

1 **TGA transcription factors and jasmonate-independent COI1**
2 **signaling regulate specific plant responses to reactive oxylipins**

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19 **Abstract**

20 Jasmonates and phytoprostanes are oxylipins that regulate stress responses
21 and diverse physiological and developmental processes. 12-Oxo-phytodienoic acid
22 (OPDA) and phytoprostanes are structurally related electrophilic cyclopentenones,
23 which activate similar gene expression profiles that are to the most part different
24 from the action of the cyclopentanone jasmonic acid (JA) and its biologically active
25 amino acid conjugates. Whereas JA-isoleucine signals through binding to COI1, the
26 bZIP transcription factors TGA2, TGA5 and TGA6 are involved in regulation of
27 gene expression in response to phytoprostanes. Here we compared root growth
28 inhibition and target gene expression after treatment with JA, OPDA or
29 phytoprostanes in mutants of the COI1/MYC2 pathway and in different TGA factor
30 mutants. Inhibition of root growth by phytoprostanes was dependent on COI1 but
31 independent of jasmonate biosynthesis. In contrast, phytoprostane-responsive gene
32 expression was strongly dependent on TGA2, TGA5 and TGA6, but not dependent
33 on COI1, MYC2, TGA1 and TGA4. Different mutant and overexpressing lines were
34 used to determine individual contributions of TGA factors to cyclopentenone-
35 responsive gene expression. Whereas OPDA-induced expression of the cytochrome
36 P450 gene *CYP81D11* was primarily regulated by TGA2 and TGA5, the glutathione-
37 S-transferase gene *GST25* and the OPDA reductase gene *OPRI* were regulated by
38 TGA5 and TGA6, but less so by TGA2. These results support the model that
39 phytoprostanes and OPDA regulate (i) growth responses, which are COI1-dependent
40 but jasmonate-independent, and (ii) lipid stress responses, which are strongly
41 dependent on TGA2, TGA5, and TGA6, differently. Identification of molecular
42 components in cyclopentenone signaling provides an insight into novel oxylipin
43 signal transduction pathways.

44 **Key words:** *Arabidopsis thaliana*, biotic and abiotic stress, class II TGA factors,
45 detoxification, lipid signaling, reactive electrophile oxylipins

46 **Abbreviations:** AOS, allene oxide synthase; JA, jasmonic acid; JAZ, JASMONATE
47 ZIM-domain; OPDA, 12-oxo-phytodienoic acid; PGA₁, prostaglandin A₁; qPCR,
48 quantitative PCR; SA, salicylic acid

49 **Introduction**

50 Oxygenation of polyunsaturated fatty acids leads to the production of
51 oxylipins, like jasmonates and phytoprostanes, via enzymatic or non-enzymatic
52 pathways (Mueller, 2004; Wasternack, 2007). Exogenous application of jasmonic
53 acid (JA) inhibits mitosis, root growth and seed germination (Swiatek *et al.*, 2002).
54 Endogenous jasmonate biosynthesis is required for development of fertile flowers
55 (Sanders *et al.*, 2000). Jasmonates also control abiotic and biotic stress responses
56 with a concomitant induction of a variety of genes related to JA biosynthesis and
57 defense (Devoto *et al.*, 2005). Biological activities have also been reported for 12-
58 oxo-phytodienoic acid (OPDA), which is a precursor of JA biosynthesis. OPDA
59 inhibits root growth and mitosis similarly to JA but induces a different set of genes
60 (Mueller *et al.*, 2008; Taki *et al.*, 2005). Endogenous OPDA was recently shown to
61 impede seed germination independent of JA biosynthesis and signaling (Dave *et al.*,
62 2011; Dave and Graham, 2012). Mutants with defects in oxylipin biosynthesis,
63 signaling, and transport were used to establish the biological functions of both
64 compounds (Dave *et al.*, 2011; Malek *et al.*, 2002; McConn and Browse, 1996;
65 McConn *et al.*, 1997; Mene-Saffrane *et al.*, 2009; Park *et al.*, 2002; Stintzi and
66 Browse, 2000; Stotz *et al.*, 2011). Such studies demonstrated that jasmonates protect
67 plants against chewing insects (Howe *et al.*, 1996; McConn *et al.*, 1997; Pieterse *et al.*,
68 2012) and modulate host-pathogen interactions (Laurie-Berry *et al.*, 2006;
69 Pieterse *et al.*, 2012; Ton *et al.*, 2002). OPDA was shown to specifically protect
70 against necrotrophic pathogens not by its virtue of being a JA precursor (Raacke *et al.*,
71 2006; Stotz *et al.*, 2011).

72 Phytoprostanes are non-enzymatically formed compounds with structural
73 similarity to OPDA (Mueller, 2004). Similarly to JA and OPDA, these compounds
74 inhibit root growth and mitosis and induce the production of secondary metabolites
75 (Mueller *et al.*, 2008). The set of genes, which is induced by phytoprostanes, shows
76 a strong overlap to the OPDA-responsive genes and only a small overlap to JA-
77 induced genes. This can be explained by the presence of an α,β -unsaturated
78 carbonyl group in OPDA and phytoprostanes, which are electrophilic
79 cyclopentenones. In contrast, JA is a non-electrophilic and chemically unreactive
80 cyclopentanone. The α,β -unsaturated carbonyl group is the reason for the higher

81 chemical reactivity, which was suggested to be crucial for the biological activity
82 (Farmer and Davoine, 2007).

83 Recently, substantial progress has been made towards understanding the
84 signal transduction pathway mediating the response to jasmonates. JA-isoleucine
85 (JA-Ile), the biologically active form of JA, is bound to the F-box protein COI1 in
86 the presence of JASMONATE ZIM-domain (JAZ) protein family members (Chini *et*
87 *al.*, 2007; Sheard *et al.*, 2010; Thines *et al.*, 2007). JAZ proteins act as negative
88 regulators of jasmonate-responsive gene expression. Binding of JA-Ile leads to the
89 degradation of JAZ proteins, resulting in the release of transcription factors like
90 MYC2, which promote the expression of jasmonate-responsive genes (Chini *et al.*,
91 2007). MYC2 was identified via positional cloning of a jasmonate-insensitive *jini1*
92 mutant allele (Berger *et al.*, 1996); *JIN1* encodes the basic helix-loop-helix
93 transcription factor MYC2 (Lorenzo *et al.*, 2004).

94 In contrast to the jasmonate signal transduction pathway, only little is known
95 about the mechanism that mediates effects of OPDA and phytoprostanes. Putative
96 binding sites for TGA transcription factors are over-represented in promoters of
97 phytoprostane-responsive genes and specifically the TGA2, TGA5 and TGA6
98 factors were shown to regulate gene expression in response to cyclopentenone
99 oxylipins (Mueller *et al.*, 2008). Induction of 30% and 60% of the genes in response
100 to OPDA and the phytoprostane PPA₁, respectively, did not occur in the *tga2 tga5*
101 *tga6* mutant, which is defective in expression of all three TGA factor genes.
102 However, the participation of other TGA factors in responses to these
103 cyclopentenones has not been tested.

104 The primary aim of this study was to uncover signaling pathways that
105 mediate effects of reactive oxylipins on plant growth and stress responses, the
106 jasmonate receptor COI1 and TGA transcription factors being of particular interest.
107 With respect to stress responses, specific contributions of individual TGA factors to
108 OPDA-dependent gene expression were determined using the cytochrome P450 gene
109 *CYP81D11*, the regulation of which was further characterized recently (Köster *et al.*,
110 2012), the glutathione-S-transferase gene *GST25* and the OPDA reductase gene
111 *OPR1*.

