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1 ***cis*-12-oxo-phytodienoic acid represses *Arabidopsis thaliana* seed germination in shade light**
2 **conditions**

3

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18

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22

23 **Highlight**

24 OPDA acts in addition to ABA to repress seed germination under far-red light conditions. The response
25 to both these phytohormones is integrated by MFT, a negative regulator of germination.

26

27 **Abstract**

28 Light-dependent seed germination is induced by gibberellins (GA) and inhibited by abscisic acid (ABA).
29 The widely accepted view of GA/ABA ratio controlling germination does not however explain the fact
30 that seeds deficient in ABA still germinate poorly under shade light conditions that repress germination.
31 In Arabidopsis, MOTHER-OF-FT-AND-TFL1 (MFT) acts as a key negative regulator of germination,
32 modulating GA and ABA responses under shade light conditions. Under full light the oxylipin cis-12-
33 oxo-phytodienoic acid (OPDA), a precursor of the stress related phytohormone jasmonic acid, interacts
34 with ABA and MFT to repress germination. Here, we show that, under shade conditions both OPDA and
35 ABA repress germination to varying extents. We demonstrate that the level of shade induced *MFT*
36 expression influences the ability of OPDA and/or ABA to fully repress germination. We also find that
37 *MFT* expression decreases with seed age and this again correlates with the response of seeds to OPDA
38 and ABA. We conclude that OPDA plays an essential role alongside ABA in repressing germination in
39 response to shade light and the combined effect of these phytohormones is integrated to a significant
40 extent through *MFT*.

41

42 **Key words**

43 OPDA, ABA, MFT, phytochrome, FR-light, shade, seed germination

44

45 **Abbreviations**

46 ABA: abscisic acid
47 FR: Far Red
48 GA: gibberellins
49 hai: hours after imbibition
50 JA: jasmonic acid
51 JA-Ile: jasmonic acid -isoleucine
52 OPDA: cis-12-oxo-phytodienoic acid
53 R: Red

54

55 **Introduction**

56 Timing of seed germination is one of the most important decision points in the life cycle of a higher
57 plant. The environmental conditions under which a seed germinates are critical for survival and
58 consequently control mechanisms that integrate environmental cues such as temperature and light
59 quality have evolved to control the timing of germination in a number of species (Smith, 2000; Linkies
60 *et al.*, 2010; Kendall *et al.*, 2011; Lee and Lopez-Molina, 2012). These cues regulate accumulation and
61 perception of gibberellins (GA) and abscisic acid (ABA) phytohormones, which promote and repress
62 seed germination respectively (Seo *et al.*, 2006; Kendall *et al.*, 2011; Shu *et al.*, 2016). ABA, acting
63 through ABA response transcription factors such as ABA-INSENSITIVE3 (ABI3), ABI4 and ABI5
64 (Finkelstein *et al.*, 1998, 2000; Clercx *et al.*, 2003), accumulates during seed development to induce a
65 physiologically dormant state, whereby newly formed seeds do not germinate even under favourable
66 environmental conditions (Graeber *et al.*, 2012; Chahtane *et al.*, 2017). Seeds gradually lose their
67 dormancy through an after-ripening process after which they can germinate if environmental conditions
68 are favourable (Holdsworth *et al.*, 2008; Smith, 2000; Jiao *et al.*, 2007).

69

70 In many plant species, including *Arabidopsis thaliana*, the probability of seedling establishment is
71 generally greater if germination occurs under direct sun (white) light, which is rich in the red (R) wave
72 length; rather than under-the-canopy light (shade), which is rich in far-red (FR) (Lee and Lopez-Molina,
73 2012). In photoblastic seeds, phytochrome photoreceptors distinguish between these different light
74 conditions on the basis of their R and FR light intensities and ratios (Shinomura, 1997; Smith, 2000;
75 Quail, 2002; Jung *et al.*, 2016). Excess R triggers GA accumulation and germination, whereas excess
76 FR, typical of shade light, triggers ABA accumulation and a block in germination (Oh *et al.*, 2006; Seo
77 *et al.*, 2006; Piskurewicz *et al.*, 2008). *Arabidopsis* has five phytochromes, (phyA-E); Clack *et al.*,
78 1994), with phyB being the main promoter of germination under sun light; while phyA is responsible for
79 germination under-the-canopy light (Shinomura *et al.*, 1994). Both phyA and phyB are synthesized as
80 inactive proteins and become active in a light-quality dependent manner. However, while short pulses of
81 R and FR light are sufficient to activate and deactivate phyB respectively; longer exposures to R and FR
82 activate phyA (Reed *et al.*, 1994; Shinomura *et al.*, 1994). Furthermore, compared to phyB, phyA
83 accumulates at high levels only after relatively long periods of seed imbibition (Lee *et al.*, 2012).

