Short title: Small molecule antagonist of JA-Ile and auxin

A small molecule antagonizes jasmonic acid perception and auxin responses in vascular and non-vascular plants

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The authors have made the following declarations about their contributions: A.C., G.R.H., N.V.R. and R.S. conceived and designed the experiments; A.C., I.M. and G.F.B. performed the experiments; A.C., I.M., G.F.B., G.R.H., N.V.R. and R.S. analyzed the data; M.B. contributed reagents/materials; A.C. and R.S. wrote the paper. A.C. and R.S. agree to serve as authors responsible for contact and ensure communication.

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One sentence summary

A chemical screen identified a molecule that antagonizes jasmonate perception by directly interfering with receptor complex formation in phylogenetically distant vascular and non-vascular plants.

Abstract

The phytohormone JA-Ile regulates many stress responses and developmental processes in plants. A co-receptor complex formed by the F-box protein COI1 (Coronatine Insensitive 1) and a JAZ (Jasmonate ZIM-domain) repressor perceives the hormone. JA-Ile antagonists are invaluable tools for exploring the role of JA-Ile in specific tissues and developmental stages, and for identifying regulatory processes of the signalling pathway. Using two complementary chemical screens, we identified three compounds that exhibit a robust inhibitory effect on both the hormone-mediated COI-JAZ interaction and degradation of JAZ1 and JAZ9 *in vivo*. One molecule, J4, also restrains specific JA-induced physiological responses in different angiosperm plants, including JA-mediated gene expression, growth inhibition, chlorophyll degradation and anthocyanin accumulation. Interaction experiments with purified proteins indicate that J4 directly interferes with the formation of the Arabidopsis (*Arabidopsis thaliana)* COI1-JAZ complex otherwise induced by JA. The antagonistic effect of J4 on COI1-JAZ also occurs in the liverwort *Marchantia polymorpha*, suggesting the mode of action is conserved in land plants. Besides JA signalling, J4 works as an antagonist of the closely-related auxin signalling pathway, preventing TIR1/Aux-IAA interaction and auxin responses *in planta,* including hormone-mediated degradation of an auxin repressor, gene expression and gravitropic response. However, J4 does not affect other hormonal pathways. Altogether, our results show that this dual antagonist competes with JA-Ile and auxin, preventing the formation of phylogenetically related receptor complexes. J4 may be a useful tool to dissect both the JA-Ile and auxin pathways in particular tissues and developmental stages since it reversibly inhibits these pathways.

78 **Introduction**

79 Plant hormones are bioactive small signalling molecules directly perceived by
80 plant receptor complexes. Phytohormones regulate, alone or in combination, multiple
81 aspects of plant development, responses to the environment and to biotic challenges
82 primarily through transcriptional reprogramming (Smith & Li, 2017). Phytohormones
83 regulate most known plant defences and different plant-interacting organisms have
84 evolved the capability to produce phytohormones or phytohormone mimics to induce
85 disease susceptibility and counteract plant defences (Robert-Seilaniantz *et al.* 2011,
86 Pieterse *et al.* 2012, Fonseca *et al*, 2018). For example, the phytotoxin coronatine
87 (COR) is a JA-Ile (jasmonoyl-L-isoleucine) functional analogue produced by
88 *Pseudomonas syringae* to increase its virulence hijacking the plant defence signalling
89 network (Kloek *et al.* 2001, Brooks *et al.* 2004, Gimenez-Ibanez and Solano 2013). In
90 addition to the evolutionary relevance, natural mimic molecules are also important tools
91 in research; for instance, the use of coronatine was determinant to the identification of
92 several components of the JA signalling cascade including the JA-Ile and COR co-
93 receptor COI1 (Coronatine Insensitive 1) (Xie *et al.* 1998, Sheard *et al.* 2010). In
94 addition, several plant hormone analogues are synthetically synthesized and exploited
95 for agricultural and research purposes. For example, several synthetic auxin analogues
96 and anti-auxins represent "classical" herbicides (Van Overbeek and Velez 1946,
97 Grossmann 2010). Mutants completely impaired in auxin perception are not viable;
98 however, auxin-related molecules, such as auxinole and PEO-IAA, are commonly
99 employed to reversibly block auxin perception and study auxin-regulated processes in
100 several plant species (Hayashi *et al.* 2012, Leyser 2018). Therefore, the identification
101 of new molecules interfering with hormonal signalling cascades may be very useful in
102 agriculture and research.

103 Chemical genomics aims to identify small molecules modifying different
104 biological processes (Norambuena *et al.* 2009, Toth and van der Hoorn 2010, Hicks
105 and Robert 2014). The structural diversity of small compounds is extraordinarily broad
106 (Dobson 2004). Large chemical libraries are screened to identify molecules affecting
107 the activity of a protein, protein families or a pathway in a spatial-temporal and
108 reversible manner. An established advantage of this methodology is to overcome the
109 common limitations of genetic mutant tools such as redundancy or lethality of essential
110 genes (Toth and van der Hoorn 2010, Hicks and Raikhel 2012). Chemical genomic
111 approaches successfully identified hormone analogues or previously unidentified
112 functions of phytohormones (Hicks and Raikhel 2012). A paradigmatic example is the
113 agonist of abscisic acid (ABA) pyrabactin, originally identified as a synthetic inhibitor
114 of growth and cell expansion (Zhao *et al.* 2007). Use of pyrabactin was instrumental in
115 discovering the redundant ABA receptors PYR/PYL (Park *et al.* 2009). Furthermore,
116 chemical genomics helped to uncover the function of strigolactone in light response
117 (Tsuchiya *et al.* 2010).
118 Jasmonates are lipid-derived phytohormones that mediate responses to abiotic
119 and biotic stress such as drought, salinity, wounding and pathogen attacks (Kazan 2015,
120 Ebel *et al.* 2018, Wasternack and Feussner 2018). In addition, jasmonates regulate the
121 biosynthesis of many secondary metabolites often involved in plant defence, such as
122 anthocyanins or terpenoids (Howe *et al.* 2018, Wasternack and Feussner 2018).
123 Jasmonates are also involved in many developmental processes including growth
124 inhibition and senescence (Howe *et al.* 2018, Wasternack and Feussner 2018).
125 Endogenous developmental processes as well as plant adaptation to environment
126 induce the accumulation of the bioactive form of the hormone, (+)-7-*iso*-JA-Ile
127 (Fonseca *et al.* 2009, Sheard*, et al.* 2010). The identification of the core JA-Ile pathway

128 components evidenced the striking mechanistic parallelism between the JA-Ile and
129 auxins pathways. Both pathways are composed by three elements, *i*) redundant
130 transcription factors regulating JA-Ile or auxins responses (e.g., MYCs or ARFs (Auxin
131 Responsive Factors), respectively), *ii*) a family of redundant repressors of the TF (i.e.
132 JAZs (JAsmonate ZIM-domain) or Aux/IAA (Auxin/Indole-3-Acetic Acid)), which
133 directly or indirectly (through the NINJA adaptor; Pauwels et al., 2010) recruits the co-
134 repressor TOPLESS, and *iii*) a co-receptor formed by an F-box protein (COI1 for JA-
135 Ile and TIR1(Transport Inhibitor Response1)/AFBs (Auxin F-Box) for auxin) and its
136 repressors targets (JAZs for JA-Ile and Aux/IAA for auxins; Chini et al. 2016; Leyser,
137 2018). In accordance to the "molecular glue" model, in both cases the hormone binding
138 to its co-receptor triggers ubiquitination and degradation of the repressor and, therefore,
139 releases the corresponding TF to activate the pathway (Chini*, et al.* 2007, Maor *et al.*
140 2007, Thines*, et al.* 2007, Chini *et al.* 2009a, Saracco *et al.* 2009, Lumba *et al.* 2010,
141 Leyser 2018, Chini *et al.* 2016, Howe*, et al.* 2018) . It is remarkable that the receptors
142 COI1 and TIR1/AFBs have a common evolutionary origin and, although strikingly
143 similar, they are adapted to perceive structurally different ligands (Bowman *et al.*
144 2017).

145 The JAZ proteins are repressors that inhibit a large number of TFs belonging to
146 several different families, including bHLH (basic helix-loop-helix), MYB, and
147 EIN3/EIL (Chini*, et al.* 2016, Howe*, et al.* 2018, Zander *et al*. 2020). The identification
148 of these components of the JA perception and signalling pathway were carried out using
149 mutant screens or reverse genetics (Xie*, et al.* 1998, Browse 2009, Chini*, et al.* 2016).
150 However, several components of the JA-signalling pathway belong to large protein
151 families with redundant functions, such as the 13 Arabidopsis JAZ repressors or the 4
152 MYC transcription activators, where genetic approaches are very laborious and their

153 success may be more limited (Chini*, et al.* 2007, Thines*, et al.* 2007, Fernandez-Calvo
154 *et al.* 2011, Qi *et al.* 2015, Guo *et al.* 2018, Zander *et al*. 2020). Therefore, specific
155 antagonist molecules of JA perception would represent excellent tools to overcome
156 redundancy and to explore the role of JA in spatiotemporal analyses. A chemical screen
157 identified jarin1 as inhibitor of jasmonate responses, impairing the synthesis of JA-Ile
158 (Meesters *et al.* 2014). In addition, coronatine-*O*-methyloxime (COR-MO) was
159 rationally designed as specific antagonist of jasmonate perception that shows strong
160 inhibiting activity of COI1-JAZ interaction, JAZ degradation and several JA-mediated
161 responses (Monte *et al.* 2014).

162 In this work, we searched for different and cheaper antagonists of JA-Ile
163 perception by chemical screens. Here we describe the identification of three
164 commercially available JA-Ile antagonists and their characterization. These molecules
165 prevent the JA-Ile-mediated COI-JAZ interaction and the degradation of JAZ1 and
166 JAZ9 *in vivo*. Moreover, one molecule (J4) exhibited JA-antagonist effects *in planta*,
167 preventing several JA-mediated responses such as gene expression, growth inhibition,
168 chlorophyll degradation and anthocyanin accumulation in Arabidopsis, tomato
169 (*Solanum licopersicum*) and *Nicotiana benthamiana*. In addition to JA signalling, J4
170 also inhibited the closely-related auxin pathway but no other hormonal pathways.
171 Furthermore, the mode of action of this molecule as direct antagonist of JA-Ile
172 perception by COI1-JAZ complexes is conserved in land plants, indicating its potential
173 use in any plant species. This commercially available compound is a potential powerful
174 tool for the pharmacological analysis and dissection of the JA and auxin signalling
175 pathways.

176

177 **Results**

178

179 *Chemical Screens*

180 The perception of the bioactive form of the JA-Ile hormone is mediated by the
181 co-receptor COI1-JAZ (Sheard*, et al.* 2010). In order to identify synthetic molecules
182 promoting or interfering with the COI1-JAZ interaction, we carried out two
183 independent chemical screens based on the interaction between COI1 and JAZ9 in
184 yeast-two-hybrid assays (Chini *et al.* 2009b, Fonseca*, et al.* 2009). Three chemical
185 libraries containing approximately 22,500 molecules (see Material and Method) were
186 tested to identify agonists and antagonists of JA-Ile perception, inducing or preventing
187 the hormone-triggered COI1-JAZ9 interaction, respectively. None of the agonist
188 compounds found could reproducibly induce COI1-JAZ9 interaction. However, 5
189 antagonist molecules were identified and confirmed (Y10, Y11 Y17, Y18 and Y20; Y
190 defines molecules identified in the Y2H screen, Figure 1A and B, Suppl. Figure S1A
191 and Supplemental Table S1), with Y10 being weaker than the other four. We also
192 confirmed that these compounds do not affect other interactions of these proteins (JAZ9
193 with itself or with NINJA) ruling out general unspecificity. We also tested whether
194 these molecules could inhibit the formation of additional COI1-JAZ complexes,
195 confirming that these five compounds prevent the COR-induced COI1-JAZ3
196 interaction (Suppl. Figure S1B).

197 Two complementary chemical screens were also carried out *in planta* exploiting
198 the rapid hormone-dependent JAZ degradation using the 35S:*JAZ1*-GUS reporter line
199 (Thines*, et al.* 2007). Approximately 15,000 compounds (see Material and Methods)
200 were tested for triggering degradation of JAZ1-GUS in the absence of JA-Ile/COR or
201 preventing COR-induced degradation of JAZ1-GUS. Again, no agonist compounds

202 were confirmed, whereas we validated 7 antagonist molecules (J1, J2, J3 J4, J9, J10
203 and J11; J defines the compounds identified in the JAZ1-GUS screen), preventing
204 hormone-induced degradation of JAZ1 (Figure 1A and C, Suppl. Figure S2 and
205 Supplemental Table S1). Since the original screen was carried out with COR, we tested
206 the candidate molecules in response JA to discard unspecific effects of COR or an effect
207 of the compound on JA conversion into JA-Ile. All J1 to J11 compounds were
208 confirmed (Suppl. Figure S2).