112 **Materials and methods**

113 **Plant material and growth conditions**

114 The *jin1* and *coil-16* mutants together with their *Arabidopsis thaliana* (L.)
115 Heynh. background Col-*gl* were those originally reported (Berger *et al.*, 1996; Ellis
116 and Turner, 2002; Nickstadt *et al.*, 2004). The *dde2-2* mutant in the background of
117 ecotype Col-0 was previously published (Malek *et al.*, 2002). The *tga6*, *tga2 tga5*,
118 and *tga2 tga5 tga6* mutants as well as the *tga1 tga4* double mutant were those
119 originally described (Kesarwani *et al.*, 2007; Zhang *et al.*, 2003). All transgenic
120 lines overexpressing *TGA2*, *TGA5* or *TGA6* were received from Prof. Christiane
121 Gatz. In addition to the previously published lines *TGA2.1*, *TGA2.2*, *TGA5.1*,
122 *TGA5.2* and *TGA6.2* (Zander *et al.*, 2010), novel *TGA5* and *TGA6* lines were tested.
123 All *tga* mutant and *TGA*-overexpressing lines were generated in the background of
124 ecotype Col-0.

125 Seedlings were grown in liquid MS (Murashige & Skoog) medium
126 containing 1% or 2% sucrose or on MS agar plates as previously described (Mueller
127 *et al.*, 2008). Seedlings were grown with a 9 h light/15 h dark cycle at 22°C under
128 fluorescent light (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

129 **Chemical treatments**

130 Seedlings grown in liquid MS medium or on MS agar plates were treated
131 with OPDA synthesized by enzymatic conversion of linolenic acid using linseed
132 acetone powder (Parchmann *et al.*, 1997), JA (Sigma-Aldrich, St. Louis, MO), the
133 phytoprostane PPA₁ (Thoma *et al.*, 2003) or the prostaglandin PGA₁ (Cayman
134 Chemical, Ann Arbor, MI).

135 **Quantitative RT-PCR analysis**

136 Total RNA from was extracted from liquid-grown seedlings using the
137 E.Z.N.A. plant RNA kit (Omega Bio-Tek, Norcross, GA). Potential DNA
138 contamination was removed using on-column digestion with DNase I. Following
139 quantification using a ND-1000 UV-Vis Spectrophotometer (NanoDrop,
140 Wilmington, DE), 1 μg of total RNA was used for cDNA synthesis using M-MLV
141 RNase H minus reverse transcriptase (Promega, Madison, WI). Real-time PCR was
142 performed using a QPCR SYBR Green Mix (Thermo Scientific, Lafayette, CO).
143 Primers are listed in Supplementary Table S1 except for *OPRI* and *Act2/8*, which

144 were already published (Ellinger *et al.*, 2010; Mueller *et al.*, 2008). Reactions were
145 performed on a Mastercycler Realplex (Eppendorf, Wesseling-Berzdorf, Germany)
146 or on a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA) with 40
147 cycles of denaturation for 15 sec at 95°C, annealing for 20 sec at 55°C, and extension
148 for 20 sec at 72°C. This program was followed by a melting curve analysis. Purified
149 RT-PCR products were used for calibration using the Relative Standard Curve
150 Method (Applied Biosystems, Carlsbad, CA). Three biological replicates were
151 used for each data point.

152 **Statistical analysis**

153 ANOVA was used for statistical analysis of root growth measurements.
154 Levene's test was used to determine homogeneity of variances. Data were
155 transformed to achieve homogeneous variances. Alternatively, data were analyzed
156 using nonparametric statistics. Two-tailed tests were used with $\alpha < 0.05$. The
157 Relative Expression Software Tool V2.0.13 (Qiagen, Hilden, Germany) was used to
158 determine the significance of pairwise comparisons of quantitative PCR data.

159 **Results**

160 **Inhibition of root growth by phytoprostanes is dependent on COI1 but** 161 **independent of jasmonate biosynthesis**

162 An effect shared by jasmonates and phytoprostanes is the inhibition of root
163 growth, which was previously measured in wild-type *A. thaliana* seedlings after
164 treatment with OPDA or PPA₁ (Mueller *et al.*, 2008). COI1 is known to mediate
165 inhibition of root growth in response to exogenous JA or JA methyl ester. To test
166 whether inhibition of root growth in response to phytoprostanes is also COI1-
167 dependent, the response of the *coi1* mutant was analyzed. Root length of *coi1*
168 seedlings on medium containing 25 μ M JA, OPDA or PPA₁ was similar to the
169 control grown on MS medium without the addition of oxylipins (Fig. 1A). This
170 demonstrates that inhibition of root growth by OPDA or phytoprostanes is dependent
171 on COI1. In addition, this result shows that growth inhibition is not based on a toxic
172 effect of cyclopentenones but on signaling processes.

173 It is not clear whether OPDA exerts the observed effect directly or indirectly
174 via JA biosynthesis because the *coi1* mutant can convert OPDA to JA. So far, COI1

175 has only been shown to bind amino acid conjugates of JA and coronatine (Katsir *et*
176 *al.*, 2008; Thines *et al.*, 2007). This raises the question whether JA-Ile mediates the
177 effect of PPA₁. To investigate the possibility that an accumulation of JA-Ile upon
178 PPA₁ treatment is responsible for the inhibition of root growth, the *dde2* mutant was
179 tested. This mutant contains a knockout allele of the allene oxide synthase (AOS)
180 gene (Malek *et al.*, 2002). As a result, the *dde2* mutant no longer produces OPDA,
181 JA, and JA-Ile (Köster *et al.*, 2012). Inhibition of root growth in the *dde2* mutant in
182 response to phytoprostane treatment was similar to the root growth inhibition
183 observed in the wild type (Table 1). This clearly shows that the inhibitory effect of
184 phytoprostanes on root growth is not mediated through OPDA or JA-Ile. These data
185 also demonstrate that COI1 plays an important role in mediating root growth
186 inhibitory effects of oxylipins other than jasmonates.

187 As mentioned above, induction of gene expression in response to
188 cyclopentenones is impaired in the *tga2 tga5 tga6* mutant. It was therefore
189 investigated whether this mutant is also insensitive to oxylipin-triggered inhibition of
190 root growth. On control medium without oxylipins, roots of the *tga2 tga5 tga6*
191 mutant were considerably shorter (54%) than wild type roots ($F_{1,132} = 230.6$, $P <$
192 0.001). Oxylipins strongly inhibited root growth. Root growth of the *tga2 tga5 tga6*
193 mutant was more sensitive to the presence of PPA₁ ($F_{1,198} = 42.4$, $P < 0.001$) and JA
194 ($F_{1,208} = 5.3$, $P = 0.023$) than wild-type roots (Fig. 1B). The difference in genotype-
195 dependent inhibition of root growth by OPDA was not significantly different. Root
196 lengths of the triple mutant were reduced to 15, 21 and 26% relative to the lengths
197 on control medium in the presence of PPA₁, OPDA and JA, respectively;
198 corresponding relative root lengths in the wild type were 56, 27 and 35%. These
199 data illustrate that the transcription factors TGA2, TGA5 and TGA6 are not required
200 for root growth inhibition in response to oxylipins. Instead, the *tga2 tga5 tga6*
201 mutant was particularly hypersensitive to PPA₁.

202 Root growth was also analyzed in *tga1 tga4*, a double mutant defective in
203 expression of TGA1 and TGA4, which represents a different class of TGA factors.
204 In contrast to the *tga2 tga5 tga6* mutant, growth phenotypes of the *tga1 tga4* mutant
205 were identical to wild type on control medium and on medium containing JA, OPDA
206 and PPA₁ (Fig. 1C). This shows that TGA1 and TGA4 are not involved in regulating
207 root growth in response to oxylipins.

