (B), on Col-0 plants treated with a mock solution, 100 µM MeJA, 100 µM
740 ABA or 1 µM ACC (C) and on control plants and plants treated with *B. cinerea* (D).
741 The hormone solutions were applied as root-drench at 5 and 2 days before
742 caterpillar feeding. One first-instar *P. rapae* caterpillar was placed on each plant and
743 allowed to feed for 7 days after which the weight was determined. Asterisks indicate
744 a statistically significant difference in comparison to Col-0 or mock-treated plants
745 (ANOVA, Dunnett post-hoc tests; *** = *P*<0.001; * = *P*<0.05, NS = not significant).
746 Error bars represent SE, *n*=8-28 plants.
747

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748 **Figure 9: Model of differential regulation of JA responses during herbivory by**
749 ***P. rapae*.**
750 Feeding by *P. rapae* stimulates the production of JAs and ABA, resulting in activation
751 of the MYC-branch and a concomitant defense response against *P. rapae*. While the
752 activation of the MYC transcription factors is dependent on ABA, the production of
753 ABA is dependent on the MYC transcription factors, resulting in a positive feedback
754 loop between ABA and the MYC2, MYC3 and MYC4 transcription factors.
755 Simultaneously, activation of the ABA pathway suppresses the ERF-branch at the
756 level of transcriptional activation at the GCC-box.
757 The ERF-branch components indicated in the model are shaded, because they are
758 not activated during the Arabidopsis-*P. rapae* interaction. Although ET has the
759 capacity to suppress the *P. rapae*-induced MYC-branch, it is not produced during the
760 Arabidopsis-*P. rapae* interaction, and thus does not play a significant role in the
761 MYC/ERF-branch interaction model during infestation.
762 Arrows indicate a stimulating effect, blocked lines indicate a suppression.
763
764 **Supplemental Figure 1: Effect of ABA treatment on *P. rapae*-induced *MYC2* and**
765 ***PDF1.2* expression.**
766 RT-qPCR analysis of *MYC2* expression at 30 h in Col-0 and *aba2-1* plants (A) and
767 *PDF1.2* gene expression at 48 h in leaves of Col-0 and *myc2,3,4* plants (B) that were
768 treated with a mock solution or with 100 μM ABA 24 h prior to infestation with *P.*
769 *rapae*. For experimental detail on timing of *P. rapae* treatment, see legend Figure 1.
770 Indicated are expression levels relative to non-infested Col-0 plants at 24 h. Different
771 letters indicate statistically significant differences between treatments of one line.
772 Indications above the brackets specify whether there is an overall statistically
773 significant difference between *aba2-1*/*myc2,3,4* and Col-0 (two-way ANOVA
774 (treatment x genotype), LSD test for multiple comparisons; ** = $P<0.01$; * = $P<0.05$;
775 NS = not significant). Error bars represent SE, $n=3$ plants.
776
777 **Supplemental Figure 2: Effect of ABA treatment on *P. rapae*-induced *ORA59***
778 **expression.**
779 RT-qPCR analysis of *ORA59* gene expression at 24 h in leaves of Col-0 and *myc2*
780 plants that were treated with a mock solution or with 100 μM ABA 24 h prior to
781 infestation with *P. rapae*. Indicated are expression levels relative to mock-treated

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782 Col-0 plants at 24 h. Different letters indicate statistically significant differences
783 between treatments of one line. Indications above the brackets specify whether there
784 is an overall statistically significant difference between *myc2* and Col-0 (two-way
785 ANOVA (treatment x genotype), LSD test for multiple comparisons; *** = $P<0.001$).
786 Error bars represent SE, $n=3$ plants.
787