209 Among the 7 compounds obtained in the screens *in planta* (J) only one was also
210 active in yeast assays, whereas among the 5 compounds identified in Y2H screens (Y)
211 only 3 were positive *in planta* (Suppl. Figure S3; despite Y10 was positive we discarded
212 this compound because of its weak activity both in yeast and plant). Therefore, we
213 confirmed 3 compounds (J4, Y11 and Y20; Figure 1A) with robust antagonistic activity
214 in both Y2H and *in vivo* assays. These three compounds prevented the JA-triggered
215 degradation of JAZ1-GUS and JAZ9-GUS *in vivo*, showing that their effect is not
216 restricted to a particular JAZ (Figure 1B). Similarly, the three compounds inhibited the
217 interaction of COI1-JAZ9 and COI1-JAZ3 in Y2H, without affecting other interactions
218 of these proteins (JAZ9 with itself or with NINJA, Figure 1C). The effect of J4 and
219 Y11 as antagonists of COR-induced COI1-JAZ9 interaction was confirmed in semi-*in*
220 *vivo* pull-down (PD) experiments between recombinant JAZ9 fused to maltose binding
221 protein (MBP) and COI1-flag expressed *in planta* (Figure 1D).

222 In summary, these results confirmed that two molecules, namely J4 and Y11,
223 interfere with the COI1 interaction with more than one JAZ co-receptor both in the
224 heterologous yeast system and in PD experiments. This suggests that these compounds
225 might compete with COR or JA-Ile for the binding to COI1 *in vivo*. However, an

226 indirect effect of these molecules acting through a regulator of COI1 or JAZ cannot be
227 discarded yet.
228
229 *Minimal active concentration of antagonist molecules of JA-Ile/COR perception*
230 Next, we estimated the minimal active concentration at which these molecules
231 could act as antagonist of the JA-Ile/COR perception. The J4 and Y20 compounds
232 prevent the formation of the COI1-JAZ receptor complex in yeast at a concentration
233 between 5 and 50 μM, similar to the concentration of COR (5 μM) used to induce COI1-
234 JAZ interaction (Suppl. Figure S4). The compound Y11 inhibits the COI1-JAZ9
235 interaction at a concentration as low as 300 nM; however, Y11 is toxic for the yeast at
236 a concentration of 1 μM as shown by the lack of yeast growth in the control JAZ9-JAZ9
237 interaction assay (Suppl. Figure S4).
238 Similarly, the J4 and Y20 molecules prevent JAZ1-degradation *in planta* at
239 minimal concentrations between 10 and 25 μM, whereas Y11 had a weaker effect
240 (Suppl. Figure S5). In summary, J4 and Y20 show an antagonistic activity on processes
241 mediated by JA-Ile and COR at a concentration close to that usually employed for
242 exogenous hormone treatments.
243
244 *Structure-activity relationship*
245 To define the active moiety of these molecules, we performed an analysis of
246 structure-activity relationship of the three identified compounds (Rosado *et al.* 2011).
247 No structurally related compounds of Y11 were available. In contrast, we found three
248 derivatives of Y20 (named Y20-L1, Y20-L2 and Y20-L3, Suppl. Figure S6A). Y20-L1
249 and Y20-L2 prevented COI1 interaction with both JAZ9 and JAZ3 at the same
250 concentration as Y20 in Y2H (Suppl. Figure S6B). Similarly, they prevented the JA-

251 induced degradation of both JAZ1-GUS and JAZ9-GUS *in planta* (Suppl. Figure S6C),
252 whereas Y20-L3 fails to show antagonistic activity in all bioassays (Suppl. Figure S6B
253 and 6C). These results suggest that the antagonistic activity of Y20 resides in the
254 minimal 3-butyryl-4-hydroxy-2H-chromen-2-one structure. Moreover, we identified
255 that either or both the hydroxy group and the butyryl lateral chain are essential for the
256 activity of this molecule as JA antagonist, as simultaneous shortening of two carbons
257 in the butyryl chain and loss of the hydroxy group in Y20-L3 abolish the activity (Suppl.
258 Figure S6A).

259 The use of J4 derivatives (Suppl. Figure S6A) showed that the different
260 substitutions of the benzene ring did not have any impact on the activity of these
261 molecules, since all of them retained the activity as JA antagonists *in planta* in the JAZ1
262 and JAZ9 degradation assays (Suppl. Figure S6C). We concluded that the 5-
263 (benzylidene)-1,3-thiazolidine-2,4-dione moiety is important for the antagonist
264 activity.

265

266 *Molecules inhibiting JA-Ile perception affect JA-mediated transcription* in vivo

267 We subsequently analysed the effect of these molecules on JA-induced gene
268 expression *in planta*. *JAZ2* and *JAZ9* are among the genes most rapidly and strongly
269 induced by JA in a COI1-dependent manner (Chini*, et al.* 2007, Thines*, et al.* 2007).
270 Therefore, we assessed the effect of these molecules on the JA-induced gene expression
271 in the *pJAZ2:GUS* and *pJAZ9:GUS* reporter lines (Monte*, et al.* 2014, Gimenez-Ibanez
272 *et al.* 2017). In basal conditions, *pJAZ2:GUS* and *pJAZ9:GUS* are expressed at very
273 low levels but they are quickly and strongly induced in response to JA and after
274 wounding stress, which stimulates the synthesis of endogenous JA-Ile (Figure 2A and
275 2B; (Monte*, et al.* 2014)). Simultaneous treatment of exogenous JA or wounding with

276 the identified molecules, and derivative compounds, strongly inhibited the JA-mediated
277 activation of both *JAZ2* and *JAZ9* expression *in planta* (Figure 2A-B and Suppl. Figure
278 S7).

279 Interestingly, the *JAZ9* transcript accumulates specifically in trichomes in basal
280 conditions (Suppl. Figure S8A). This basal expression is abolished in the *coi1-30*
281 background, which indicates that perception of endogenous JA-Ile is required for the
282 specific *JAZ9* expression in the trichomes (Suppl. Figure S8B). We assessed the
283 inhibitory effect of the identified molecules on the trichome tissue specific expression
284 of *JAZ9*. Interestingly, only the compound J4 repressed the JA-regulated expression of
285 *JAZ9* in the trichomes (Suppl. Figure S8C). To further assess the inhibitory effect of J4
286 on additional JA-markers, we analysed the expression of the JA-induced *OPR3*, *TAT3*
287 and *JAZ10* genes. Indeed, exogenous treatment with J4 partially prevents the
288 transcriptional activation of these markers by JA (Suppl. Figure S9A).

289 To test if the JA-inhibitory activity of J4 is conserved across angiosperm plants,
290 we tested the effect of J4 on JA-regulated gene expression in two species of the Asterid
291 clade, tomato and *Nicotiana benthamiana*. Similar to the results in Arabidopsis,
292 exogenous treatment with J4 significantly prevents the JA-induced transcriptional
293 activation of several marker genes in both tomato and *N. benthamiana* plants (Figure 2
294 C-D).

295 These results show that these three compounds can prevent transcriptional
296 activation induced by both endogenous and exogenous JA. Moreover, J4 can also
297 inhibit the expression of a marker gene mediated by endogenous JA-Ile in a specific
298 tissue as well as in different angiosperm plants.

299

300 *Physiological effect of the identified antagonists* in planta

301 Jasmonate induced several physiological responses, such as growth inhibition,
302 anthocyanin accumulation and reduction of chlorophylls, among others (Wasternack
303 and Feussner 2018). In order to assess the effect of the identified molecules on JA-
304 regulated physiological responses, we grew plants in presence of JA and the identified
305 molecules. The concurrent treatment of the J4 compound at concentrations as low as 5
306 μM could partially prevent the root growth-inhibition, chlorophyll degradation and
307 anthocyanin accumulation induced *in planta* by JA (10 μM; Figure 3). In contrast, Y11
308 and Y20 failed to prevent JA-mediated responses *in planta* (Suppl. Figure S10). We
309 also determined the half maximal inhibitory concentration (IC50) of J4. For a
310 concentration of 15 μM of JA in the JAZ1-GUS degradation assay, the IC50 of J4 was
311 17.66 μM (Suppl. Figure S11). To study if J4 conserved its JA-inhibitory activity in
312 different plants, we tested the effect of J4 on JA-induced chlorophyll and carotenoids
313 degradation in *N. benthamiana*. Exogenous treatment of *N. benthamiana* with J4
314 significantly prevents the JA-induced degradation of both chlorophyll and carotenoids
315 *in planta* (Suppl. Figure S12).
316 Altogether, these data confirm that J4 acts as an antagonist of the JA pathway
317 and inhibits several hallmark JA-mediated responses *in planta*.
318
319 *Specificity of J4*
320 To study J4 specificity, we analyzed possible inhibitory side effects of J4 on the
321 ubiquitin/proteasome process that would in turn stabilize the JAZ proteins. Therefore,
322 we analysed the effect of J4 on other hormonal pathways regulated by the
323 ubiquitin/proteasome system, similarly to the JA pathway. First, we monitored the
324 gibberellin-mediated proteasome degradation of RGA-GFP (Repressor of GA) and the
325 constitutive proteasome-dependent degradation of EIN3-GFP (Ethylene-Insensitive-3),

326 which is inhibited by ethylene or its precursor ACC (Silverstone *et al.* 2001, Guo and
327 Ecker 2003). As shown in Figure 4A, J4 did not prevent the GA-triggered degradation
328 of RGA. Similarly, J4 did not substantially inhibit the constitutive degradation of EIN3
329 in the absence of ACC (Figure 4B). Secondly, we assessed the effects of J4 on the
330 turnover of the auxin Aux/IAA repressors, whose degradation depends on the activity
331 of the F-box TIR1, the receptor of auxins and the closest homolog of COI1 (Chico *et*
332 *al.* 2008). In contrast to GA and ethylene, J4 partially inhibited the auxin-mediated
333 degradation of the dII-VENUS auxin-repressor marker and the auxin-repressor IAA1
334 (Figure 4C and 4D). To further assess the effect of J4 on the auxin-pathway, we tested
335 the impact of this molecule on the auxin transcriptional marker *DR5:GUS*. Exogenous
336 indole-3-acetic acid (IAA) treatment highly induced the expression of *DR5:GUS*,
337 whereas the anti-auxin TIBA (2,3,5-triiodobenzoic acid) and J4 could partially inhibit
338 the IAA-mediated *DR5:GUS* expression (Figure 5A). PD analyses in Fig 5B shows that
339 IAA induces the interaction between TIR1 and IAA7 whereas J4 partially inhibited this
340 interaction, similarly to the effect of the specific auxin perception inhibitor auxinole
341 (Figure 5B). Finally, J4 also impairs the auxin-regulated gravitropic response in root
342 (Figure 5C-D).

343 These results show that J4 does not generally affect ubiquitin/proteasome-
344 regulated systems (UPS) but is not a fully specific antagonist of JA-Ile perception,
345 having also an inhibitory effect on the auxin pathway.