208 **Regulation of phytoprostane-responsive genes is dependent on class II TGA**
209 **factors but not on COI1 and MYC2**

210 The results on COI1-dependent inhibition of root growth by phytoprostanes
211 prompted us to also investigate whether induction of phytoprostane-responsive genes
212 is dependent on COI1. A limited analysis of this latter oxylipin response was
213 previously documented in *coi1* mutant and wild type plants using northern
214 hybridization with two probes, one for the cytochrome P450 gene *CYP81D11*, which
215 responds to diverse stimuli (Köster *et al.*, 2012; Matthes *et al.*, 2010; Mueller *et al.*,
216 2008), and the other one for the OPDA reductase genes *OPR1/2*, which are
217 phytoprostane-responsive but also up-regulated after OPDA and JA treatment
218 (Mueller *et al.*, 2008). To challenge these previous findings, a more comprehensive
219 analysis was performed using an independent method. Quantitative RT-PCR
220 analysis of the above mentioned genes as well as the glutathione-S-transferase genes
221 *GST6* and *GST25*, which are related to detoxification, and the *TolB*-like gene was
222 performed; all three genes are phytoprostane-responsive; *GST6* and *TolB*-like genes
223 also show some up-regulation after OPDA treatment (Mueller *et al.*, 2008). To
224 discriminate effects of different classes of oxylipins, the MYC2 transcription factor
225 mutant *jin1* and expression of the vegetative storage protein gene *VSP1*, which is not
226 responsive to phytoprostanes but shows COI1-dependent induction after JA
227 treatment, were tested.

228 Relative to wild type, induction of all tested phytoprostane-responsive genes
229 by PPA₁ or OPDA was not reduced in the *jin1* and *coi1* mutants (Fig. 2). The trend
230 of the previously reported reduced induction of *CYP81D11* in the *coi1* mutant by
231 reactive oxylipins (Mueller *et al.*, 2008) was confirmed; methodological differences
232 are likely responsible for quantitative differences between northern hybridization and
233 quantitative RT-PCR because *CYP81D11* belongs to a gene family with 15 members
234 (Bak *et al.*, 2011). Up-regulation of *VSP1* and *CYP81D11* after JA treatment was
235 clearly reduced in both mutants. Reduction of *VSP1* induction was stronger in the
236 *coi1* mutant than in the *jin1* mutant, which is in agreement with published data
237 (Benedetti *et al.*, 1995; Berger *et al.*, 1996). The *jin1* mutant has a small effect on
238 *VSP1* expression because MYC2 acts in concert with MYC3 and MYC4 to regulate
239 the expression of *VSP1* (Fernandez-Calvo *et al.*, 2011). Together, these data show

240 that, in contrast to inhibition of root growth, induction of the tested phytoprostane-
241 responsive genes is not dependent on CO11.

242 It was previously shown by microarray and northern analysis that induction
243 of *CYP81D11* and *OPR1/2* genes by oxylipins is reduced in the *tga2 tga5 tga6*
244 mutant (Mueller *et al.*, 2008). To compare the response of the triple mutant to
245 exogenous JA and reactive oxylipins, target gene expression was analyzed by
246 quantitative RT-PCR. To determine whether class II TGA factors specifically
247 regulate oxylipin-induced gene expression, the class I TGA factor mutant *tga1 tga4*
248 was tested.

249 The *tga2 tga5 tga6* mutant exhibited lower induction of *CYP81D11*, *GST25*,
250 *OPR1* and *TolB*-like by PPA₁ and OPDA in comparison to the wild type.
251 Expression of *GST6* showed a tendency to lower induction than in wild type,
252 especially after treatment with OPDA (Fig. 3). These results are consistent with
253 published data on *CYP81D11*, *OPR1*, *TolB*-like, and *GST6* expression (Mueller *et*
254 *al.*, 2008). In addition, the induction of all tested genes by JA was lower relative to
255 wild type. This result confirms the previous conception that, besides their
256 involvement in responses to OPDA and phytoprostanes, TGA2, TGA5 and TGA6
257 mediate responses to exogenous JA (Köster *et al.*, 2012; Mueller *et al.*, 2008). In
258 contrast to the triple mutant, induction of all tested genes was not reduced in the *tga1*
259 *tga4* mutant. This suggests that TGA1 and TGA4 are not necessary for oxylipin
260 responses.

261 **Differential regulation of phytoprostane-responsive genes in *tga6*, *tga2 tga5*, and** 262 ***tga2 tga5 tga6* mutants**

263 To test the individual contributions of TGA2, TGA5, and TGA6 to
264 cyclopentenone-regulated *CYP81D11*, *OPR1* and *GST25* expression, *tga6*, *tga2*
265 *tga5*, and *tga2 tga5 tga6* mutants were used. In addition to OPDA, *A. thaliana*
266 seedlings grown in MS medium were challenged with prostaglandin A₁ (PGA₁), a
267 commercially available and structurally related cyclopentenone, which was
268 previously shown to covalently bind to AtGST6 (Dueckershoff *et al.*, 2008).

269 *CYP81D11* was induced 60- to 70-fold after treatment of wild-type seedlings
270 for 4 h with OPDA or PGA₁ (Fig. 4). *CYP81D11* reached more than 70% of the
271 wild-type induction level in the *tga6* mutant irrespective of the stimulus, suggesting

272 that the absence of TGA6 does not have a significant effect on cyclopentenone-
273 induced expression of this gene. Basal *CYP81D11* levels did not differ between the
274 *tga6* mutant and wild type, but basal expression levels were reduced >4-fold in the
275 *tga2 tga5* and *tga2 tga5 tga6* mutants. Both OPDA- and PGA₁-stimulated
276 expression of *CYP81D11* was significantly reduced in the *tga2 tga5* double mutant,
277 reaching less than 20% of induced wild-type levels. A further reduction in oxylin-
278 induced *CYP81D11* expression occurred in the *tga2 tga5 tga6* mutant, reaching less
279 than 3% of wild type expression, which was not significantly different from
280 uninduced wild-type levels. TGA6 therefore exerts a significant effect on
281 *CYP81D11* expression in the absence but not in the presence of TGA2 and TGA5.

282 *OPR1* expression increased 10- and 21-fold after treatment of wild-type
283 seedlings with OPDA and PGA₁, respectively (Fig. 4). Basal *OPR1* levels did not
284 vary much between mutant and wild-type seedlings. In the *tga6* mutant, expression
285 of *OPR1* reached only 46% and 26% of wild-type levels after induction with OPDA
286 and PGA₁, respectively. The response to PGA₁ was significantly reduced, indicating
287 that TGA6 plays an essential role in *OPR1* induction. Up-regulation of *OPR1* by
288 OPDA reached 26% of wild-type levels in the *tga2 tga5* mutant. Induction of *OPR1*
289 by PGA₁ was significantly less in the *tga2 tga5* mutant, reaching only 10% of wild-
290 type levels. OPDA- and PGA₁-responsive expression of *OPR1* was further
291 decreased in the *tga2 tga5 tga6* mutant.

292 *GST25* was induced 16- and 5-fold after treatment of wild-type plants with
293 OPDA and PGA₁, respectively (Fig. 4). *GST25* expression reached 57% and 45% of
294 wild-type levels in the *tga6* mutant after induction with OPDA and PGA₁,
295 respectively. Cyclopentenone-induced *GST25* expression levels were very similar in
296 the *tga6* and *tga2 tga5* mutant, suggesting that induced *GST25* expression is
297 regulated similarly by TGA2 and TGA5 and by TGA6. The induction level in the
298 *tga2 tga5 tga6* mutant was below 3% relative to wild type and did not differ from
299 uninduced wild-type levels. Quantitative differences in *GST25* or *OPR1* induction
300 levels among experiments (as compared to Fig. 2 and 3) are likely attributed to
301 subtle changes in plant growth conditions.

302 **Separate effects of three TGA factors on OPDA-induced gene expression**

303 To further examine the contribution of individual TGA factors to OPDA-
304 induced gene expression, *TGA2*-, *TGA5*-, or *TGA6*-overexpressing *A. thaliana* lines
305 (Zander *et al.*, 2010) were used. TGA protein expression was readily detected in
306 crude extracts from overexpressing plants (Supplementary Fig. S1). TGA protein
307 expression varied among overexpressing lines but did not substantially alter the
308 induction of target gene expression (Supplementary Fig. S2 and S3).