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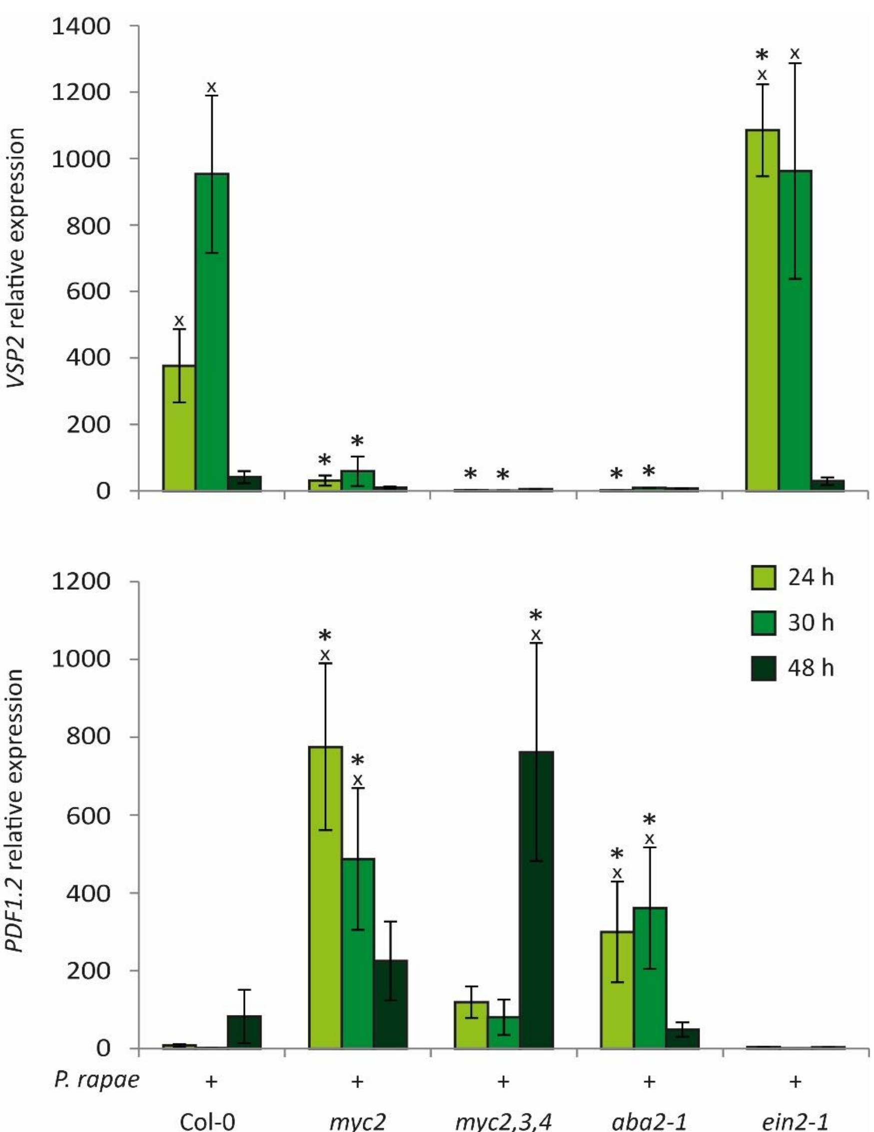

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1014 Figure 1

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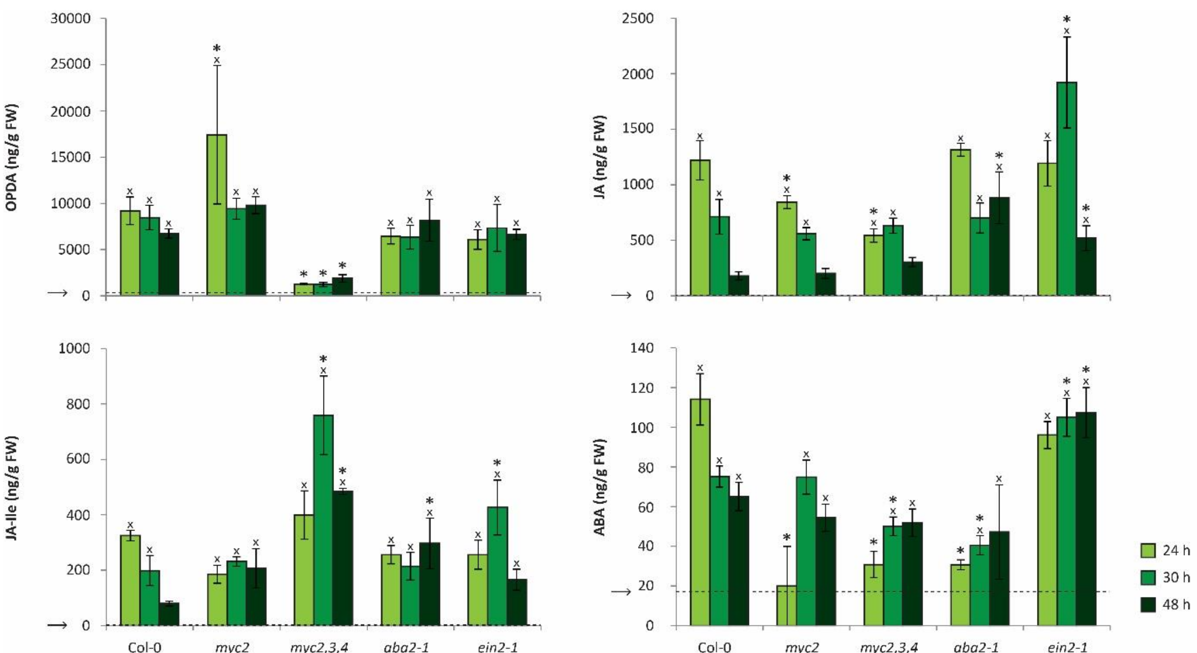