346

347 *J4 mode of action: A direct antagonist of hormone receptor complex*

348 Previous results suggest a possible mode of action of J4 as a direct competitor
349 with JA-Ile or COR for induction of COI1-JAZ complex (or competition with auxin in
350 the case of TIR1-IAA complex). To further study the J4 mode of action on the JA-

351 pathway, we checked if this molecule interferes directly with the assembly of the COI1-
352 JAZ perception complex *in vitro*, in the absence of other plant proteins. For that, we
353 used a heterologous baculovirus–insect cell expression system to express and purify the
354 COI1-flag protein. Since purified COI1 is quite unstable and mostly inactive, we also
355 expressed in insect cells a key component of the SCF^{COI1} complex, the adaptor
356 Arabidopsis SKP-like protein 1 (ASK1), which stabilizes and maintains the bioactivity
357 of COI1 (Li *et al*. 2017). In the presence of ASK1, COR promotes the interaction
358 between the purified COI1 and several recombinant JAZ-MBP proteins (JAZ1, JAZ2,
359 JAZ3 and JAZ9). J4 efficiently inhibits the COR-induced formation of all the tested
360 COI1-JAZ complexes (Figure 6A). To test if the mode of action of J4 is conserved
361 across land plants, we tested the effect of J4 on the MpCOI1-MpJAZ co-receptor from
362 the bryophyte *Marchantia polymorpha* (Monte *et al*. 2018). This co-receptor is the most
363 different COI1-JAZ complex known from that of Arabidopsis, which even perceives a
364 different ligand (dn-OPDA instead of JA-Ile), and diverged evolutionarily more than
365 450 million years ago (Bowman *et al*. 2017; Monte *et al*. 2018). The MpCOI1 and
366 MpASK1 proteins were expressed and purified in insect cells and their hormone
367 induced interaction with *E. coli*-purified MBP-MpJAZ protein was tested in PD
368 experiments. As shown in Figure 6B, dn-OPDA induces the interaction of the purified
369 MpCOI1 and MpJAZ, similar to the effect of JA-Ile on Arabidopsis COI1-JAZs. In
370 contrast, treatment with J4 substantially inhibited the dn-OPDA-dependent formation
371 of the MpCOI1-MpJAZ complex (Figure 6B). We subsequently analysed the effect of
372 J4 *in planta* by studying the OPDA-regulated transcriptional expression of several
373 OPDA marker genes such as Mp*JAZ*, Mp*DIR*, Mp*PAT* and Mp*BHLH4* (Monte et al.,
374 2018; Monte et al., 2019; Peñuelas et al., 2019). Indeed, exogenous pre-treatment with

375 J4 partially prevents the transcriptional activation of these marker genes by OPDA
376 (Figure 6C and Suppl. Figure S13).
377 Altogether, these results show that J4 directly interferes with the hormone-
378 triggered establishment of the COI1-JAZ receptor complexes of vascular and non-
379 vascular plants in absence of any other plant proteins. In addition, J4 has an *in vivo*
380 inhibitory effect on the jasmonates-mediated responses in both vascular and non-
381 vascular plants. Finally, the absence of putative regulators of COI1 or JAZ proteins in
382 these assays (Figure 6A-B) indicate that J4 has a direct effect on the formation of the
383 hormone-promoted COI1-JAZ complexes, likely competing with the ligands for the
384 binding pocket.
385

386 **Discussion**

387

388 To date, the role of JA regulating plant development and adaptation to biotic
389 and abiotic stresses has been primarily analysed using classical genetic approaches
390 relying on mutants impaired in the biosynthesis or response to JA (Browse 2009, Chini,
391 *et al.* 2016). However, these loss-of-function genetic tools hold some intrinsic
392 limitations such as functional redundancy, sterility and lethality (Xie, *et al.* 1998,
393 Sanders *et al.* 2000, Browse 2009, Fernandez-Calvo, *et al.* 2011, Qi, *et al.* 2015, Chini
394 *et al.* 2018b). Thus, the identification of molecules that specifically prevent JA-Ile-
395 perception represent excellent tools to explore the role of JA in specific plant stages or
396 tissues, avoiding these limitations. For example, specific auxin inhibitors were critical
397 tools to understand the role of auxins in various stages of plant development and
398 different plant species (Hayashi, *et al.* 2012, Leyser 2018). Using the auxin inhibitor
399 naxillin researchers defined a previously unknown function for root cap in root
400 branching by specifically stimulating the accumulation of active endogenous auxin
401 only in the root cap (De Rybel *et al.* 2012).

402 Molecules antagonizing JA-perception would allow the manipulation of the JA
403 pathway in a specific tissue or developmental stage in a reversible manner. Ideally, the
404 antagonists identified in Arabidopsis should be easily transferred to other plant species
405 and might clarify the role of JA-Ile in different plants. The two-step approach reported
406 here strongly minimizes the identification of false hits, one of the most problematic
407 constraints in chemical screens (Hicks and Robert 2014, Serrano *et al.* 2015). Thus, we
408 identified only three molecules (among the more than 20,000 compounds screened)
409 with robust JA-Ile antagonistic activity in both bioassays (Figure 1). Further
410 biochemical and physiological analyses confirmed J4 as the only compound with robust

411 JA-Ile antagonistic activity on several transcriptional and physiological JA-responses
412 *in planta* at concentrations similar to that of JA (Figure 3 and Suppl. Figure S9).
413 The toxicity and secondary effects of current agrochemicals can seriously affect
414 the environment and, potentially, animal and human health (Enserink *et al.* 2013,
415 Lamberth*, et al.* 2013). Multiple, unspecific or pleiotropic effects are rather common
416 among compounds identified in chemical screens (Fonseca *et al.* 2014b, Serrano*, et al.*
417 2015). Exceptionally, chemical screens also identified molecules with very specific
418 targets and limited or none off-target effects. For example, Kynurenine, identified as
419 inhibitor of the ethylene responses in root tissues (He*, et al.* 2011), is the first specific
420 inhibitor of auxin biosynthesis binding to the substrate pocket of the TAA1/TAR
421 aminotransferases family but no other related aminotransferases.
422 Here, we showed that J4 acts as a specific and dual inhibitor of the closely-
423 related jasmonate and auxin signalling pathways by antagonizing the hormone-induced
424 formation of the co-receptor complexes. Moreover, this inhibitory activity is
425 evolutionarily conserved in land plants (at least between liverworts and eudicots) since
426 J4 also inhibits the formation of the Marchantia MpCOI1-dn-OPDA-MpJAZ complex.
427 Jasmonate and auxin pathways share strikingly similar receptors (COI1 and TIR1),
428 which have a common evolutionary origin and share many conserved residues
429 (Bowman *et al*. 2017). Thus, we propose that J4 can partially mimic dn-OPDA, JA-Ile
430 and auxin to enter the COI1/TIR1 binding pocket, but cannot stablish the complex due
431 to a lack of interaction with the JAZ/Aux-IAA co-receptors.
432 Therefore, J4 may be considered a specific antagonist of two related receptors
433 that, however, do not affect other hormonal pathways or general proteasome-related
434 mechanisms. Our results support the use of J4 as a commercially available inhibitor of
435 JA- and auxin pathways in a particular tissue or developmental stage, useful in plant

436 research and for agronomic purposes. J4 could also be useful in dissecting JA/auxin
437 crosstalk. However, the concurrent inhibition of these two hormonal pathways may be
438 undesirable in some circumstances. Therefore, additional structure activity
439 relationships (SAR) analysis may be carried out to identify molecules structurally
440 related J4 that potentially act as specific inhibitors of the JA- or auxin-pathway.

441 An alternative to chemical screen is the rational design of antagonist molecules
442 specifically binding to the active pocket of key proteins. This approach, based on
443 structural information of receptor-ligand complexes, is extensively exploited in medical
444 research but just emerging in the agrochemical field (Lamberth*, et al.* 2013). For
445 instance, the rational design of auxin analogues successfully obtained receptor
446 antagonists useful in evolutionary distant plants species and that overcome the
447 redundancy of auxin receptors (Hayashi *et al.* 2008, Hayashi*, et al.* 2012). Similarly,
448 based on the crystal structure of the COI1-JAZ co-receptor, we designed a COR-
449 derivative (COR-MO) with a very strong and specific activity preventing COI-JAZ
450 interaction (Monte *et al.* 2014). Despite rational design being a very efficient and
451 specific approach, it requires a deep knowledge of the structures of receptor-ligand
452 complexes. In addition, the synthesis of rational-designed molecules is often very
453 complex and expensive. A limited amount of compounds is therefore suitable only for
454 specific, small-scale laboratory assays and not for agronomic use. Moreover, their
455 specificity may prevent their use in different species. For instance, bryophytes such as
456 *Marchantia polymorpha* do not synthesise JA-Ile, and the bioactive jasmonate and
457 ligand of the Marchantia COI1-JAZ co-receptor is dn-OPDA (Monte *et al.* 2018). The
458 Marchantia COI1-JAZ fails to bind JA-Ile or COR, and therefore, COR-MO cannot be
459 used as JA-inhibitor in non-vascular plants (Monte*, et al.* 2018). In contrast, J4 is able
460 to block all COI1-JAZ complexes tested (from Marchantia and Arabidopsis),

461 suggesting that J4 is potentially active in all plants, from bryophytes to angiosperm.
462 This broad activity of J4 underscores the high agronomic potential of this compound
463 and its derivates because they can be active in all crops. Therefore, the "unbiased"
464 chemical screen using commercially-available chemically diverse libraries is an
465 informative approach.

466 Finally, the robustness of the reported screening system encourages to
467 undertake additional screens using newly accessible "natural compound libraries" to
468 identify natural molecules directly perturbing the COI1-JAZ co-receptor complex.
469 Natural JA-Ile agonists could represent either novel activators of hormone biosynthesis
470 or different active forms of the plant hormone, modulating specific COI1-JAZ
471 complexes. Besides, the *in planta* optimized screen described here open the way to
472 identify natural molecules affecting previously unknown post-transcriptional
473 regulatory mechanisms of the key components of the JA signalling pathway, the JAZ
474 repressors.
475

476 **Material & Methods**
477

478 **Plant materials, growth conditions and transgenic plants**

479 *Arabidopsis thaliana* Col-0 is the genetic background of wild type and
480 transgenic lines used throughout the work. Seeds were surface sterilized and kept at
481 4°C in the dark for 48 h, then grown at 23°C with a 16-hour day cycle for 6 days, as
482 previously described (Chini*, et al.* 2018b, Chico *et al.* 2020). *Nicotiana benthamiana*
483 and tomato seeds were germinated and grown in the same conditions. Vertically grown
484 seedlings were germinated in the same conditions. The transgenic line 35S:JAZ1-GUS
485 was generated by (Thines*, et al.* 2007). The generation of transgenic plants expressing
486 35S:JAZ9-GUS were previously described (Monte *et al*. 2014). The *pJAZ9:GUS* and
487 *pJAZ2:GUS* reporter lines were also previously described (Monte *et al*. 2014, Gimenez-
488 Ibanez *et al*. 2017). The homozygous *pJAZ9:GUS* line was crossed with the loss-of-
489 function *coi1-30* mutant and the double homozygous *pJAZ9:GUS coi1-30* line was
490 isolated.
491

492 **Yeast-two-hybrid assays**

493 The growth, handling and transformation of yeast were as previously described
494 (Chini*, et al.* 2009b, Chini 2014). Briefly, the described plasmids were co-transformed
495 into *Saccharomyces cerevisiae* AH109 cells following standard LiAC protocols to
496 assess protein interactions. Successfully transformed colonies were identified on yeast
497 synthetic dropout lacking Leu and Trp. 3 days after transformation, yeast colonies were
498 grown in selective -WL liquid media for 6/7 hours and the cell density was adjusted to
499 3 x 10^7 cells/ml (OD_{600} = 1). 4 µl of the cell suspensions were plated out on yeast
500 synthetic dropout lacking Ade, His, Leu and Trp to test protein interaction
501 (supplemented with coronatine and antagonist compounds as indicated). Plates were

502 incubated at 28ºC for 2 to 4 days. As positive control, the yeast suspensions were also
503 plated on -AHWL media containing coronatine and the appropriate volume of DMSO.
504
505 **Chemical libraries and compounds**
506 In the COI1-JAZ Y2H screen, we tested 22,680 compounds from three chemical
507 libraries: the 2,320 molecules of MicroSource (from MDSI,
508 http://www.msdiscovery.com/spectrum.html), the approximately 20,000 compounds of
509 the 20 K DIVERSet™ (from ChemBridge, http://www.chembridge.com) and the 360
510 bioactive compounds described by (Drakakaki *et al.* 2011). In the JAZ1-GUS screen,
511 compounds from the libraries MicroSource and 360 bioactive, and a subset of the 20 K
512 DIVERSet™ were employed. All chemicals were stored at -20°C as 10 mg/ml stocks
513 in 100% DMSO and supplemented to a final concentration of 50–100 µM based on the
514 molecular mass of each compound.
515
516 **GUS staining and visualization**
517 The visualization of GUS (β-glucuronidase) in the four JAZ1-GUS, JAZ9-
518 GUS, *pJAZ2:GUS* and *pJAZ9:GUS* marker lines was carried out as previously
519 described (Chini 2014, Chini *et al.* 2018a). JAZ1-GUS and JAZ9-GUS seeds were
520 germinated in MS plates congaing 1% agar, kept in vertical for 7 days, transferred to 2
521 ml Johnson's liquid media supplemented with the described chemicals and incubated in
522 an orbital shaker at 100 rpm, as described in (Chini 2014). Seedlings of the *pJAZ9:GUS*
523 and *pJAZ2:GUS* lines were germinated in liquid MS media in an orbital shaker at 100
524 rpm. Chemical treatments were carried out 6 days after germination.
525 After the described treatments, samples were placed in staining solution 50 mM
526 phosphate buffer (pH 7), 0.1 % (v/v) Triton X-100 (Sigma), 1 mM X-Gluc