309 OPDA treatment of wild-type seedlings increased *CYP81D11* expression 93-
310 fold (Fig. 5). This level of induction was consistent across experiments in the wild-
311 type background Col-0 (Fig. 3 and 4), but induction of *CYP81D11* appeared to be
312 quantitatively lower in the genotype Col-*gl* (Fig. 2). No induction of *CYP81D11* by
313 OPDA was observed in the *tga2 tga5 tga6* mutant, which served as the genetic
314 background for all three lines overexpressing TGA factors. *CYP81D11* expression
315 was significantly increased after OPDA treatment of *TGA2.1*- and *TGA5.1*-
316 overexpressing lines by 46% and 23% of wild-type levels, respectively. However,
317 OPDA induction of *CYP81D11* was not significant in the *TGA6.3*-overexpressing
318 line, reaching only 12% of wild-type levels. These results support the *tga* mutant
319 data (Fig. 4) and demonstrate that TGA6 is not sufficient for induced *CYP81D11*
320 expression.

321 Effects of *TGA2.1*, *TGA5.1* and *TGA6.3* overexpression on OPDA-induced
322 expression of *OPRI* and *GST25* were similar and distinct from *CYP81D11*.
323 Overexpression of each of the three transcription factors overcame the lack of *OPRI*
324 and *GST25* induction after OPDA treatment in the *tga2 tga5 tga6* mutant. Although
325 TGA2 made a significant contribution to OPDA-induced expression of *OPRI* and
326 *GST25*, the effects of TGA5 and TGA6 were quantitatively larger.

327 Based on data from both mutant and transgenic seedlings, the response of
328 *CYP81D11* to OPDA is regulated directly or indirectly by TGA2 and TGA5. In
329 contrast, TGA5 and TGA6 make a quantitatively larger contribution to OPDA-
330 induced expression of *OPRI* and *GST25* than TGA2. These data suggest that at least
331 two classes of OPDA-regulated genes exist.

332 Discussion

333 **COI1 mediates root growth inhibition in response to phytoprostanes**
334 **independent of jasmonates**

335 Whereas root growth was not inhibited by JA, OPDA or PPA₁ in the *coi1* mutant
336 (Fig. 1A), the AOS mutant *dde2* was fully sensitive to phytoprostane treatment
337 (Table 1). This finding illustrates that root growth in this JA- and OPDA-deficient
338 mutant is dependent on COI1 and that COI1 mediates jasmonate-independent
339 responses to an electrophilic oxylipin. While similar JA-Ile-independent COI1-
340 mediated responses were previously documented (Adams and Turner, 2010; Köster
341 *et al.*, 2012; Ralhan *et al.*, 2012; Ribot *et al.*, 2008; Stotz *et al.*, 2011), the underlying
342 mechanism has not been resolved. Based on these published results, apparently two
343 jasmonate-independent COI1 pathways exist. Unlike the *opr3* mutant, *aos* and *coi1*
344 mutants are impaired in defense responses against the necrotrophic ascomycete
345 *Sclerotinia sclerotiorum* (Stotz *et al.*, 2011) and during wound-induced expression of
346 *AtPHO1;H10* (Ribot *et al.*, 2008), suggesting that OPDA mediates JA-Ile-
347 independent COI1 responses. On the other hand, ethylene-dependent inhibition of
348 root growth (Adams and Turner, 2010), susceptibility to *Verticillium longisporum*
349 (Ralhan *et al.*, 2012) and induction of *CYP81D11* in response to xenobiotics (Köster
350 *et al.*, 2012) are altered in the *coi1* but not in the *aos* mutant, suggesting that in this
351 case COI1 exerts its effects independently of OPDA. Elegant grafting experiments
352 showed that susceptibility to *V. longisporum* is dependent on a COI1-specific
353 recognition event in the root (Ralhan *et al.*, 2012), suggesting that this organ may
354 also play a role in mediating oxylipin responses. In analogy, we now show that the
355 phytoprostane PPA₁ signals through COI1 independently of OPDA and JA
356 biosynthesis.

357 COI1 interacts with JAZ1, JAZ3, JAZ6, JAZ9 and JAZ10 in a JA-Ile- and
358 coronatine-dependent manner (Chung and Howe, 2009; Melotto *et al.*, 2008; Sheard
359 *et al.*, 2010). Although OPDA does not facilitate interactions of COI1 with JAZ1,
360 JAZ3 and JAZ9 (Chung and Howe, 2009; Melotto *et al.*, 2008), the possibility
361 cannot be excluded that cyclopentenones may promote interactions between COI1
362 and other JAZ proteins. JA-Ile induces 10 of the 12 JAZ family members as part of
363 a negative feedback loop (Chini *et al.*, 2007). Analysis of transcript profiling in
364 response to the phytoprostane PPA₁ (Mueller *et al.*, 2008) did not indicate regulation
365 of JAZ genes by this compound. Alternatively, binding of phytoprostanes to COI1
366 may facilitate interactions with other proteins that are not related to JAZ proteins but
367 nevertheless act as co-receptors of COI1.

368 **TGA factors 2, 5 and 6 activate oxylipin-responsive gene expression but impede**
369 **inhibition of root growth by oxylipins**

370 The TGA factors 2, 5 and 6 were shown to act as redundant members of the
371 class II TGA factors during the establishment of systemic acquired resistance, which
372 is regulated by the salicylic acid (SA) pathway (Zhang *et al.*, 2003). In addition,
373 these transcription factors are involved in regulating gene expression in response to
374 the jasmonate/ethylene pathway (Zander *et al.*, 2010). This pathway is important for
375 resistance to necrotrophic pathogens and the *tga2 tga5 tga6* mutant is more
376 susceptible to *Botrytis cinerea* than wild-type plants (Zander *et al.*, 2010). A
377 possible explanation for this hypersusceptibility is perhaps reduced
378 jasmonate/ethylene signaling and a strongly reduced expression of genes related to
379 detoxification (Mueller *et al.*, 2008), leading to a reduced and slower metabolism of
380 phytoprostanes and other toxic compounds. This is supported by results showing
381 that in the *tga2 tga5 tga6* mutant cell death is elevated after treatment with tert-butyl
382 hydroperoxide (Supplementary Fig. S4) and that sensitivity to xenobiotics is
383 increased relative to wild type (Fode *et al.*, 2008). Collectively, these data suggest
384 that these three TGA factors play an important role in detoxification responses of
385 plants.

386 The fact that the *tga2 tga5 tga6* mutant still responded to oxylipins with a
387 reduction in root growth (Fig. 1) suggests that this response is not dependent on
388 these transcription factors. Although the growth of the triple mutant was reduced on
389 MS agar medium relative to wild type, inhibition of root growth by PPA₁ was
390 quantitatively larger in the *tga2 tga5 tga6* mutant than in the wild type. The
391 hypersensitivity of the triple mutant to a phytoprostane seems to support the
392 proposed antagonism between these three TGA factors and MYC2 affecting ORA59
393 expression and jasmonate/ethylene-related gene expression (Zander *et al.*, 2010).