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1016 Figure 2

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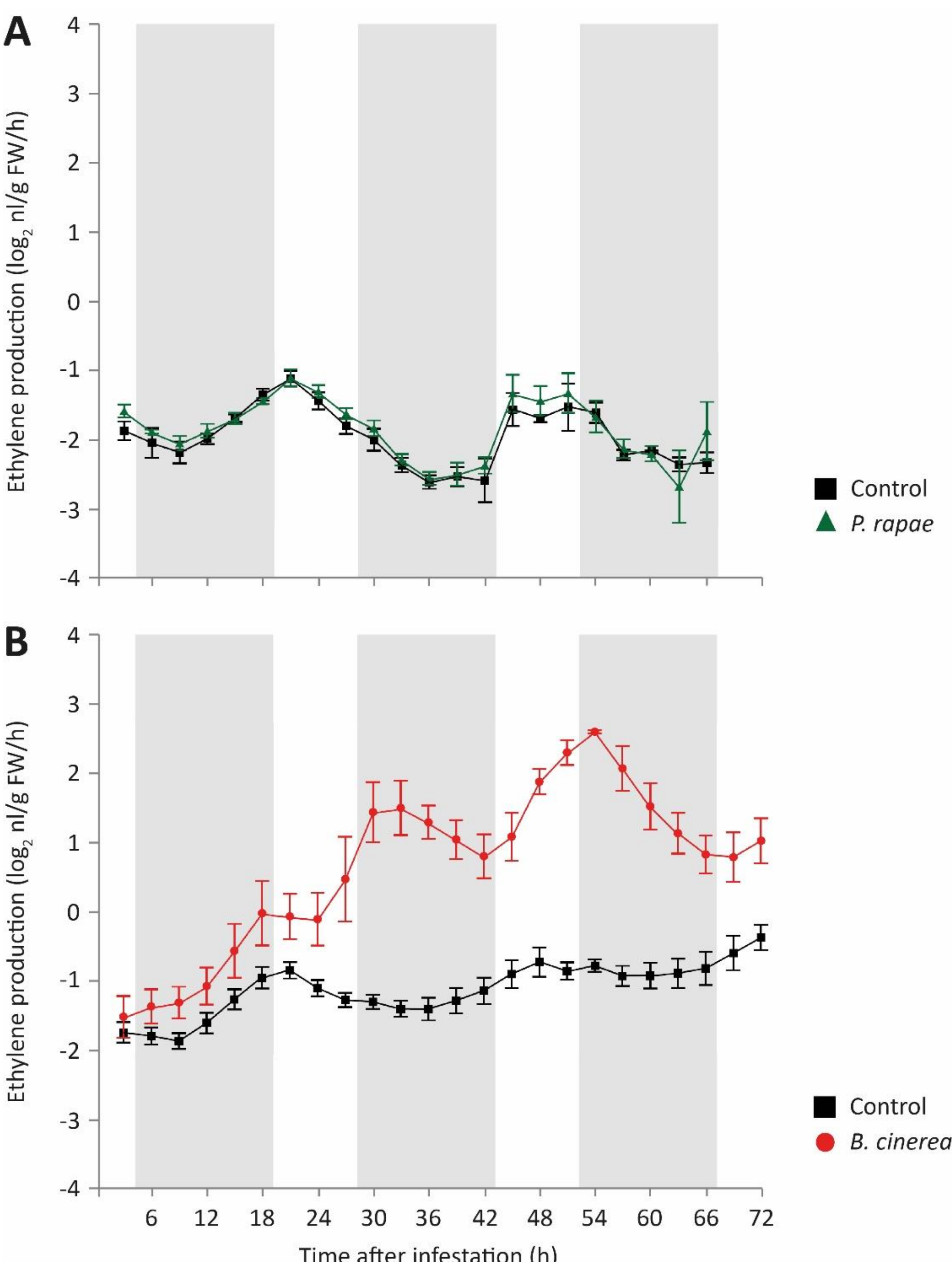