527 (Glycosynth), 1 mM K-ferrocyanide (Sigma), 1 mM K-ferricyanide (Sigma) and
528 incubated at 37°C overnight. After staining, the seedlings were washed in of 75%
529 ethanol to eliminated chlorophyll. JAZ1-GUS seedlings were otherwise treated as
530 described above and roots were collected. Protein extract was employed for
531 fluorometric quantification of GUS activity using a Spectra Max M2 flouorometer
532 (Molecular Devices) (Monte*, et al.* 2014).
533
534 **Degradation of RGA-GFP and EIN3-GFP**
535 pRGA:GFP-RGA, 35S:EIN3-GFP and 35S:HA-IAA1 (Silverstone*, et al.* 2001,
536 Guo and Ecker 2003) seeds were germinated in 2 ml liquid MS media in an orbital
537 shaker at 100 rpm and the described compounds were added directly to the media 6
538 days after germination. After 2 hours, 20 plants of the same size were collected and
539 proteins were extracted as previously described (Fonseca and Solano 2013). Samples
540 were denatured, loaded on 9% SDS–PAGE gels, transferred to nitrocellulose
541 membranes (Bio-Rad) and incubated with anti-GFP-HRP antibody (Milteny Biotec).
542 Blots were developed using ECL (Pierce).
543
544 **Protein extracts and pull-down assays**
545 MBP-JAZ fusion proteins were generated as previously described (Fonseca*, et*
546 *al.* 2009). Pull-down experimental procedure is extensively described by (Fonseca and
547 Solano 2013). Briefly, 35:COI1-flag seedlings were ground in liquid nitrogen and
548 homogenized in extraction buffer. For PD experiments, 6 µg of resin-bound MBP
549 fusion protein was added to 1 mg of total protein extract and incubated for 1 hour at
550 4 °C with rotation in presence of coronatine and the indicated compounds or DMSO
551 control. After washing, samples were denatured, loaded on 8% SDS–PAGE gels,

552 transferred to nitrocellulose membranes and incubated with anti-flag antibody (Sigma).
553 To confirm equal protein loading, 7 µl of MBP-fused protein of each sample was run
554 into SDS-PAGE gels and stained with Coomassie blue.
555
556 **Recombinant protein expression by baculovirus–insect cell expression systems.**
557 The expression of Arabidopsis and Marchantia ASK1 and COI1 in Sf9 insect
558 cells was carried out as previously described (Sheard *et al*. 2010; Li *et al*. 2017;
559 Takaoka *et al*. 2018). Briefly, pFast-HIS-flag-COI1 and pFastBac1-flag-ASK1 vectors
560 were transformed into DH10Bac competent cell and further transposed into the bacmid.
561 Blue/white selection were used to identify colonies containing the recombinant bacmid.
562 The recombinant bacmid was transfected into Sf9 insect cells using Lipofectamine
563 (Invitrogen) according to the manufacturers' instructions. After the titter check, the
564 isolated P1 recombinant baculovirus was further amplified to generate P2 and P3
565 stocks. P3 recombinant baculoviruses were added to a large cultured Sf9 cells at a
566 volume ratio of 4.5:1000. 50 µl of insect cell culture supernatant for ASK1 and 50 µl
567 for AtCOI1 or 50 µl for MpCOI1 were employed, together with 100 µl of PD buffer,
568 for pull-down assays in presence of AtJAZ-MBP or MpJAZ proteins expressed in *E.*
569 *coli* as reported above and extensively described by (Fonseca and Solano 2013).
570
571 **Root measurement, Anthocyanin and Chlorophyll quantification**
572 Root growth inhibition assay was carried as previously described (Fonseca *et*
573 *al.* 2014a). 3-day-old Arabidopsis seedlings germinated in vertical plates were
574 transferred onto vertical Johnson medium in presence of JA with or without 5 µM J4
575 for 10 days and root length of 15 to 50 seedlings was measured. Roots were quantified
576 using ImageJ software. Values represent mean and the error bars standard deviation.

577 The same growing conditions were employed to measure chlorophylls using 13-
578 day-old Arabidopsis seedlings grown for10 days in presence 10 µM JA with or with 5
579 µM J4. *Nicotiana benthamiana* seeds were germinated and grown at 23°C in a 16-hour
580 day cycle. *N. benthamiana* seedlings were germinated and grown in vertical MS plates
581 for 7 days; seedlings were then transferred in 5 ml liquid MS media supplemented with
582 25 µM JA with or without 10 µM J4. Three days later, the aerial part of 5 to 7 seedlings
583 was collected for each measurement and 4 to 6 independent replicates were analysed.
584 Chlorophyll measurements were performed as previously described (Fonseca*, et al.*
585 2014a). Acetone 80% (V/V) was used for extraction and absorbance at 645 and 663 nm
586 was measured in a spectrophotometer (Spectra Max M2 Molecular Devices). For
587 carotenoids measurements, samples were extracted overnight in 95% ethanol and
588 absorbance at 470, 645 and 663 nm was measured in a spectrophotometer. Values
589 represent mean and the error bars correspond to standard deviation. The experiment
590 was repeated three with similar results.
591 Anthocyanin quantification was carried as previously described (Fonseca*, et al.*
592 2014a). Briefly, seedlings grown in vertical Johnson plates for 5 days and transferred
593 to liquid Johnson media supplemented with 10 µM JA with or without 2.5 µM J4. Two
594 days later, the aerial parts of 10 to 15 seedlings were pooled for each replicate and
595 anthocyanin quantification was performed using a spectrophotometer. Four
596 independent replicates (seedling pools) were measured for each sample. Values
597 represent mean and the error bars standard deviation. The experiment was repeated at
598 least three times with similar results.
599
600 **Quantitative RT-PCR**

601 Quantitative RT-PCR was performed using biological samples of tissue pooled
602 from 10–15 Arabidopsis seedlings. RNA was extracted and purified using a Plant Total
603 RNA isolation kit (Favorgen), including on-column DNase digestion to remove
604 genomic DNA contamination. cDNA was synthesized from 1 µg total RNA with a high-
605 capacity cDNA reverse transcription kit (Applied Biosystems). Gene amplification was
606 caried out using 4 µl from a 1:10 cDNA dilution, 4 µl of EvaGreen® qPCR Mix Plus
607 (Solis BioDyne) and gene-specific primers (Supplemental Table S2). Quantitative PCR
608 was performed in 96-well optical plates in a HT 7900 Real Time PCR system (Applied
609 Biosystems) using standard thermocycler conditions. Relative expression values given
610 as the means of three or four technical replicates (from 10–15 Arabidopsis seedlings)
611 relative to the mock wild-type control using ACT8 as the housekeeping gene. Data were
612 analysed using One-way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$).
613
614 **Accession Numbers**
615 Sequence data from this article can be found in the GenBank/EMBL data libraries under
616 accession numbers_. *AtJAZ1*/*AT1G19180* (gene ID 838501), *AtJAZ3*/*AT3G17860*
617 (821055), *AtCOI1*/*AT2G39940* (818581), *MpJAZ*/*Mapoly0097s0021*/*Mp6g06230*
618 (PTQ32535) and *MpCOI1*/*Mapoly0025s0025*/ *Mp2g26590* (PTQ43322).
619
620 **Supplemental Data**
621 **Supplemental Figure S1. Inhibition of COR-induced COI1-JAZ interactions in**
622 **yeast two- hybrid assays.**
623
624 **Supplemental Figure S2. Molecules antagonizing the JA-mediated degradation**
625 **of JAZ1-GUS protein *in planta*.**

626

627 **Supplemental Figure S3. Confirmation of the antagonistic effect of the 12**
628 **identified molecules on COI1-JAZ interaction in Y2H and JAZ1 degradation *in***
629 ***planta*.**

630

631 **Supplemental Figure S4. Minimal concentrations of antagonist molecules**
632 **required to prevent COR-mediated COI1/JAZ interactions in Y2H.**

633

634 **Supplemental Figure S5. Quantification of the inhibition of JAZ1-GUS**
635 **degradation by antagonist molecules *in vivo*.**

636

637 **Supplemental Figure S6. Inhibition of COI1-JAZ interactions in Y2H and of JAZ**
638 **degradation *in planta* by derivate molecules of J4 and Y20.**

639

640 **Supplemental Figure S7. Inhibition of *JAZ2 and JAZ9* expression of derivate**
641 **molecules of J4 and Y20.**

642

643 **Supplemental Figure S8. Specific expression of *JAZ9* in trichomes requires COI1**
644 **and it is inhibited by J4 treatment.**

645

646 **Supplemental Figure S9. J4 partially inhibits JA-mediated transcriptional**
647 **activation in several angiosperm plants.**

648

649 **Supplemental Figure S10. Y11 and Y20 do not prevent JA-mediated responses *in***
650 ***planta*.**

651 **Supplemental Figure S11. Half maximal inhibitory concentration (IC50) of J4 in**
652 **JA-promoted JAZ1-GUS degradation.**
653
654 **Supplemental Figure S12. Antagonistic effect of the compound J4 on JA-induced**
655 **responses in tobacco plants.**
656
657 **Supplemental Figure S13. J4 partially inhibits OPDA-mediated transcriptional**
658 **activation.**
659
660 **Supplemental Table S1. List of all identified molecules including their basic**
661 **information.**
662
663 **Supplemental Table S2. List of primers sequences used for qPCR analyses.**
664
665

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682 **Figure legends:**

683

684 Figure 1. **Identification of molecules with JA-Ile antagonist activity in yeast two-**
685 **hybrid, *in planta* and in the hormone-dependent formation of the receptor**
686 **complex COI1-JAZ9**.

687 (A) Chemical structure of the JA-antagonist compounds.

688 (B) Yeast cells co-transformed with pGAD-JAZ9 (preys) and pGBK-COI1, pGBK-
689 NINJA or pGBK-JAZ9 (baits) were selected and subsequently grown on yeast synthetic
690 drop-out lacking Leu and Trp (-LW) as a transformation control, or on selective media
691 lacking Ade, His, Leu and Trp (-AHLW) to test protein interactions. COI1 interaction
692 with JAZ9 or JAZ3 is detected only in presence 5 mM or 20 mM coronatine
693 respectively. Antagonist molecules inhibit COI1-JAZ interaction in presence of COR
694 (J4 and Y20 were used at 15 μM, and Y11 at 300 nM). Compounds were dissolved in
695 DMSO; therefore, an equivalent volume of DMSO was used in the negative control
696 (labelled as -). As control, we tested that the antagonist molecules did not interfere with
697 the interaction between JAZ9 and NINJA or JAZ9 dimerization.

698 (C) 6-day-old JAZ1-GUS and JAZ9-GUS seedlings were concurrently treated with 2.5
699 μM JA and the indicated molecules (J4 and Y20 were used at 25 μM, and Y11 at 50
700 μM). Jasmonic acid triggers the degradation of JAZ-GUS protein, whereas the addition
701 of antagonist molecules prevented JAZ degradation. Compounds were dissolved in
702 DMSO; a volume of DMSO equivalent to that in the compounds was used in the
703 negative control (identified as -).

704 (D) Immunoblot analysis of COI1-flag/JAZ9-MBP interaction performed with anti-flag
705 antibodies to COI1-flag protein recovered from MBP-JAZ9 and extracts of transgenic
706 *COI1-flag* plants. MBP was employed as negative control. The COI1-flag/JAZ9-MBP

707 interaction is dependent on the presence of COR (0.5 μM). Compounds were dissolved
708 in DMSO; therefore, a volume of DMSO equal to that in 0.5 μM of the compounds was
709 used as control. The compounds Y11 and J4 reduce COI1-flag/JAZ9-MBP interaction
710 (at 100 and 500 μM). The lower panel shows Coomassie blue staining of the MBP and
711 the JAZ9-MBP after cleavage with Factor Xa.
712
713 Figure 2. **Antagonistic molecules prevent the JA-induced expression of several *JAZ***
714 **genes *in planta*.**
715 (A) Representative seedlings (N > 30) of the *pJAZ2:GUS* and *pJAZ9:GUS* line were
716 concurrently treated with 5 μM JA for 75 minutes and the indicated molecules (J4 at 10
717 μM, Y20 at 25 μM, and Y11 at 100 μM) for 3 hours. Jasmonic acid triggers the
718 expression of *JAZ*, whereas the addition of antagonist molecules could prevent *JAZ*
719 transcriptional activation. Compounds were dissolved in DMSO; an equivalent volume
720 of DMSO was used in the negative control (identified as -) in A and B. White bars are
721 equal to 1 mm in A and B.
722 (B) Seedlings of *pJAZ2:GUS* and *pJAZ9:GUS* were wounded several times and
723 concurrently treated with the indicated compounds (J4 at 10 μM, Y20 at 25 μM, and
724 Y11 at 100 μM) for 2 hours. The mechanical wounding induces the *JAZ* expression
725 whereas the JA-antagonists can prevent wound-induced *JAZ* expression.
726 (C-D) Gene expression analysis of JA-marker genes in *Solanum lycopersicum*,
727 moneymaker cultivar, (C) and *Nicotiana benthamiana* (D) plants in response to JA
728 treatment and/or the J4 molecule. Tomato (C) plants were pre-treated with DMSO (-;
729 untreated control), 50 or 100 μM J4 for 1 hour and then with 50 μM JA for 1 hour.
730 Sl*αTUB4* was used as housekeeping control gene. *Nicotiana benthamiana* plants were
731 pre-treated with DMSO (-; untreated control), 50 or 100 μM J4 for 1 hour and then with