394 **TGA-specific regulation of phytoprostane-responsive target genes**

395 The putative detoxification genes *CYP81D11*, *OPR1*, and *GST25* responded
396 differently to TGA2, TGA5 and TGA6. *CYP81D11* varied from *GST25* and *OPR1*
397 in the level of induction by cyclopentenones but also in the specificity of induction
398 by different TGA factors. Cyclopentenone-induced expression of *CYP81D11* was
399 more strongly regulated by TGA2 and TGA5 than by TGA6 (Fig. 4 and 5). At the

400 most, overexpression of TGA factors resulted in an OPDA induction of ~50%
401 relative to wild-type levels (Fig. 5). Thus, overexpression of single TGA factors
402 results in partial induction of *CYP81D11* expression, raising the possibility that TGA
403 factors may become limiting due to the heterodimerization requirements of these
404 transcription factors. In contrast, overexpression of TGA5 or TGA6 in the
405 background of the *tga2 tga5 tga6* mutant resulted in wild-type levels of *GST25* and
406 *OPR1* expression after OPDA treatment (Fig. 5), suggesting that individual TGA
407 factors can be sufficient for the induction of these genes. These results show that
408 control of gene expression by TGA factors varies among target genes. In contrast to
409 the results presented here, SA-induced expression of *PR1* is blocked in the *tga2 tga5*
410 *tga6* mutant, but wild-type induction levels are reached in *tga6* and *tga2 tga5*
411 mutants, which demonstrates transcription factor redundancy with respect to *PR1*
412 expression (Zhang *et al.*, 2003). On the other hand, expression of *PDF1.2* after
413 induction with methyl-JA and ACC is similar in wild-type and *tga6* mutant plants,
414 whereas stimulus-induced expression is equally low in *tga2 tga5* and *tga2 tga5 tga6*
415 mutants (Zander *et al.*, 2010). Thus, expression of *PDF1.2* under these conditions is
416 strictly dependent on TGA2 and TGA5. However, TGA factors indirectly regulate
417 *PDF1.2* expression (Zander *et al.*, 2010).

418 Unlike *GST25*, which is exclusively regulated by TGA2, TGA5 and TGA6,
419 *CYP81D11* was recently shown to be co-regulated by these TGA factors and COI1
420 (Köster *et al.*, 2012). Sequence analysis of the *OPR1* promoter provides no evidence
421 for the presence of a MYC2-responsive G-box, also suggesting a fundamental
422 difference in regulation of *CYP81D11* versus *GST25* and *OPR1* genes.

423 **Contrast of the responses to COI1 or TGA2, TGA5 and TGA6**

424 COI1 as well as TGA2, TGA5 and TGA6 induce related but distinct defense
425 responses. For instance, susceptibilities of both *coil* and *tga2 tga5 tga6* mutants to
426 *B. cinerea* are elevated relative to wild type (Thomma *et al.*, 1998; Zander *et al.*,
427 2010). Likewise, induction of *PDF1.2* expression after *B. cinerea* inoculation is
428 severely reduced in both types of mutants (Guo and Stotz, 2007; Zander *et al.*,
429 2010). However, *coil* and *tga2 tga5 tga6* mutants differ in cis-jasmone-responsive
430 gene expression patterns (Matthes *et al.*, 2010), demonstrating clear differences in
431 these signal transduction pathways. This is not surprising because class II TGA
432 factors were shown to indirectly activate the jasmonate/ethylene pathway that is

433 controlled by COI1 (Zander *et al.*, 2010). Given that COI1 also fulfills distinct roles
434 in regulation of responses to JA and to pathogens via combinatorial
435 jasmonate/ethylene signaling, differences in observed physiological (Fig. 1) and
436 defense responses (Fig. 2 and 3) can be reconciled.

437 Whereas PPA₁ activates the expression of stress and detoxification genes,
438 this compound down-regulates the expression of genes that contribute to cell growth
439 and division (Mueller *et al.*, 2008), which may explain the fact that roots respond to
440 phytoprostanes with growth inhibition (Fig. 1). Moreover, root growth inhibition in
441 response to phytoprostanes is lessened by TGA2, TGA5 and TGA6 possibly because
442 these proteins may influence the repression of gene expression associated with
443 growth and division. In contrast, COI1 exerts a negative effect on root growth in
444 response to cyclopentenones, although this receptor is only known to bind JA-Ile and
445 coronatine.

446 Collectively, these data strongly suggest the existence of two phytoprostane
447 signaling pathways (Fig. 6). One pathway regulates the expression of detoxification
448 genes and is influenced positively by both COI1 and class II TGA factors. The
449 second pathway inhibits root growth, which is mediated by COI1 but negatively
450 influenced by the TGA factors. This proposed model can be reconciled with a
451 previously published model on the antagonism between class II TGA factors and
452 MYC2 (Zander *et al.*, 2010).

453 **Acknowledgements**

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Table 1. Oxylin-mediated root growth inhibition in the allene oxide synthase mutant *dde2* and wild-type (Col-0) *A. thaliana*.

	Col-0			<i>dde2</i>		
	Control	25 μ M JA	25 μ M PPA ₁	Control	25 μ M JA	25 μ M PPA ₁
Length (mm)	21.9 \pm 1.8	6.7 \pm 1.8	10.2 \pm 1.7	24.4 \pm 1.9	7.2 \pm 1.7	12.2 \pm 1.8
% Length	100	31	47	100	30	50

Sterilized seeds of Col-0 and *dde2-2* were grown on vertically oriented square Petri dishes containing MS medium supplemented with 2 % (w/v) sucrose and oxylin in a final concentration of 25 μ M. Control treatments contained the solvent methanol (<2%). Root length was determined after 7 d. Shown are means and \pm 95% confidence intervals of 14 to 16 seedlings. Mann-Whitney U tests revealed no significant effect of genotypes on treatment ($P \leq 0.129$).

Figure 1: Inhibition of root growth by oxylipins in different mutants. Seedlings of *coil-16* (A), *tga2 tga5 tga6* (B) and *tga1 tga4* (C) were grown together with their corresponding wild types on vertically oriented MS agar plates containing phytoprostane A₁ (PPA₁), 12-oxo phytodienoic acid (OPDA), jasmonic acid (JA) in a final concentration of 25 μM, or the solvent <2% methanol (control or Cont.). Root lengths were measured after 8 d of growth. Shown are means of 20 seedlings ± 95% confidence intervals. Letters indicate significant differences among means. Independent experiments (six for *tga2 tga5 tga6*, four for *coil* and *tga1 tga4*) were performed with similar results.

Figure 2. Expression of oxylipin-responsive genes in the wild type and in mutants of the jasmonate pathway, *coil* (left column) and *jin1* (right column). Seedlings were grown for 10 days in MS medium containing 2% sucrose under short day conditions. The medium was exchanged for 75 μM phytoprostane A₁ (PPA₁), 75 μM 12-oxo phytodienoic acid (OPDA), 75 μM jasmonic acid (JA), or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the glutathione-S-transferase genes *GST6* and *GST25*, the OPDA reductase gene *OPR1*, the *TolB*-like gene and the gene encoding vegetative storage protein1 *VSP1* are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild-type control treatment was set to 1 and all other data was expressed relative to it. Presented are means and standard deviations of three independent experiments with different biological replicates.

Figure 3. Expression of oxylipin-responsive genes in the wild type and in *tga2 tga5 tga6* (left column) and *tga1 tga4* mutants (right column). Seedlings were grown for 10 days in MS medium containing 2% sucrose under short day conditions. The medium was exchanged for 75 μM phytoprostane A₁ (PPA₁), 75 μM 12-oxo phytodienoic acid (OPDA), 75 μM jasmonic acid (JA), or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the glutathione-S-transferase genes *GST6* and *GST25*, the OPDA reductase gene *OPR1* and the *TolB*-like gene are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control.

Expression of the wild type control treatment was set to 1 and all other data was expressed relative to it. Presented are means and standard deviations of three independent experiments with different biological replicates.

Figure 4. Expression of oxylipin-responsive genes in wild type and *tga* mutants. Seedlings were grown for 10 days in MS medium containing 1% sucrose under short day conditions. The medium was exchanged for 75 μ M 12-oxo phytodienoic acid (OPDA), 75 μ M prostaglandin A₁ (PGA₁), or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the OPDA reductase gene *OPRI*, and the glutathione-S-transferase gene *GST25* are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild type control treatment was set to 1 and all other data was expressed relative to it. Means and standard errors of three biological replicates are shown. Significant differences among means indicated by letters were determined using the Relative Expression Software Tool V2.0.13 (Qiagen, Hilden, Germany).