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1018 Figure 3

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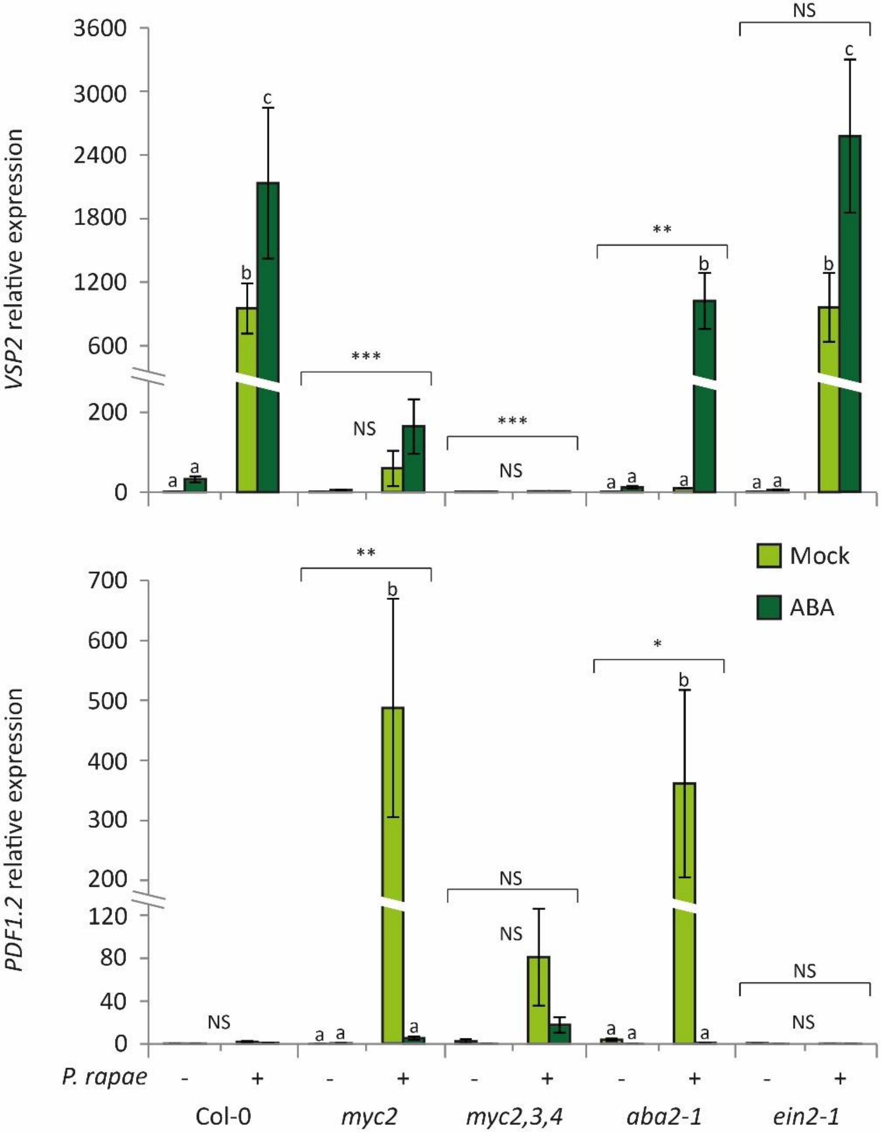