732 25 μM JA for 1 hour. Nb*βACT* was used as housekeeping control gene. Experiments
733 were repeated three times with similar results.
734 One-way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define the
735 significant differences in gene expression. Each biological sample consisted of tissue
736 pooled from 10-15 plants. Data show mean ± SD of three to four technical replicates.
737
738
739 Figure 3. **Antagonistic effect of the compound J4 on JA-induced responses *in***
740 ***planta*.**
741 (A) 13-day-old WT seedlings grown for 10 days on vertical plates in presence of 10
742 μM JA with or without 5 μM J4. White bar stands for 1 cm.
743 (B) Root growth inhibition by 10 μM JA of 13-day-old seedlings in presence or absence
744 of 5 μM J4. J4 was dissolved in DMSO; therefore, an equivalent volume of DMSO was
745 used in the negative control (identified as -) in A-D.
746 (C) Anthocyanin accumulation, shown as Absorbance (530) per gram of plant fresh
747 weigh (FW), in 7-day-old WT seedlings grown for 2 days in presence of 10 μM JA with
748 or without 2.5 μM J4.
749 (D) chlorophyll a and b content of 13-day-old WT seedlings grown for 10 days on
750 vertical plates in presence of 10 μM JA with or without 5 μM J4.
751 (B, C and D) Bars represent the average value and error bars the standard deviation.
752 Asterisks indicate statistically significant values of JA-treated plants according to a
753 Student's t-test ($p < 0.05$).
754
755 Figure 4. **The compound J4 does not affect the ubiquitin/proteasome system.**

756 (A) Immunoblot analysis of the effect of antagonists on RGA-GFP (Repressor of GA)
757 stability (with anti GFP antibodies). pRGA:GFP-RGA seedlings were concurrently
758 treated for 2 hours with 10 μM gibberellic acid (GA_3) and J4 at 10 μM. GA_3 induces
759 RGA degradation and J4 fails to prevent RGA destabilization. J4 was dissolved in
760 DMSO; an equivalent volume of DMSO was used in the negative control (labelled as -
761) in A-D.
762 (B) Immunoblot analysis of the effect of antagonists on EIN3-GFP (Ethylene-
763 Insensitive-3) stability (with anti GFP antibodies). Seedlings of the 35S:EIN3-GFP line
764 were treated for 2 hours with the J4 at 10 μM or 10 μM of the ethylene precursor ACC.
765 EIN3 is continuously degraded and stabilized by ACC. The synthetic proteasome
766 inhibitor MG132 (at 100 μM) was included as positive control.
767 (C-D) Effect of J4 the stability of the on dII-VENUS (with anti GFP antibodies) auxin
768 sensor and IAA1-HA (with anti HA antibodies) *in planta*. Immunoblot analysis of
769 35S:dII-VENUS and 35S:IAA1-HA seedlings of the dII-VENUS auxin repressor
770 marker (C) or the auxin repressor IAA1 (D) treated for 1 hour with 1 μM IAA in
771 absence or presence of J4 at 10 μM. The first band shows constitutive expression of the
772 dII-VENUS auxin marker (C) or IAA1-HA (D).
773
774 Figure 5. **Effect of the compound J4 on several auxin-regulated responses.**
775 (A) Effect of J4 on auxin-induced *DR5:GUS* reporter gene expression. 6-d-old
776 seedlings were treated with IAA (5 μM) alone and in presence of the auxin inhibitor
777 TIBA (100 μM) or J4 (10 μM) for 90 minutes. J4 was dissolved in DMSO, therefore
778 an equivalent volume of DMSO was used as negative control (defined as -) in A-D.
779 (B) Immunoblot analysis of TIR1-myc/IAA7-GST interaction performed with anti-myc
780 antibodies to TIR1-myc protein recovered from IAA7-GST and extracts of transgenic

781 *TIR1-myc* plants. IAA (5 μM) induces the interaction between the auxin co-receptors
782 TIR1-myc and IAA7-GST, whereas the specific auxin-perception inhibitor auxinole
783 and J4 (at 100 μM) partially inhibits the TIR1-IAA7 complex formation.
784 (C-D) Effect of J4 on auxin-mediated gravitropic response. 4-day-old seedlings grown
785 on MS plates were transferred to control DMSO (-), NPA (1 μM) or J4 (2.5 μM) plates
786 and then rotated 135º from the gravity vector (arrow 1 vs 2). Photographs were taken
787 24 hours after re-orientation (C). Bar lengths represent the percentage of seedlings (n =
788 28 to 32 seedlings for each treatment) growing at the indicated orientation 24 hours
789 after re-orientation (D).
790
791 Figure 6. **J4 interferes with the hormone-dependent formation of several COI1-**
792 **JAZ receptor complexes**.
793 (A) Immunoblot analysis of AtCOI1-flag/AtJAZ-MBP interaction performed with anti-
794 flag antibodies to COI1-flag proteins recovered from MBP-AtJAZ columns. ASK1-
795 flag and COI1-flag were expressed in Sf9 insect cells, whereas AtJAZ were expressed
796 in *E. coli*. The AtCOI1-flag/AtJAZ-MBP interaction is dependent on the presence of
797 COR (0.5 μM); the compound J4 (at 100 and 200 μM) inhibits the hormone-dependent
798 AtCOI1-flag/AtJAZ-MBP interaction. J4 was dissolved in DMSO; therefore, an
799 equivalent volume of DMSO was used as negative control (labelled as -) in A-C. The
800 lower panel shows Coomassie blue staining of the AtJAZ-MBP after cleavage with
801 Factor Xa. Asterisks indicate the JAZ-MBP bands of the expected size in the Coomassie
802 panel of each pull-down assay.
803 (B) Immunoblot (antiflag antibody) of recovered MpCOI1-flag after pull-down
804 reactions using recombinant MpJAZ-MBP protein alone (-) or with dinor-OPDA.
805 MpASK1-flag and MpCOI1-flag were expressed in Sf9 insect cells, and MpJAZ was

806 expressed in *E. coli*. The MpCOI1-flag/MpJAZ-MBP interaction depends on the
807 presence of dn-OPDA (50 μM). The J4 compound (at 100 and 200 μM) inhibits the
808 hormone-dependent MpCOI1-flag/MpJAZ-MBP interaction. Bottom, Coomassie blue
809 staining of MpJAZ-MBP after cleavage with Factor Xa.
810 (C) Gene expression analysis of Mp*DIR* (Dirigent-like protein) and Mp*BHLH4* (basic
811 helix-loop-helix 14) in Tak-1 Marchantia plants in response to 10 μM OPDA for 1 hour;
812 plants pre-treated with DMSO (-, untreated control), 15 or 30 μM J4 (+) for 1 hour.
813 Mp*ACT* was used as housekeeping control gene. One-way ANOVA with post-hoc
814 Tukey HSD Test ($p < 0.01$) analyses define the significant differences in gene
815 expression. Each biological sample consisted of tissue pooled from 5-8 plants. Data
816 show mean ± SD of four technical replicates.
817
818

819 **Supplementary Material**

820

821 Supplemental Figure S1. **Inhibition of COR-induced COI1-JAZ interactions in**
822 **yeast two- hybrid assays.**
823 Yeast cells co-transformed with pGAD-JAZ9 or pGAD-JAZ3 (preys) and pGBK-
824 COI1, pGBK-NINJA or pGBK-JAZ9 (baits) were grown on yeast synthetic drop-out
825 lacking Leu and Trp (-LW), as control or on selective media lacking Ade, His, Leu and
826 Trp (-AHLW), to test protein interactions. COI1 interaction with JAZ9 (A) and JAZ3
827 (B) is detected only in presence 5 and 20 μM COR respectively. This figure shows the
828 Y2H interaction in presence of COR and the indicated antagonist molecules (Y10 at
829 100 μM; Y17 at 25 μM; Y18 at 20 μM; Y20 at 15 μM; Y11 at 300 nM). Compounds
830 were dissolved in DMSO, therefore as negative control an equivalent volume of DMSO
831 was used (labelled as -). As control, we tested that the antagonist molecules did not
832 interfere with the interaction between JAZ9 and NINJA or JAZ9 dimerization.

833

834 Supplemental Figure S2. **Molecules antagonizing the JA-mediated degradation of**
835 **JAZ1-GUS protein *in planta*.**
836 Roots of 7-day-old JAZ1-GUS seedlings concurrently treated with 5 μM JA and 100
837 μM of the indicated molecules (J1, J2, J3, J4, J9, J10, J11) for 1 hour. JA treatment
838 triggers the degradation of JAZ1-GUS, whereas the addition of the identified molecules
839 could prevent JAZ1 destabilization. Compounds were prepared in DMSO; as negative
840 control contained an equivalent volume of DMSO (defined as -).
841 The original chemical screen was carried out using COR whereas the JA was employed
842 in the secondary confirmation assays to discard unspecific effects of COR or an effect
843 of the compound on JA conversion into JA-Ile.

844

845 Supplemental Figure S3. **Confirmation of the antagonistic effect of the 12 identified**
846 **molecules on COI1-JAZ interaction in Y2H and JAZ1 degradation *in planta*.**
847 (A-B) Yeast cells co-transformed with pGAD-JAZ9 or pGAD-JAZ3 (preys) and
848 pGBK-COI1, pGBK-NINJA or pGBK-JAZ9 (baits) were selected and subsequently
849 grown on yeast synthetic drop-out lacking Leu and Trp (-LW), as a transformation
850 control or on selective media lacking Ade, His, Leu and Trp (-AHLW), to test protein
851 interactions. COI1 interaction with JAZ9 (A) and JAZ3 (B) is induced only in presence
852 5 and 20 μM COR respectively. This figure shows the Y2H interaction in presence of
853 COR and the indicated antagonist molecules (J1 and J9 at 100 μM; J10 at 50 μM; J4 at
854 15 μM; J2 at 10 μM; J3 at 10 nM, J11 at 5 nM). As control, we tested that the antagonist
855 molecules did not interfere with the interaction between JAZ9 and NINJA or JAZ9
856 dimerization. Compounds were dissolved in DMSO, therefore as negative control an
857 equivalent volume of DMSO was used (labelled as -) in A-C.
858 (C) Roots of 7-day-old JAZ1-GUS seedlings concurrently treated with 2 μM JA and
859 100 μM of the indicated molecules (Y10, Y11, Y17, Y18, Y20) for 1 hour. JA treatment
860 triggers the degradation of JAZ1-GUS, whereas the addition of most antagonist
861 molecules could prevent JAZ1 destabilization.
862

863 Supplemental Figure S4. **Minimal concentrations of antagonist molecules required**
864 **to prevent COR-mediated COI1/JAZ interactions in Y2H**.
865 Yeast cells co-transformed with pGAD-JAZ9 (prey) and pGBK-COI1, pGBK-NINJA
866 or pGBK-JAZ9 (baits) were selected and subsequently grown on yeast synthetic drop-
867 out lacking Leu and Trp (-LW), as a transformation control or on selective media
868 lacking Ade, His, Leu and Trp (-AHLW), to test protein interactions. COI1-JAZ9

869 interaction is detected only in presence 5 μM coronatine. A range of concentration of
870 antagonistic molecules was used to define the minimal concentration required to inhibit
871 COI1-JAZ interaction: 25 μM J4 (A), 300 nM Y11 (B) and 5 μM Y20 (C). Compounds
872 were dissolved in DMSO; negative controls carry an equivalent volume of DMSO was
873 used (defined as -) in A-C. As control, we tested that these molecules did not interfere
874 with the interaction between JAZ9 and NINJA or JAZ9 dimerization.
875
876 Supplemental Figure S5. **Quantification of the inhibition of JAZ1-GUS degradation**
877 **by antagonist molecules *in vivo*.**
878 Twenty to thirty 7-day-old seedlings were incubated in medium containing 50 nM COR
879 with or without indicated compounds for 1 hour. Fluorometric GUS quantification of
880 roots of Arabidopsis JAZ1-GUS line is shown. Relative GUS activity is shown
881 normalizing 100% value to mock treatment (absence of COR). Compounds were
882 dissolved in DMSO, therefore as negative control an equivalent volume of DMSO was
883 used (labelled as -). Columns represent mean of 6 readings and error bars are standard
884 deviations. One-way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define
885 the significant differences in JAZ1-GUS degradation.
886
887 Supplemental Figure S6. **Inhibition of COI1-JAZ interactions in Y2H and of JAZ**
888 **degradation *in planta* by derivate molecules of J4 and Y20**.
889 (A) Chemical structures of the described J4 and Y20 molecules. Oxygen atoms are
890 highlighted in red, nitrogen in blue, sulphur in yellow and fluorine in purple.
891 (B) COR induces the interaction between the COI1 receptor and JAZ co-receptors in
892 Y2H assay, whereas most Y20 derivate molecules prevent the COR-dependent COI1-
893 JAZ interaction. Yeast cells co-transformed with pGAD-JAZ9 or pGAD-JAZ3 (preys)