Figure 5. Expression of oxylipin-responsive genes in wild-type, *tga2 tga5 tga6* mutant, and *TGA*-overexpressing plants. *TGA* overexpression occurred in the background of the *tga2 tga5 tga6* mutant. Seedlings were grown for 10 days in MS medium containing 1% sucrose under short day conditions. The medium was exchanged for 75 μ M 12-oxo phytodienoic acid (OPDA) or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the OPDA reductase gene *OPRI*, and the glutathione-S-transferase gene *GST25* are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild type control treatment was set to 1 and all other data was expressed relative to it. Means and standard errors of three biological replicates are shown. Significant differences among means indicated by letters were determined using the Relative Expression Software Tool V2.0.13 (Qiagen, Hilden, Germany).

Figure 6. Tentative model explaining the observed effects of cyclopentenone oxylipins on root growth and expression of detoxification genes. Roles of the jasmonate receptor COI1 and TGA transcription factors in mediating oxylipin

signaling are highlighted. Individual contributions of TGA factors were determined for induction of detoxification genes but not for root growth inhibition. The *CYP81D11* promoter is primarily regulated by TGA2 and TGA5. *GST25* and *OPR1* promoters are primarily regulated by TGA5 and TGA6, although TGA2 does also contribute to the expression of these genes. MYC2 was previously shown to activate *CYP81D11* expression (Köster *et al.*, 2012) presumably by binding to a G-box in the promoter sequence.

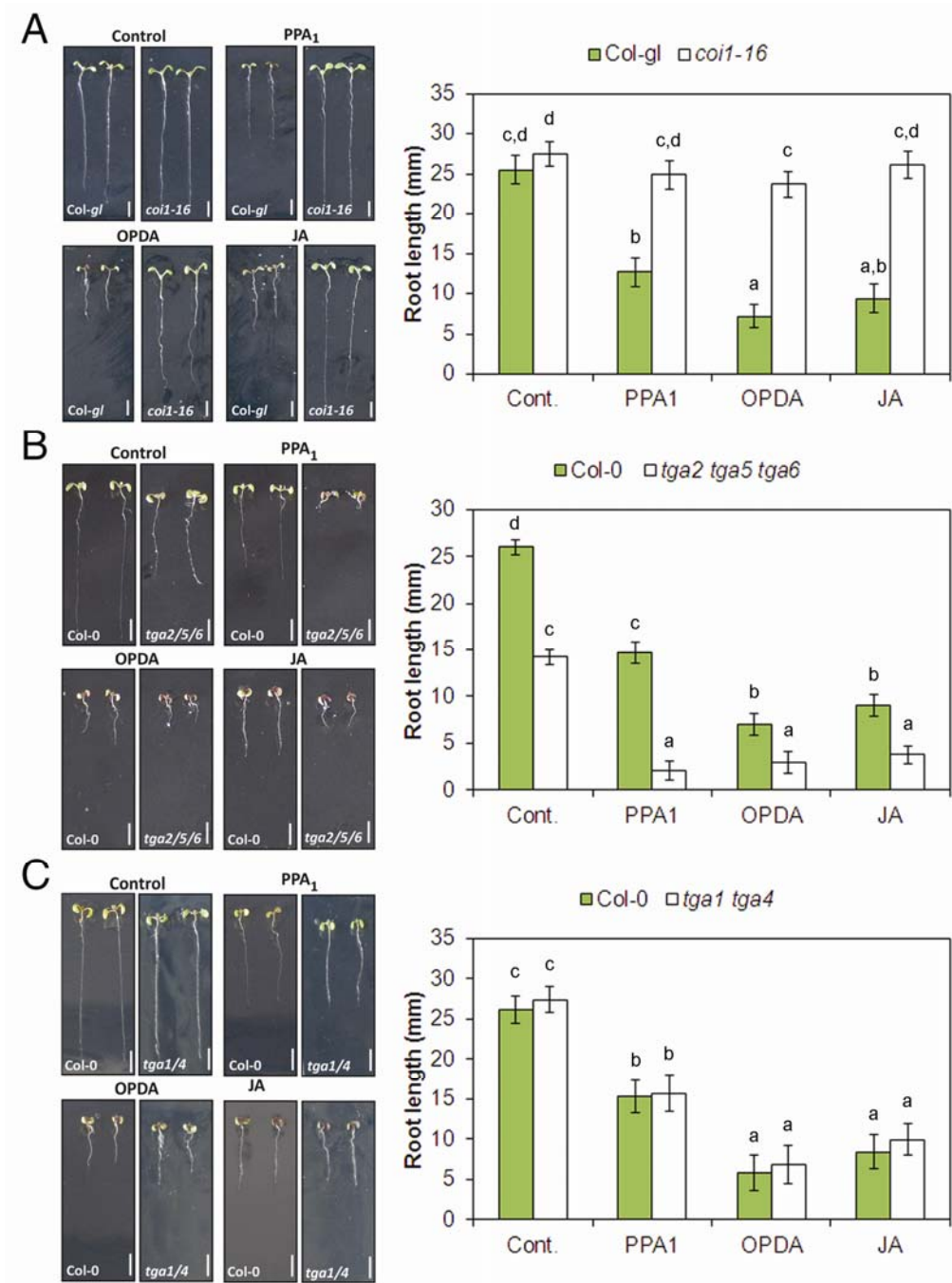


Figure 1: Inhibition of root growth by oxylipins in different mutants. Seedlings of *coi1-16* (A), *tga2 tga5 tga6* (B) and *tga1 tga4* (C) were grown together with their corresponding wild types on vertically oriented MS agar plates containing phytoprostane A₁ (PPA₁), 12-oxo phytodienoic acid (OPDA), jasmonic acid (JA) in a final concentration of 25 μ M, or the solvent <2% methanol (control or Cont.). Root lengths were measured after 8 d of growth. Shown are means of 20 seedlings \pm 95% confidence intervals. Letters indicate significant differences among means. Independent experiments (six for *tga2 tga5 tga6*, four for *coi1* and *tga1 tga4*) were performed with similar results.