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1020 Figure 4

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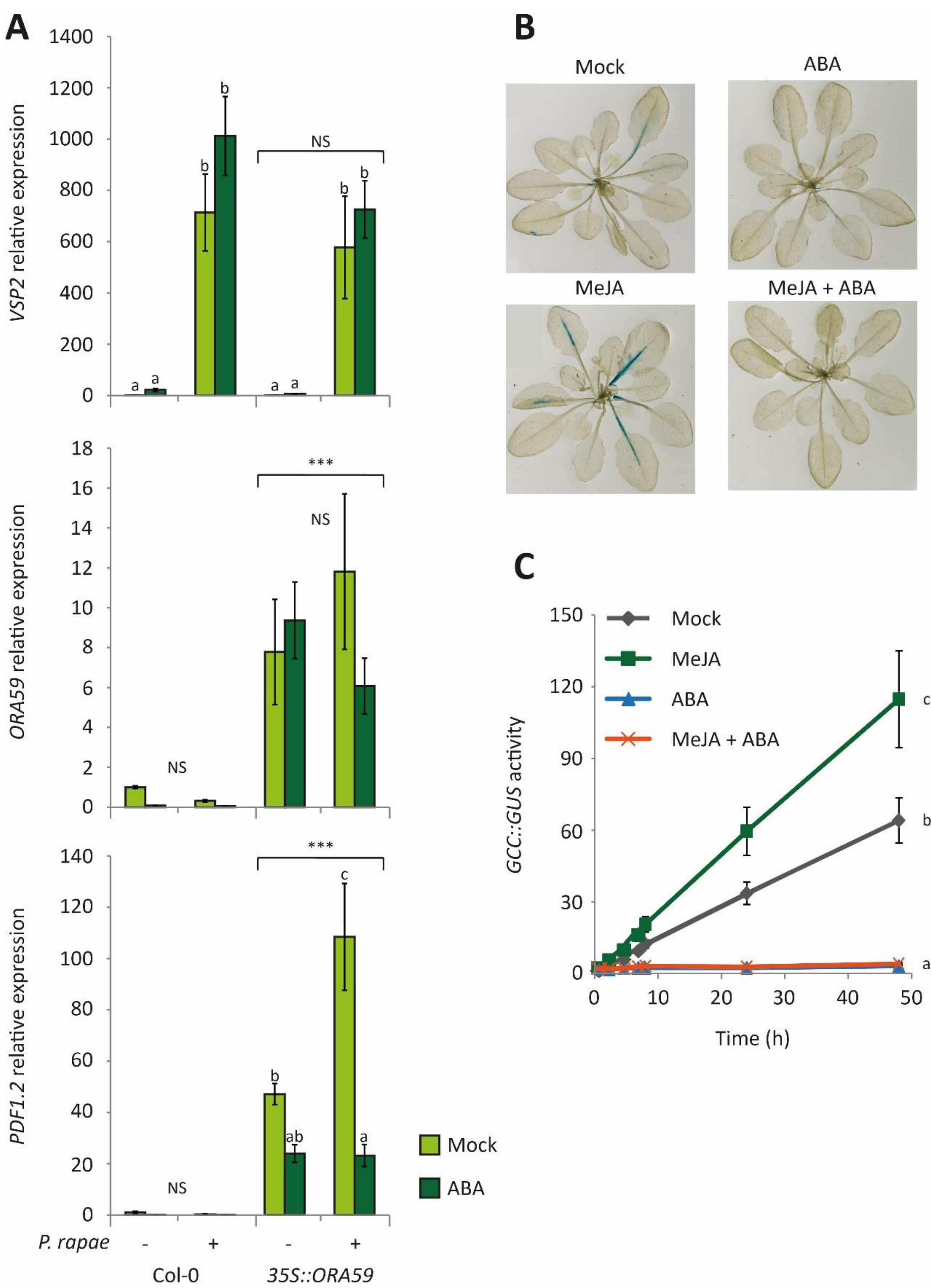