894 and pGBK-COI1, pGBK-NINJA or pGBK-JAZ9 (baits) were grown on yeast synthetic
895 drop-out lacking Leu and Trp (-LW), as control or on selective media lacking Ade, His,
896 Leu and Trp (-AHLW), to test protein interactions. COI1 interaction with JAZ9 and
897 JAZ3 is detected only in presence 5 and 20 μM coronatine respectively. Y20 derivate
898 molecules were employed at 25 μM. As control, we tested that these molecules did not
899 interfere with the interaction between JAZ9 and NINJA or JAZ9 dimerization.
900 Compounds were prepared in DMSO; as negative control an equivalent volume of
901 DMSO was used (labelled as -).
902 (C) The figure show roots of 6-day-old JAZ1-GUS and JAZ9-GUS seedlings
903 concurrently treated with 2 μM jasmonic acid and 100 μM of the indicated molecules.
904 JA induces the degradation of JAZ1 and JAZ9, whereas most J4 and Y20 derivate
905 molecules could prevent JAZ degradation.
906
907 Supplemental Figure S7. **Inhibition of *JAZ2 and JAZ9* expression of derivate**
908 **molecules of J4 and Y20**.
909 (A) Roots of seedlings of the *pJAZ2:GUS* marker line concurrently treated for 75
910 minutes with 5 μM JA and the indicated molecules (J4, Y20, Y20-L1 and Y20-L2 at
911 10 μM; J4-L1, J4-L2 and J4-L3 at 25 μM; Y20-L3 at 100 μM). JA triggers the
912 expression of *JAZ2*, whereas most molecules could prevent the *JAZ* transcriptional
913 activation. Compounds were dissolved in DMSO, therefore as negative control an
914 equivalent volume of DMSO was used (labelled as -).
915 (B) Seedlings of the *pJAZ9:GUS* line were concurrently wounded and treated for 3
916 hours the indicated molecules (100 μM). Mechanical wounding induces the
917 accumulation of endogenous JA-Ile and the expression of *JAZ9*, whereas the addition
918 of most molecules could inhibit the transcriptional activation of *JAZ9*.

919

920 Supplemental Figure S8. **Specific expression of *JAZ9* in trichomes requires COI1**
921 **and it is inhibited by J4 treatment**.
922 (A-B) Leaves of 10-day-old *pJAZ9:GUS* seedlings in WT (A) and *coi1-30* (B)
923 background are shown. Expression of *JAZ9* in trichomes requires *COI1*.
924 (C) *pJAZ9:GUS* seedlings were treated over-night with the indicated compounds at 100
925 μM. Only the J4 compound can inhibit the trichome-specific *JAZ9* expression.
926 Compounds were prepared in DMSO, so an equivalent volume of DMSO was used as
927 negative control (labelled as -).
928

929 Supplemental Figure S9. **J4 partially inhibits JA-mediated transcriptional**
930 **activation in Arabidopsis.**
931 Gene expression analysis of *JAZ10*, *OPR3* and *TAT3* in wild-type (Col-0) plants in
932 response to 12.5 μM JA for 45 minutes; plants pre-treated with DMSO (-; untreated
933 control) or J4 (+) for 1 hour. *ACT8* was used as housekeeping control gene. One-way
934 ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define the significant
935 differences gene expression. Each biological sample consisted of tissue pooled from
936 10-15 plants. Data show mean ± SD of three to four technical replicates.
937 (A),
938

939 Supplemental Figure S10. **Y11 and Y20 do not prevent JA-mediated responses *in***
940 ***planta***.
941 (A) 13-day-old WT seedlings germinated in MS media for 3 days and then grown for
942 10 days in vertical plate in presence of 10 μM JA and 2.5 μM Y11 or Y20. Compounds

943 were dissolved in DMSO, therefore an equivalent volume of DMSO was used as
944 negative control (labelled as -) in. A-C. Lines represent 1 cm.
945 (B) Root growth inhibition by 15 μM JA of 13-day-old WT seedlings grown for 10
946 days in vertical plate in presence or absence of 2.5 μM Y11 or Y20. Results are
947 expressed as mean +/- SD of 25-30 plants. One-way ANOVA with post-hoc Tukey
948 HSD Test ($p< 0.01$) analyses define the significant differences.
949 (C) Anthocyanin accumulation, shown as Absorbance (530) per gram of plant fresh
950 weigh (FW), in 8-day-old WT seedlings grown for 2 days in presence of 50 μM JA with
951 or without 2.5 μM Y11 or Y20. Results are expressed as mean +/- SD. One-way
952 ANOVA with post-hoc Tukey HSD Test ($p< 0.01$) analyses define the significant
953 differences.
954
955 Supplemental Figure S11. **Half maximal inhibitory concentration (IC50) of J4 in**
956 **JA-promoted JAZ1-GUS degradation.**
957 (A) Quantification of GUS activity (arbitrary unit U per μg protein per h) in roots of 7-
958 d-old JAZ1-GUS plants. Seedlings (N =20 to 30) were pretreated with the indicated
959 concentrations of J4 for 1 h and then treated with 15 μM JA for 1 hour. Results shown
960 are the mean ± s.d. of seven replicates.
961 (B) Relative quantification of JAZ1-GUS. Untreated control was set as 100% of GUS
962 activity in roots of 7-d-old JAZ1-GUS plants. Seedlings (N =20 to 30) were pretreated
963 for 1 hour with the indicated concentrations of J4 and then with 15 μM JA for an
964 additional hour. Results shown are the mean ± s.d. of seven replicates. The plain line
965 connects the experimental values, whereas the dotted line is the trendline.
966

967 Supplemental Figure S12. **Antagonistic effect of the compound J4 on JA-induced**
968 **responses in *Nicotiana benthamiana* plants.**
969 Chlorophyll a and b (A) and carotenoids (B) content of 10-day-old *N. benthamiana*
970 seedlings (N = 7 plants) germinated in vertical plates and then grown for 3 days in
971 presence of 25 µM JA with or without 10 µM J4. The experiments were repeated three
972 times with similar results.
973 A and B, Bars represent the average value and error bars the standard deviation. One-
974 way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define the significant
975 differences.
976
977 Supplemental Figure S13. **J4 partially inhibits OPDA-mediated transcriptional**
978 **activation.**
979 Gene expression analysis of Mp*PAT* and Mp*JAZ* in Tak-1 Marchantia plants in
980 response to 10 µM OPDA for 1 hour; plants pre-treated with DMSO (-; untreated
981 control), 15 or 30 µM J4 (+) for 1 hour. Mp*ACT* was used as housekeeping control
982 gene. One-way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define the
983 significant differences in gene expression. Each biological sample consisted of tissue
984 pooled from 5-8 plants. Data show mean ± SD of four technical replicates.
985
986 **Supplemental Table S1.** List of all identified molecules including their basic
987 information.
988
989 **Supplemental Table S2.** List of primers sequences used for RT-qPCR analyses.
990
991

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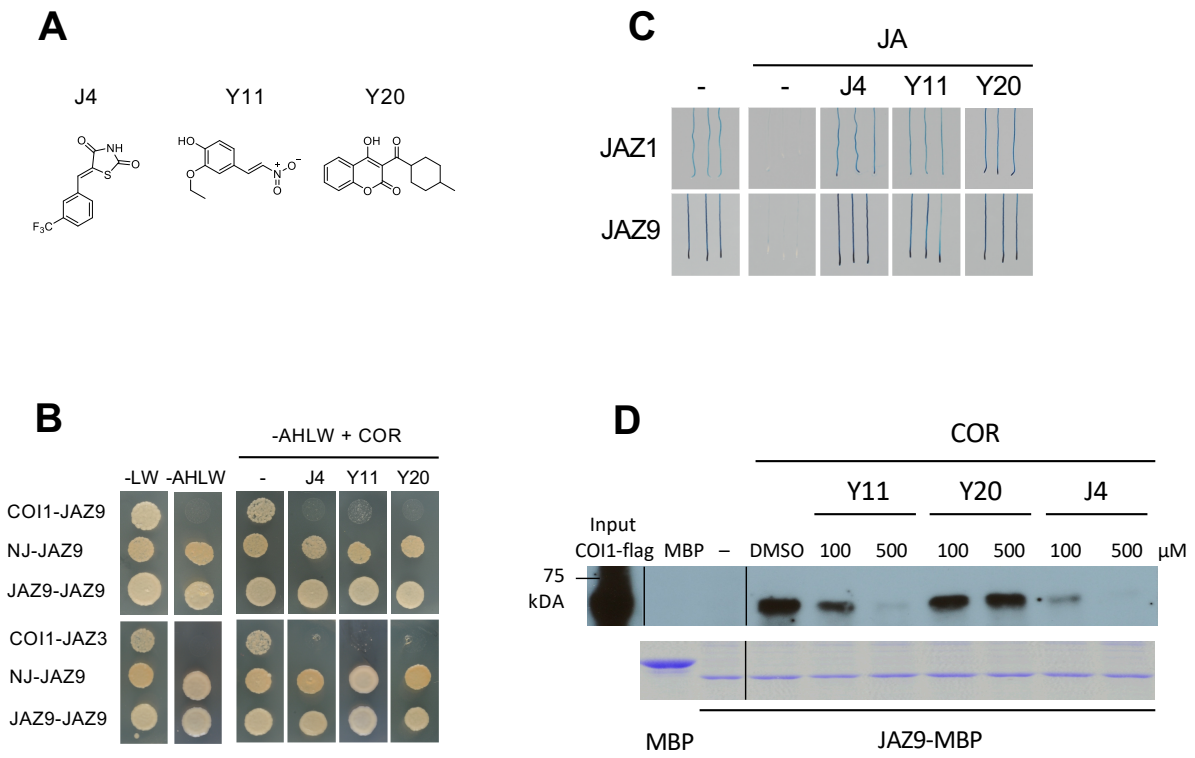


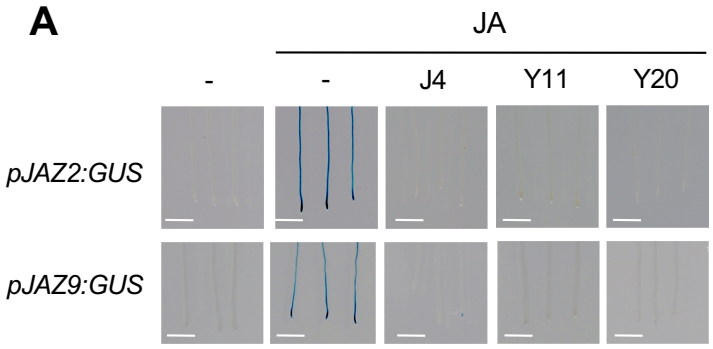
Figure 1. **Identification of molecules with JA-Ile antagonist activity in yeast two-hybrid, *in planta* and in the hormone-dependent formation of the receptor complex COI1-JAZ9**.

(A) Chemical structure of the JA-antagonist compounds.

(B) Yeast cells co-transformed with pGAD-JAZ9 (preys) and pGBK-COI1, pGBK-NINJA or pGBK-JAZ9 (baits) were selected and subsequently grown on yeast synthetic drop-out lacking Leu and Trp (-LW), as a transformation control or on selective media lacking Ade, His, Leu and Trp (-AHLW), to test protein interactions. COI1 interaction with JAZ9 or JAZ3 is detected only in presence 5 µM or 20 µM coronatine respectively. Antagonist molecules inhibit COI1-JAZ interaction in presence of COR (J4 and Y20 were used at 15 µM and Y11 at 300 nM). Compounds were dissolved in DMSO, therefore an equivalent volume of DMSO was used in the negative control (labelled as -). As control, we tested that the antagonist molecules did not interfere with the interaction between JAZ9 and NINJA or JAZ9 dimerization.

(C) 6-day-old JAZ1-GUS and JAZ9-GUS seedlings were concurrently treated with 2.5 µM JA and the indicated molecules (J4 and Y20 were used at at 25 µM and Y11 at 50 µM). Jasmonic acid triggers the degradation of JAZ-GUS protein, whereas the addition of antagonist molecules could prevent JAZ degradation. Compounds were dissolved in DMSO; a volume of DMSO equivalent to that in the compounds was used in the negative control (identified as -).