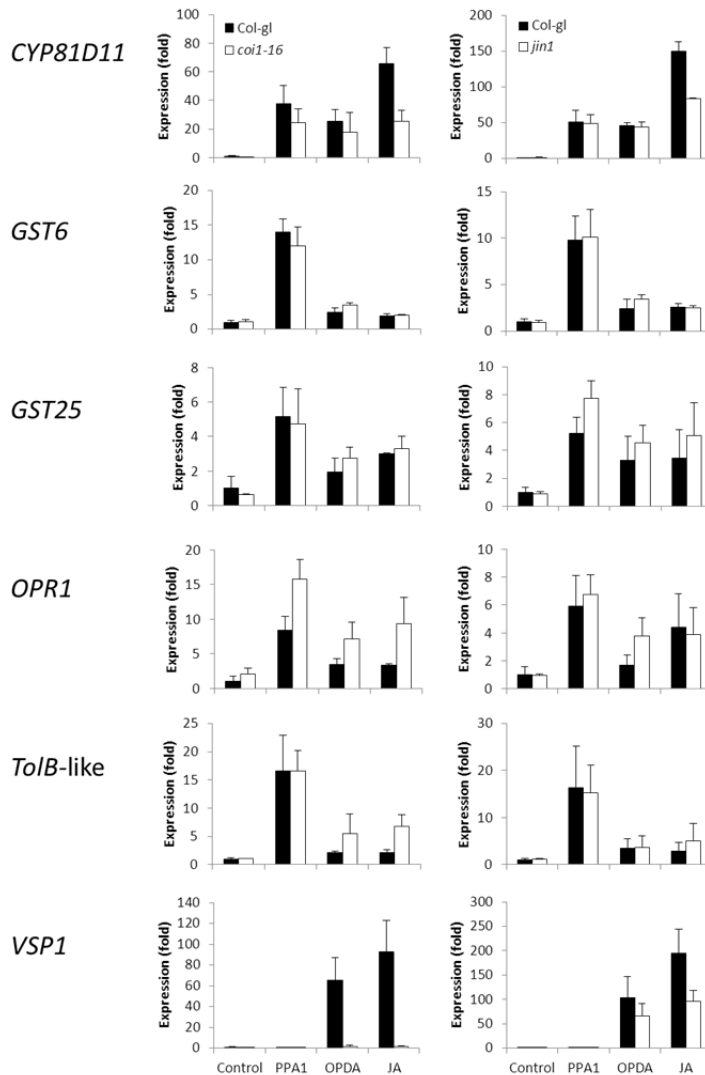


Figure 2. Expression of oxylinpin-responsive genes in the wild type and in mutants of the jasmonate pathway, *coi1* (left column) and *jin1* (right column). Seedlings were grown for 10 days in MS medium containing 2% sucrose under short day conditions. The medium was exchanged for 75 μ M phytoprostane A₁ (PPA₁), 75 μ M 12-oxo phytodienoic acid (OPDA), 75 μ M jasmonic acid (JA), or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the glutathione-S-transferase genes *GST6* and *GST25*, the OPDA reductase gene *OPR1*, the *TolB*-like gene and the gene encoding vegetative storage protein1 *VSP1* are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild-type control treatment was set to 1 and all other data was expressed relative to it. Presented are means and standard deviations of three independent experiments with different biological replicates.

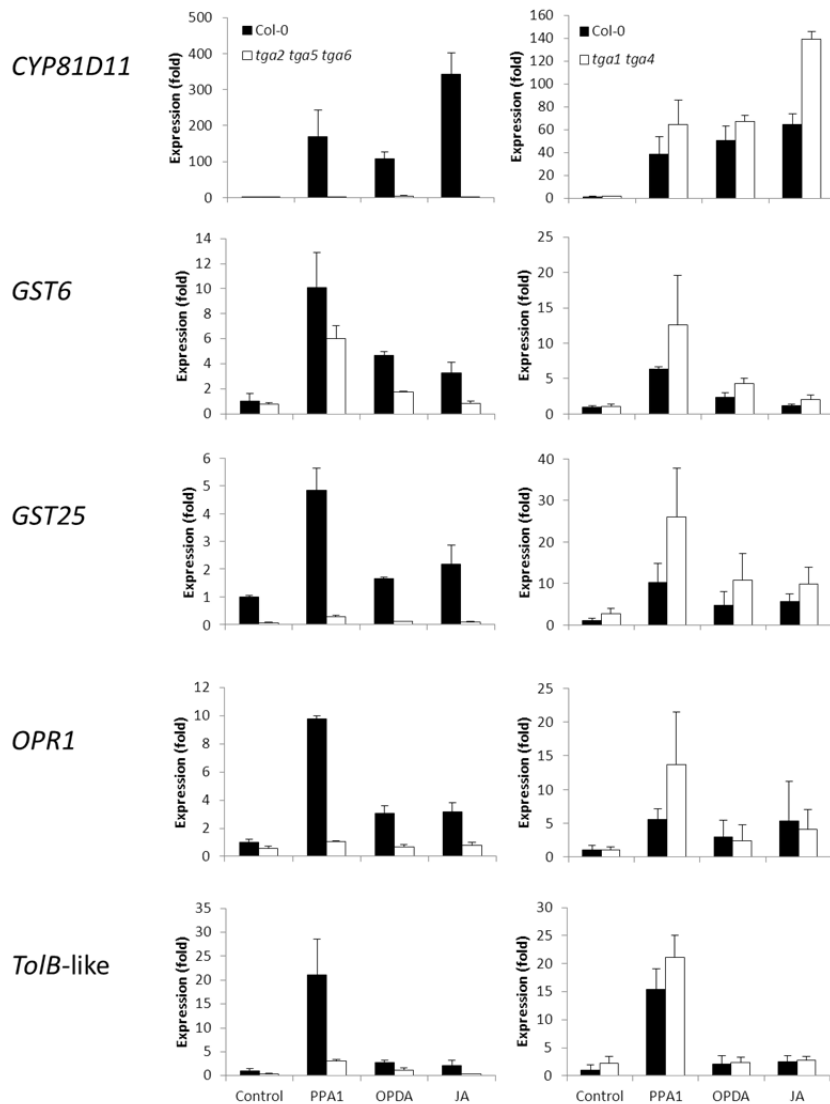


Figure 3. Expression of oxylipin-responsive genes in the wild type and in *tga2 tga5 tga6* (left column) and *tga1 tga4* mutants (right column). Seedlings were grown for 10 days in MS medium containing 2% sucrose under short day conditions. The medium was exchanged for 75 μ M phytoprostane A₁ (PPA₁), 75 μ M 12-oxo phytodienoic acid (OPDA), 75 μ M jasmonic acid (JA), or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the glutathione-S-transferase genes *GST6* and *GST25*, the OPDA reductase gene *OPR1* and the *TolB*-like gene are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild type control treatment was set to 1 and all other data was expressed relative to it. Presented are means and standard deviations of three independent experiments with different biological replicates.