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1022 Figure 5

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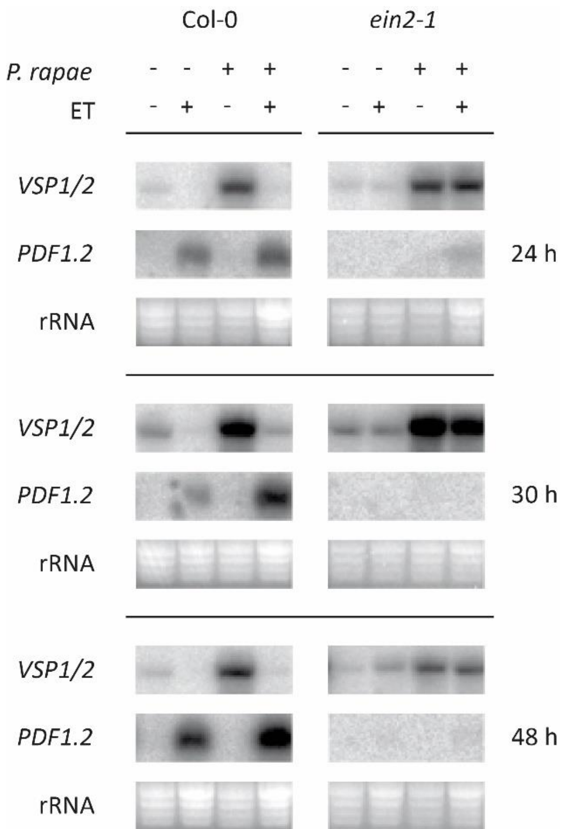

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1024 Figure 6

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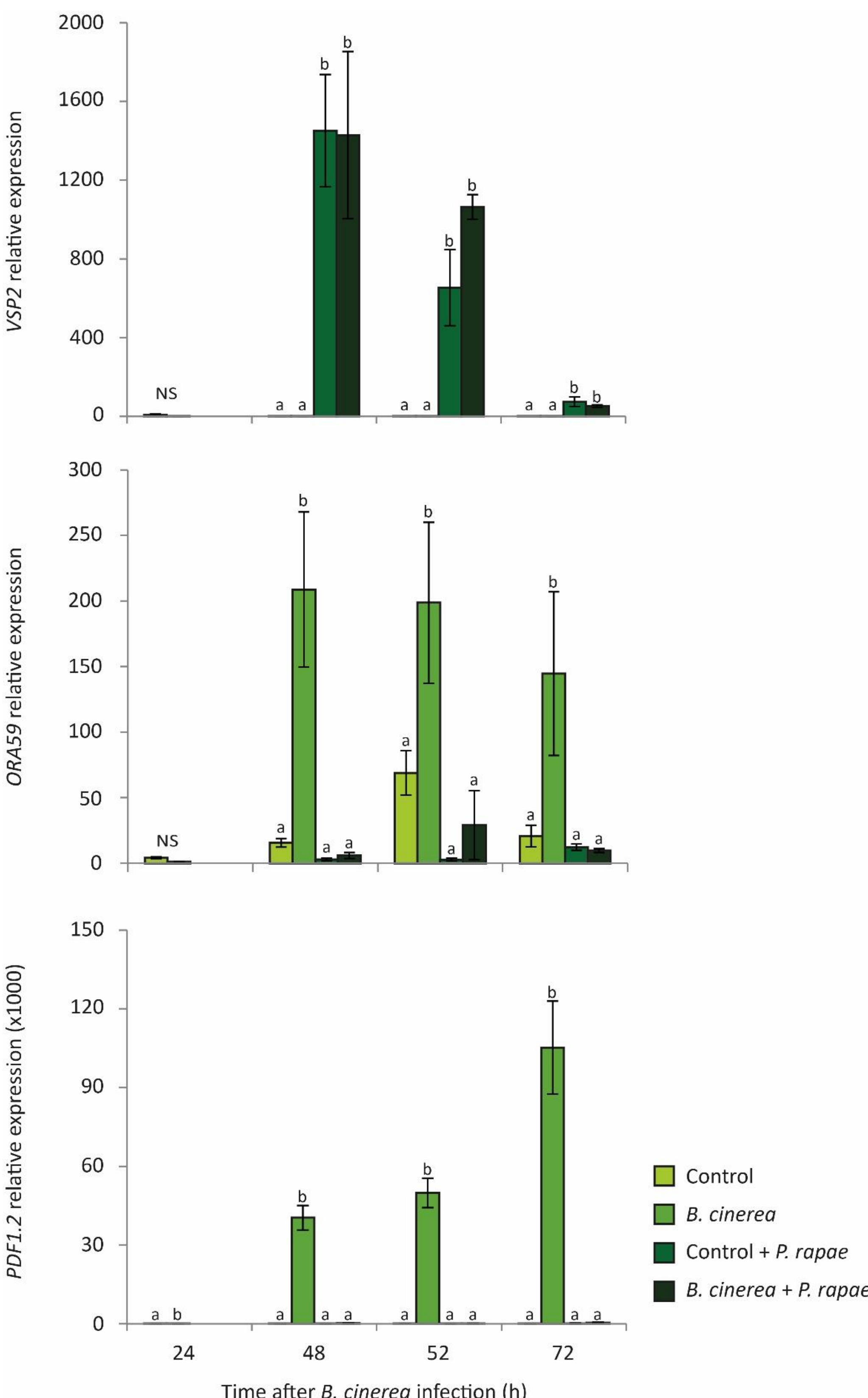