(D) Immunoblot analysis of COI1-flag/JAZ9-MBP interaction with anti flag antibodies of recovered COI1-flag protein from MBP-JAZ9 and extracts of transgenic *COI1-flag* plants. MBP was employed as negative control. The COI1-flag/JAZ9-MBP interaction is dependent on the presence of COR (0.5 µM). Compounds were dissolved in DMSO, therefore the volume of DMSO equals to that in 0.5 mM of the compounds was used as control. The compounds Y11 and J4 reduce COI1-flag/JAZ9-MBP interaction (at 100 and 500 µM). The lower panel shows Coomassie blue staining of the MBP and the JAZ9-MBP after cleavage with Factor Xa.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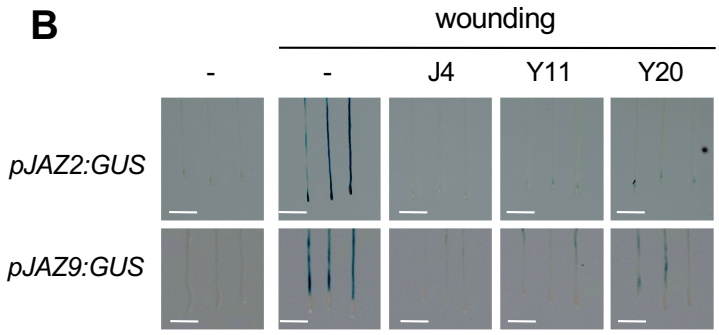
JA
-
-
J4
Y11
Y20
pJAZ2:GUS
pJAZ9:GUS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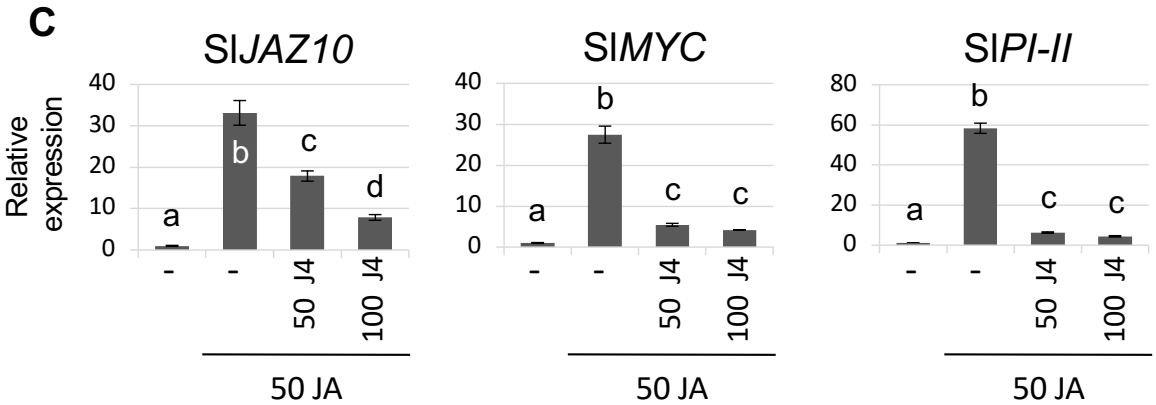
J4
Y11
Y20
pJAZ2:GUS
pJAZ9:GUS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SlJAZ10
SlMYC
SlPI-II
Relative expression
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30
20
10
0
80
60
a
b
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d
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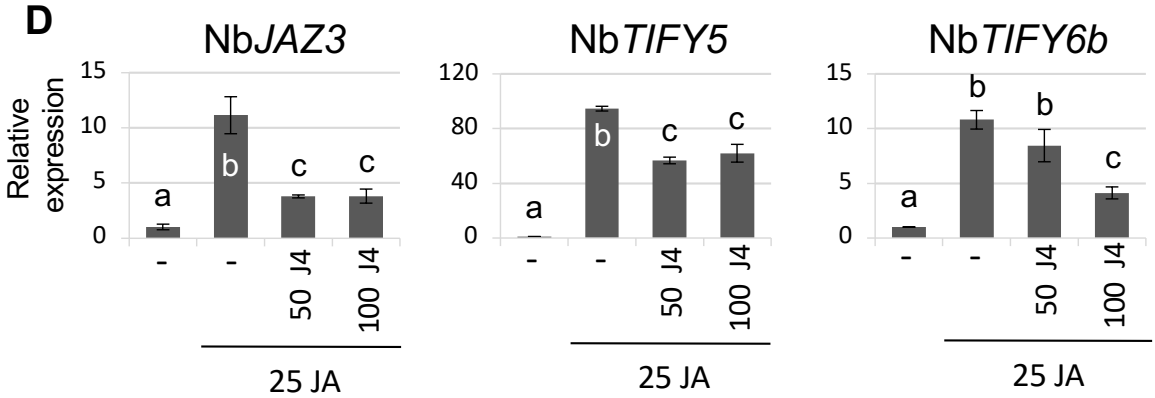
50 J4
100 J4
50 JA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NbTIFY5
NbTIFY6b
Relative expression
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10
5
0
120
80
40
a
b
c
-
50 J4
100 J4
25 JA

Figure 2. **Antagonistic molecules prevent the JA-induced expression of several *JAZ in planta***.

(A) Representative seedlings (N > 30) of the *pJAZ2:GUS* and *pJAZ9:GUS* line were concurrently treated with 5 µM JA and the indicated molecules (J4 at 10 µM, Y20 at 25 µM and Y11 at 100 µM) for 75 minutes and 3 hours respectively. Jasmonic acid triggers the expression of *JAZ*, whereas the addition of antagonist molecules could prevent *JAZ* transcriptional activation. Compounds were dissolved in DMSO; an equivalent volume of DMSO was used in the negative control (identified as -) in A and B. White bars are equal to 1 mm in A and B.

(B) Seedlings of *pJAZ2:GUS* and *pJAZ9:GUS* were wounded several times and concurrently treated with the indicated compounds (J4 at 10 µM, Y20 at 25 µM and Y11 at 100 µM) for 2 hours. The mechanical wounding induces the *JAZ* expression whereas the JA-antagonists can prevent wound-induced *JAZ* expression.

(C-D) Gene expression analysis of JA-marker genes in Moneymaker cultivar of *Solanum lycopersicum* (C) and *Nicotiana benthamiana* (D) plants in response JA treatment and/or the J4 molecule. (C) Tomato plants were pre-treated with DMSO (-; untreated control) 50 or 100 µM J4 for 1 hour and then with 50 µM JA for 1 hour. *SlαTUB4* was used as housekeeping control gene. (D) Tobacco plants were pre-treated with DMSO (-; untreated control) 50 or 100 µM J4 for 1 hour and then with 25 µM JA for 1 hour. Nbβ*ACT* was used as housekeeping control gene. Experiments were repeated three times with similar results.

One-way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define the significant differences gene expression. Each biological sample consisted of tissue pooled from 10-15 plants. Data show mean ± SD of three to four technical replicates.

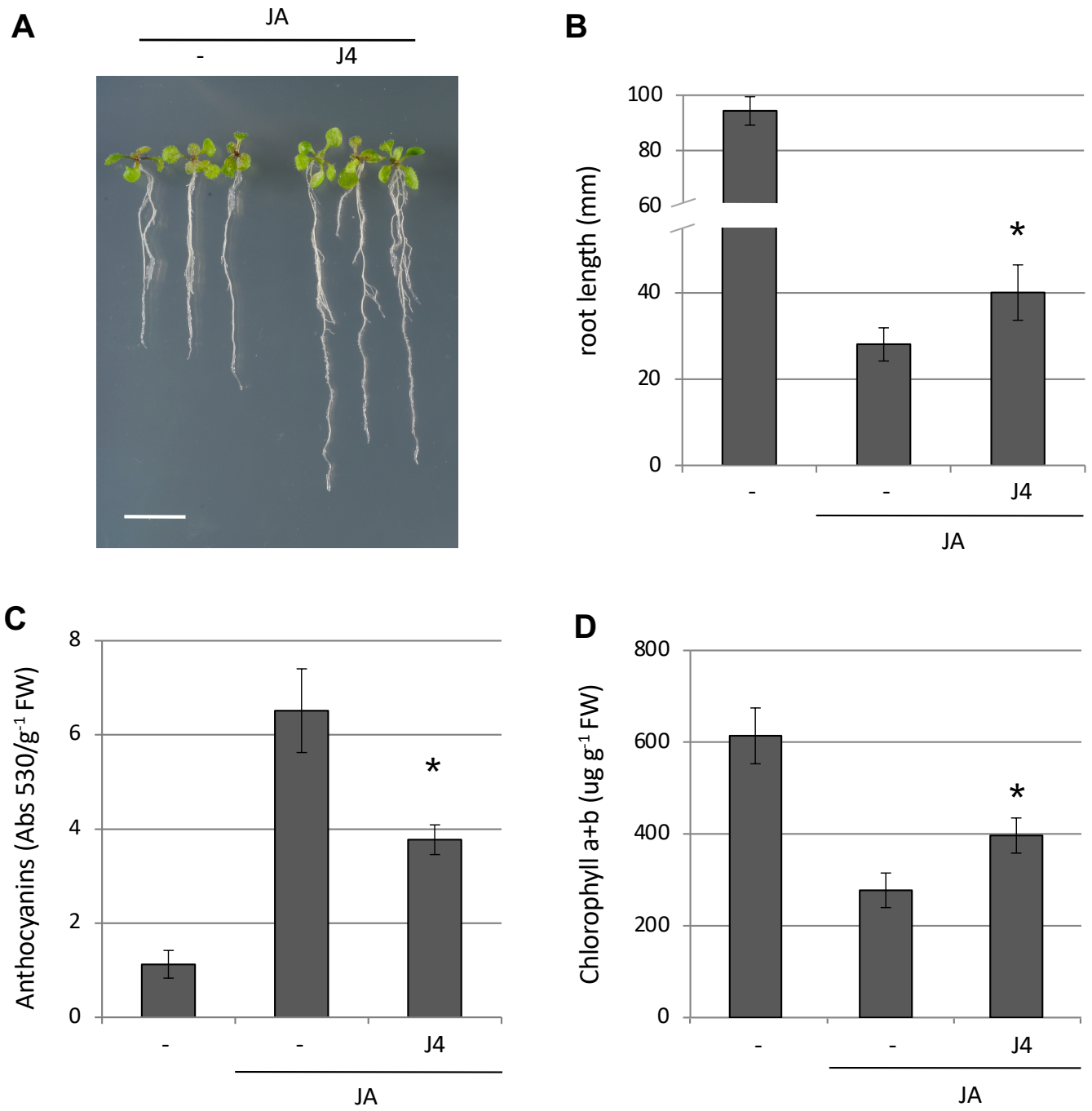

Figure 3. **Antagonistic effect of the compound J4 on JA-induced responses *in planta*.**

(A) 13-day-old WT seedlings grown for 10 days in vertical plates in presence of 10 µM JA with or without 5 µM J4. White bar stands for 1 cm.

(B) Root growth inhibition by 10 µM JA of 13-day-old seedlings in presence or absence of 5 µM J4. J4 was dissolved in DMSO, therefore an equivalent volume of DMSO was used in the negative control (identified as -) in A-D.

(C) Anthocyanin accumulation in 7-day-old WT seedlings grown for 2 days in presence of 10 µM JA with or without 2.5 µM J4.

(D) chlorophyll a and b content of 13-day-old WT seedlings grown for 10 days in vertical plates in presence of 10 µM JA with or without 5 µM J4.

(B, C and D) Bars represent the average value and error bars the standard deviation. Asterisks indicate statistically significant values of JA-treated plants applying a Student's t-test ($p < 0.05$).

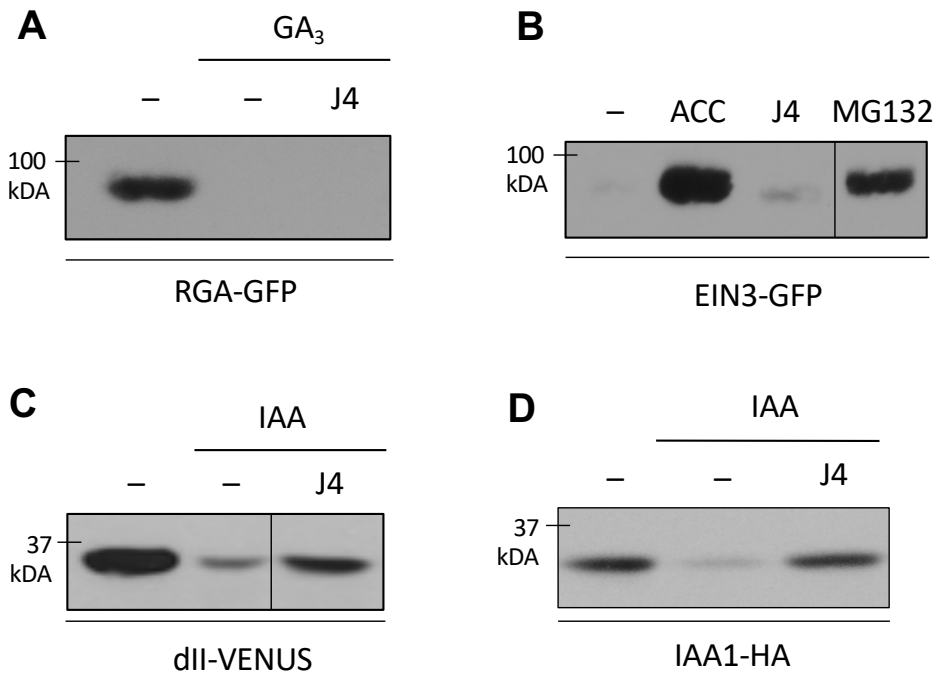

Figure 4. **The compound J4 does not affect the ubiquitin/proteasome system.**

(A) Immunoblot analysis of the effect of antagonists on RGA-GFP stability with anti GFP antibodies. pRGA::GFP-RGA seedlings were concurrently treated for 2 hours with 10 µM gibberellic acid (GA_3) and J4 at 10 µM. GA_3 induces RGA degradation and J4 fails to prevent RGA destabilization. J4 was dissolved in DMSO; an equivalent volume of DMSO was used in the negative control (labelled as -) in A-D.