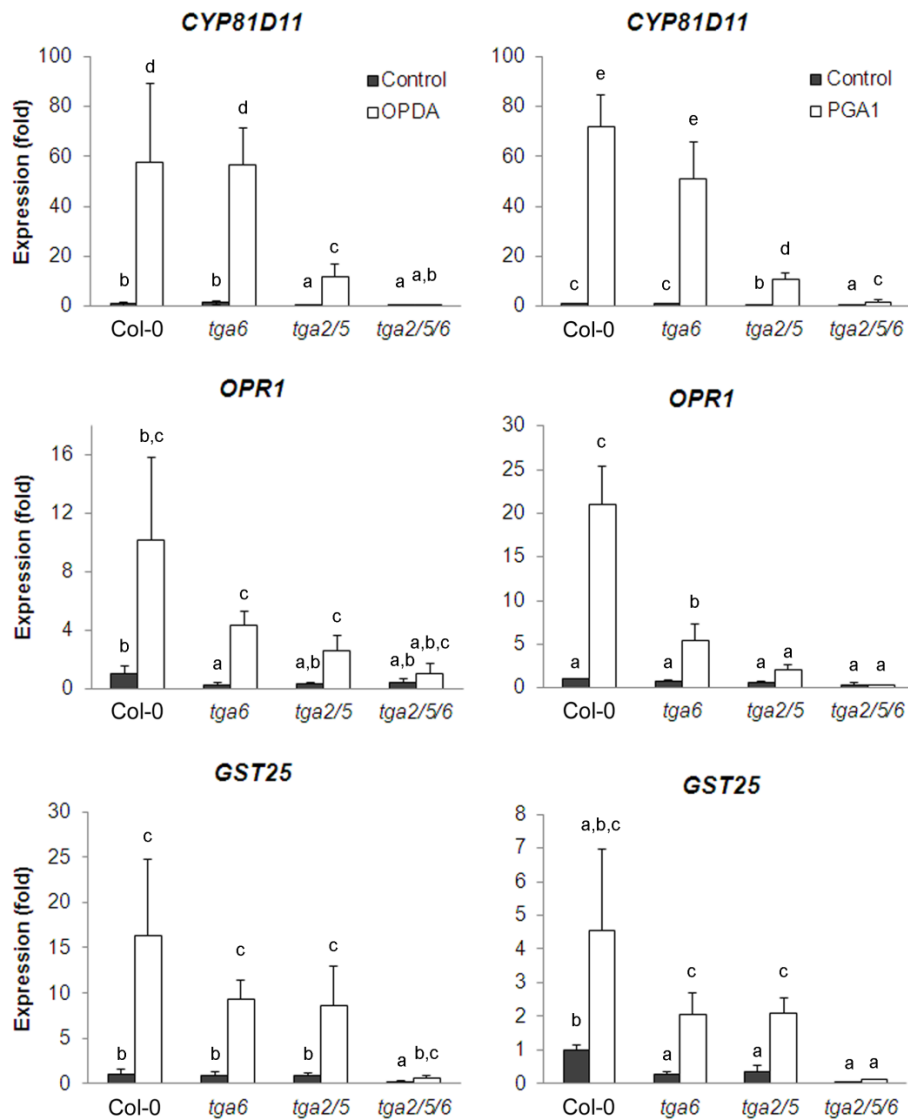


Figure 4. Expression of oxylipin-responsive genes in wild type and *tga* mutants. Seedlings were grown for 10 days in MS medium containing 1% sucrose under short day conditions. The medium was exchanged for 75 μ M 12-oxo phytodienoic acid (OPDA), 75 μ M prostaglandin A₁ (PGA₁), or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the OPDA reductase gene *OPR1*, and the glutathione-S-transferase gene *GST25* are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild type control treatment was set to 1 and all other data was expressed relative to it. Means and standard errors of three biological replicates are shown. Significant differences among means indicated by letters were determined using the Relative Expression Software Tool V2.0.13 (Qiagen, Hilden, Germany).

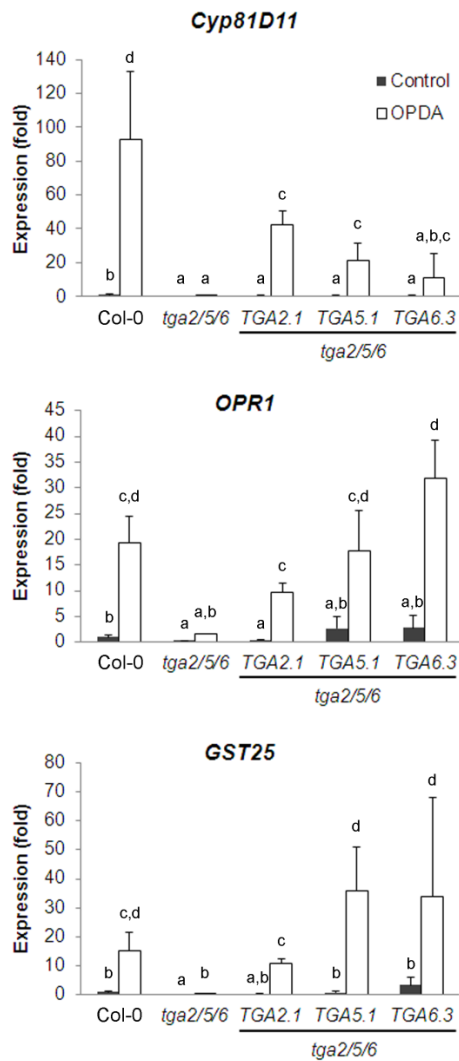


Figure 5. Expression of oxylinin-responsive genes in wild-type, *tga2 tga5 tga6* mutant, and *TGA*-overexpressing plants. *TGA* overexpression occurred in the background of the *tga2 tga5 tga6* mutant. Seedlings were grown for 10 days in MS medium containing 1% sucrose under short day conditions. The medium was exchanged for 75 μ M 12-oxo phytodienoic acid (OPDA) or the solvent 0.5% methanol (control). After a treatment for 4 h, RNA was extracted, converted into cDNA, and amplified using quantitative RT-PCR. Expression of the cytochrome P450 gene *CYP81D11*, the OPDA reductase gene *OPR1*, and the glutathione-S-transferase gene *GST25* are shown. Expression was normalized to the actin gene *Act2/8*, which was used as constitutively expressed internal control. Expression of the wild type control treatment was set to 1 and all other data was expressed relative to it. Means and standard errors of three biological replicates are shown. Significant differences among means indicated by letters were determined using the Relative Expression Software Tool V2.0.13 (Qiagen, Hilden, Germany).

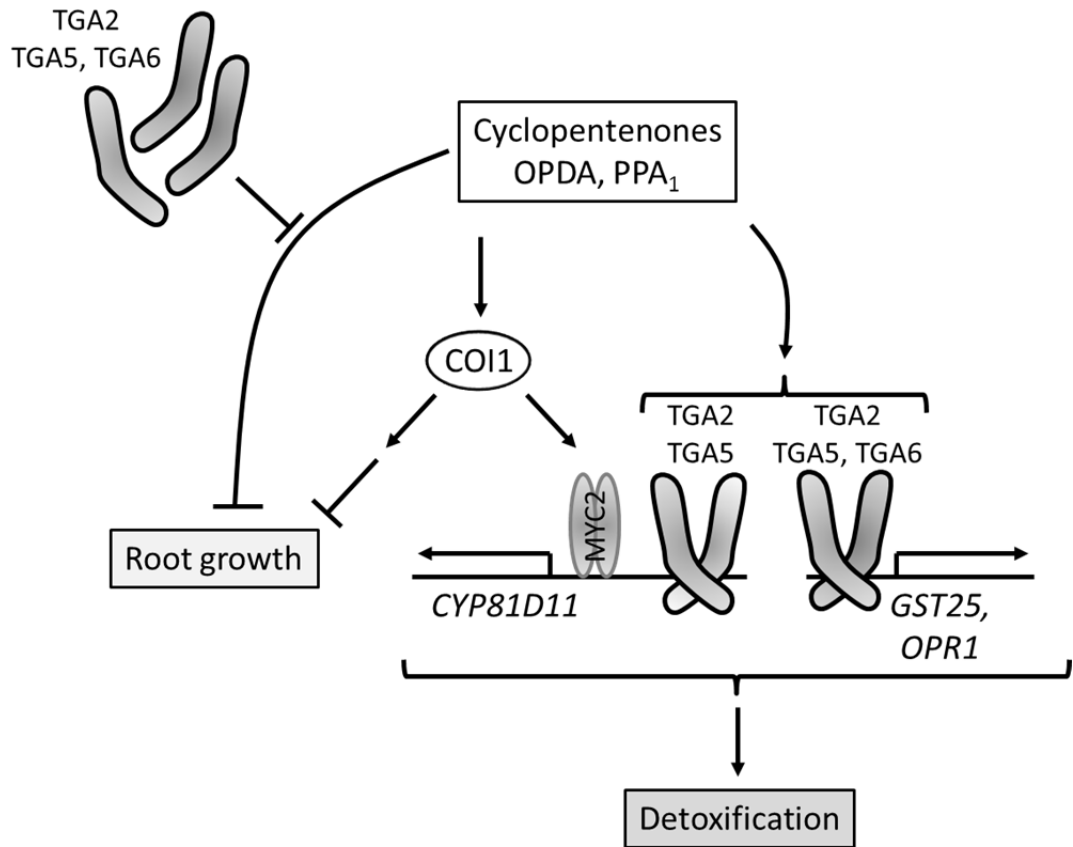


Figure 6. Tentative model explaining the observed effects of cyclopentenone oxylipins on root growth and expression of detoxification genes. Roles of the jasmonate receptor COI1 and TGA transcription factors in mediating oxylin signaling are highlighted. Individual contributions of TGA factors were determined for induction of detoxification genes but not for root growth inhibition. The *CYP81D11* promoter is primarily regulated by TGA2 and TGA5. *GST25* and *OPR1* promoters are primarily regulated by TGA5 and TGA6, although TGA2 does also contribute to the expression of these genes. MYC2 was previously shown to activate *CYP81D11* expression (Köster *et al.*, 2012) presumably by binding to a G-box in the promoter sequence.