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1026 Figure 7

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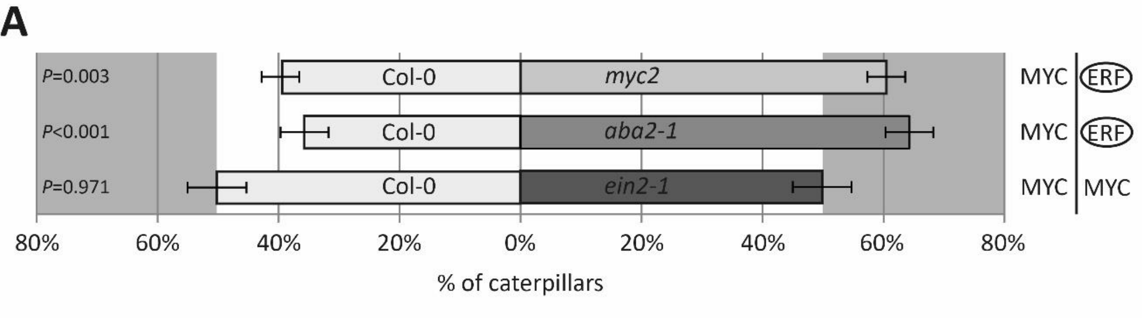

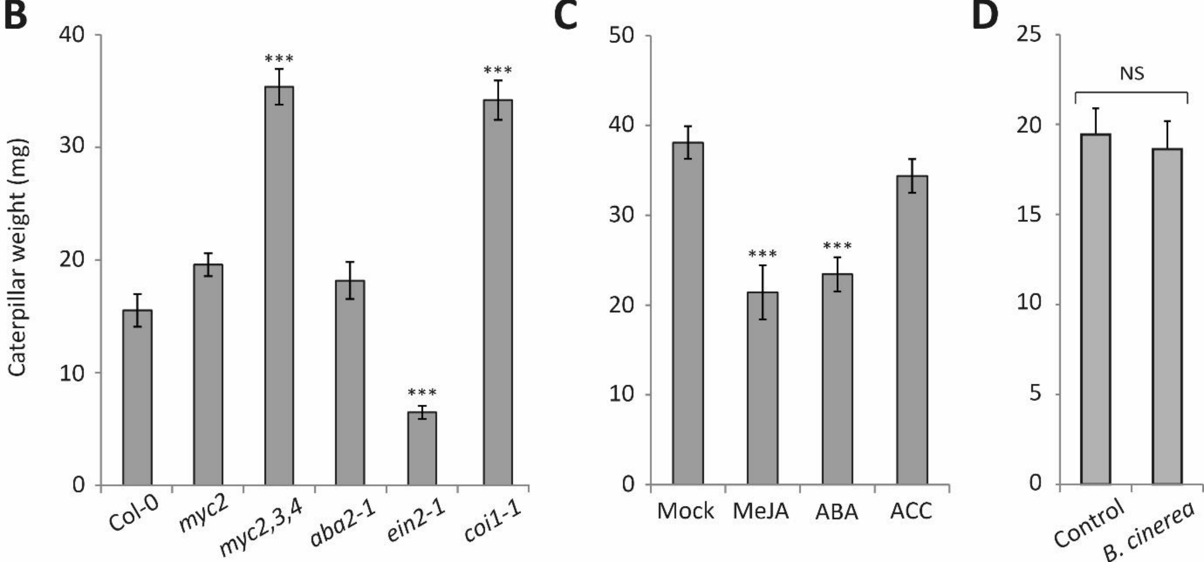

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1028 Figure 8

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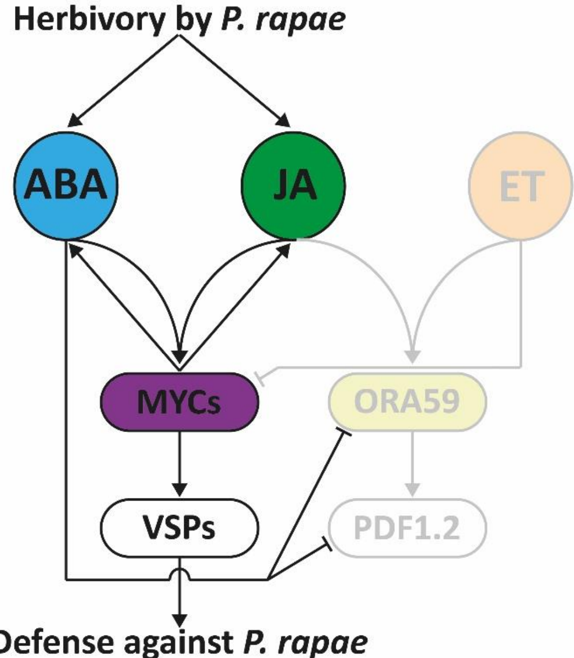