(B) Immunoblot analysis of the effect of antagonists on EIN3-GFP stability with anti GFP antibodies. Seedlings of the 35S::EIN3-GFP line were treated for 2 hours with the J4 at 10 µM or 10 µM of the ethylene precursor ACC. EIN3 is continuously degraded and stabilized by ACC. The synthetic proteasome inhibitor MG132 (at 100 µM) was included as positive control.

(C-D) Effect of J4 the stability of the on dII-VENUS (with anti GFP antibodies) auxin sensor and IAA1-HA (with anti HA antibodies) *in planta*. Immunoblot analysis of 35S::dII-VENUS and 35S::IAA1-HA seedlings of the dII-VENUS auxin repressor marker (A) or the auxin repressor IAA1 (B) treated for 1 hour with 1 µM IAA in absence or presence of J4 at 10 µM. The first line shows constitutive expression of the dII-VENUS auxin marker (C) or IAA1-HA (D).

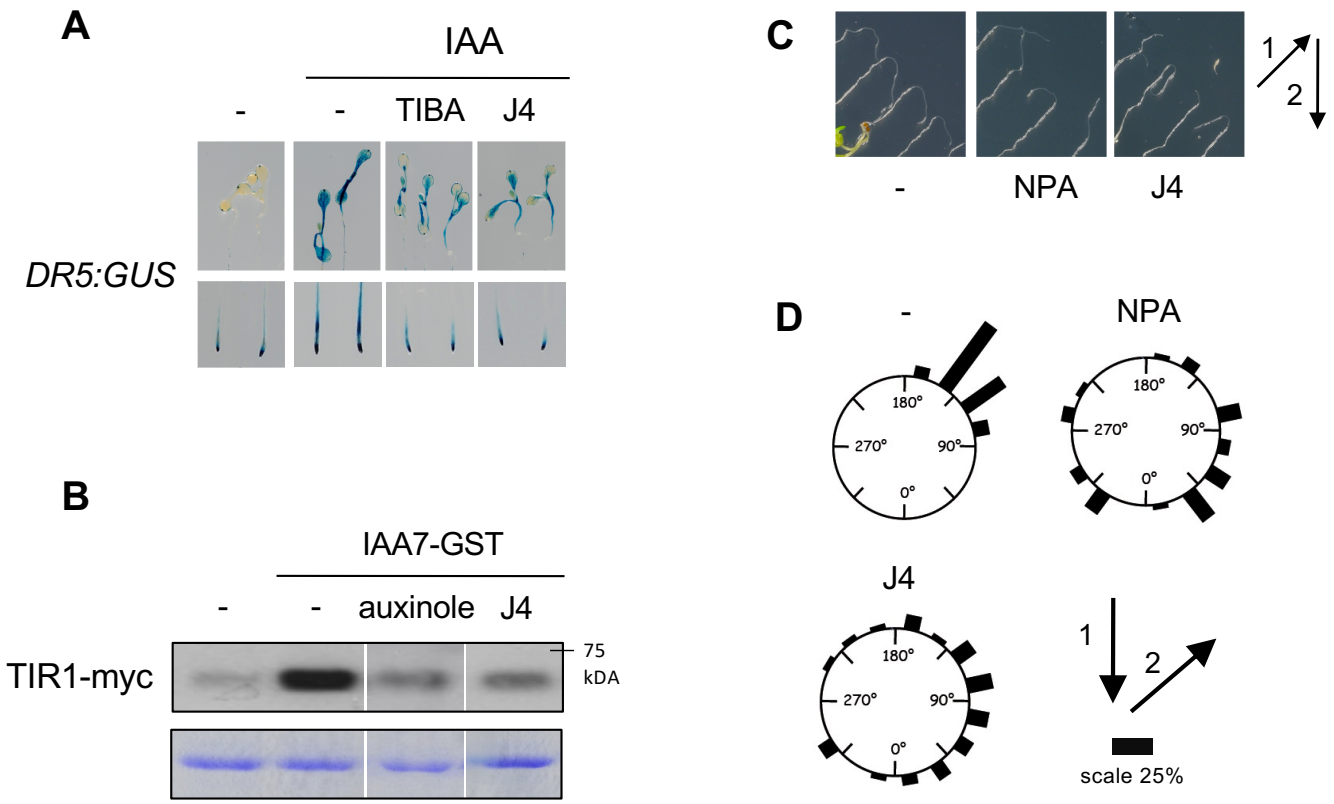

Figure 5. **Effect of the compound J4 on several auxin-regulated responses.**

(A) Effect of J4 on auxin-induced *DR5:GUS* reporter gene expression. 6-d-old seedlings were treated with IAA (5 µM) alone and in presence of the auxin inhibitor TIBA (100 µM) or J4 (10 µM) for 90 minutes. J4 was dissolved in DMSO, therefore an equivalent volume of DMSO was used as negative control (defined as -) in A-D.

(B) Immunoblot analysis of TIR1-myc/IAA7-GST interaction with anti-myc antibodies of recovered TIR1-myc protein from IAA7-GST and extracts of transgenic *TIR1-myc* plants. IAA (5 µM) induces the interaction between the auxin co-receptors TIR1-myc and IAA7-GST, whereas the specific auxin-perception inhibitor auxinole and J4 (at 100 µM) partially inhibit the TIR1-IAA7 complex formation. The lower panel represents Coomassie blue staining of recombinant IAA7-GST protein.

(C-D) Effect of J4 on auxin-mediated gravitropic response. 4-day-old seedling grown on MS plates were transferred control DMSO (-), NPA (1 uM) or J4 (2.5 uM) plates and then rotated 135º from the gravity vector (arrow 1 vs 2). Photographs were taken 24 hours after re-orientation (C). Bar lengths represent the percentage of seedlings (n = 28 to 32 seedlings for each treatment) growing at the indicated orientation 24 hours after re-orientation (D).

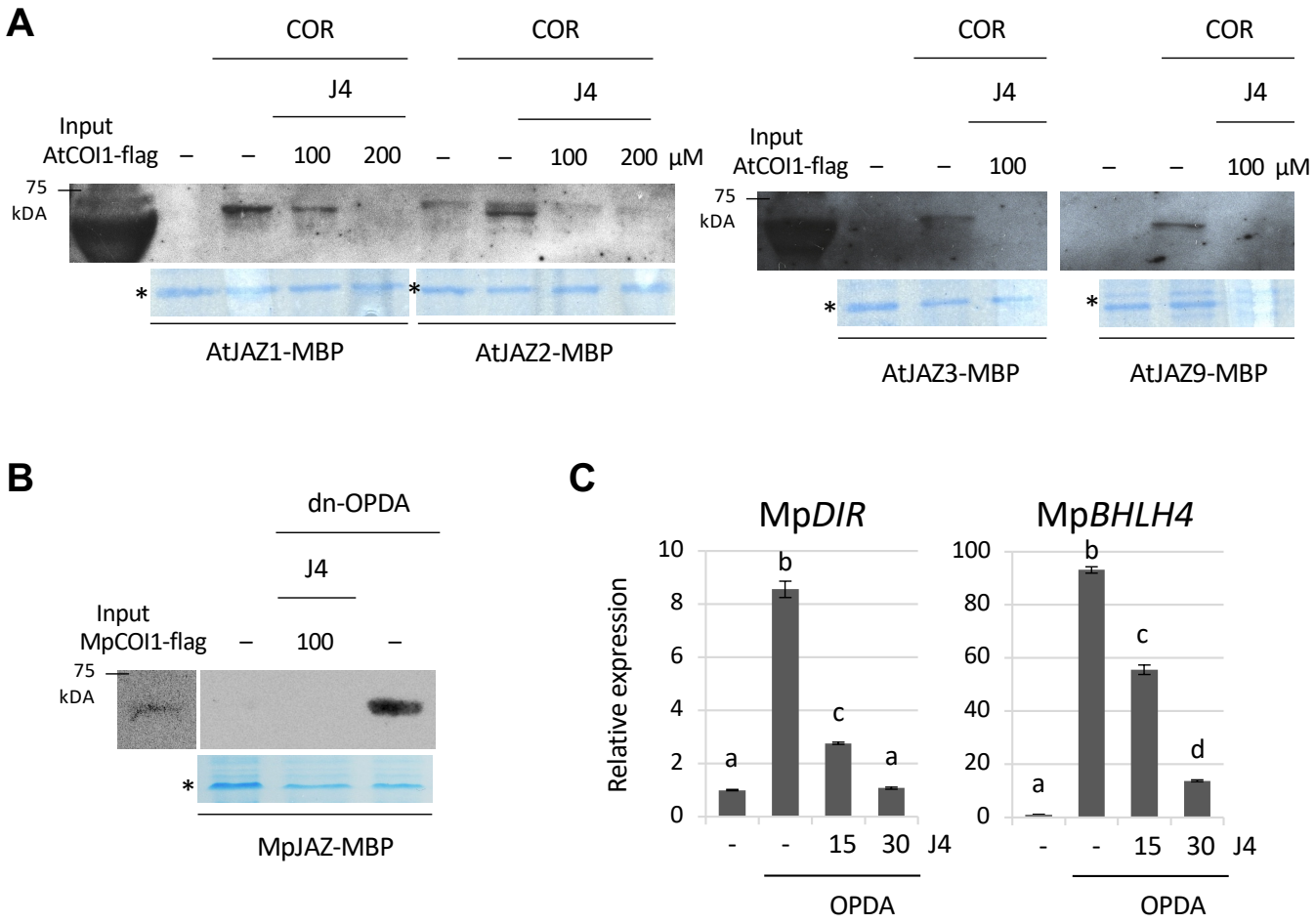

Figure 6. **J4 interferes with the hormone-dependent formation of several COI1-JAZ receptor complexes and it inhibits OPDA-mediated responses *in planta*.**

(A) Immunoblot analysis of AtCOI1-flag/AtJAZ-MBP interaction performed with anti-flag antibodies to COI1-flag recovered from MBP-AtJAZ columns. ASK1-flag and COI1-flag were expressed in Sf9 insect cells, whereas AtJAZ were expressed in *E. coli*. The AtCOI1-flag/AtJAZ-MBP interaction is dependent on the presence of COR (0.5 µM); the compound J4 (at 100 and 200 µM) inhibits the hormone-dependent AtCOI1-flag/AtJAZ-MBP interaction. J4 was dissolved in DMSO; therefore, an equivalent volume of DMSO was used as negative control (labelled as -) in A-C. The lower panel shows Coomassie blue staining of the AtJAZ-MBP after cleavage with Factor Xa. Asterisks indicate the JAZ-MBP bands of the expected size in the Coomassie panel of each pull-down assay.

(B) Immunoblot (antiflag antibody) of recovered MpCOI1-flag after pull-down reactions using recombinant MpJAZ-MBP protein alone (-) or with dinor-OPDA. MpASK1-flag and MpCOI1-flag were expressed in Sf9 insect cells, and MpJAZ was expressed in *E. coli*. The MpCOI1-flag/MpJAZ-MBP interaction depends on the presence of dn-OPDA (50 µM). The J4 compound (at 100 and 200 µM) inhibits the hormone-dependent MpCOI1-flag/MpJAZ-MBP interaction. Bottom, Coomassie blue staining of MpJAZ-MBP after cleavage with Factor Xa.

(C) Gene expression analysis of Mp*DIR* (Dirigent-like protein) and Mp*BHLH4* (basic helix-loop-helix 14) in Tak-1 Marchantia plants in response to 10 µM OPDA for 1 hour; plants pre-treated with DMSO (-, untreated control), 15 or 30 µM J4 (+) for 1 hour. Mp*ACT* was used as housekeeping control gene. One-way ANOVA with post-hoc Tukey HSD Test ($p < 0.01$) analyses define the significant differences gene expression. Each biological sample consisted of tissue pooled from 5-8 plants. Data show mean ± SD of four technical replicates.