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1030 Figure 9

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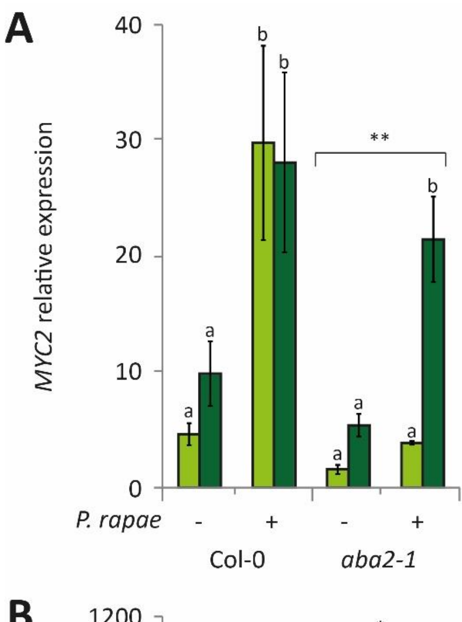

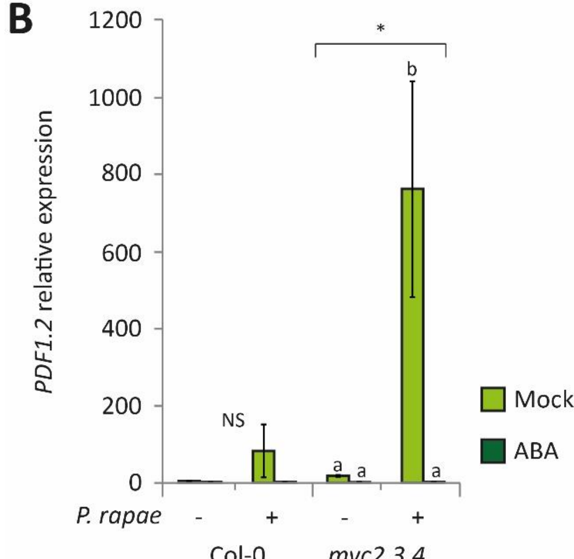

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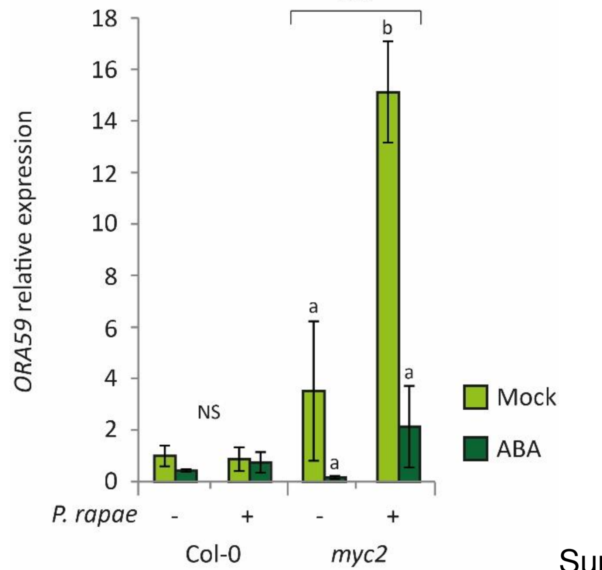

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