84

85

86 Upon activation, both phyA and phyB induce degradation of the transcription factor PHYTOCHROME
87 INTERACTING FACTOR1 (PIF1, formerly known as PIL5) (Shen *et al.*, 2005; Park *et al.*, 2012).

88 Upon phytochrome inactivation, PIF1 accumulates and regulates transcription of many genes, including
89 SOMNUS (SOM), which encodes a CCCH- type zinc finger protein that is part of the phytochrome
90 signal transduction pathway controlling genes involved in regulating ABA and GA levels ultimately
91 leading to high ABA:GA ratios to repress germination (Oh *et al.*, 2004; Oh *et al.*, 2007; Kim *et al.*,
92 2008; Kim *et al.*, 2016; Park *et al.*, 2011). We showed recently that PIF1 and SOM also promote
93 *MOTHER-OF-FT-AND-TFL1* (*MFT*) expression and that *MFT* plays a key role in repressing
94 germination by modulating ABA and GA responses (Vaistij *et al.*, 2018). Furthermore, PIF1 stimulates
95 the expression of *GAI* and *RGA*, which encode growth repressing DELLA proteins (Oh *et al.*, 2004; Oh
96 *et al.*, 2007; Piskurewicz *et al.*, 2008; Piskurewicz *et al.*, 2009). GA promotes germination by targeting
97 destruction of the DELLA proteins through the 26S-proteasome. Under FR conditions, the DELLA
98 proteins RGL2, GAI, and RGA repress germination by stimulating the expression of ABA biosynthetic
99 genes, further increasing the ABA:GA ratio (Piskurewicz *et al.*, 2008; Piskurewicz *et al.*, 2009; Lee *et*
100 *al.*, 2012).

101

102 The phytohormone jasmonic acid (JA) and its precursor *cis*-12-oxo-phytodienoic acid (OPDA) are
103 oxilipins derived from linolenic acid (Wasternack and Song, 2016). The biologically active conjugated
104 JA-isoleucine (JA-Ile) form is involved in responses to biotic and abiotic stress as well as in many other
105 biological processes including seed germination (Linkies and Leubner-Metzger, 2012; Wasternack and
106 Hause, 2013; Wasternack and Strnad, 2015; Singh *et al.*, 2017). OPDA also exhibits signalling
107 properties, some of which are shared with JA-Ile, but others are distinct (Goetz *et al.*, 2012; Guo *et al.*,
108 2014; Savchenko and Dehesh, 2014; Bosh *et al.*, 2014; Dave *et al.*, 2011). Previously we characterised
109 the role of oxilipins in seed dormancy. We did this by analyzing mutant seeds defective in: (i) *ALLENE*
110 *OXIDE SYNTHASE* (*AOS*), which encodes a cytochrome P450 oxidase enzyme involved in one of the
111 final steps of OPDA biosynthesis inside plastids (Park *et al.*, 2002); (ii) *PXA1* (also known as *CTS* and
112 *PED3*), which encodes an ABC-type transporter that imports OPDA into peroxisomes (Zolman *et al.*,
113 2001; Footitt *et al.*, 2002; Hayashi *et al.*, 2002); and (iii) *12-OXOPHYTODIENOIC ACID REDUCTASE*
114 (*OPR3*), which is involved in the conversion of OPDA to JA in peroxisomes (Stintzi and Browse, 2000).
115 It has been determined that seeds of the *aos* mutant, which is compromised in OPDA and JA/JA-Ile

116 accumulation, are less dormant than wild-type seeds; whereas seeds of the *pxa1-1* and *opr3-1* single
117 mutants, which over-accumulate OPDA but are deficient in JA/JA-Ile, are more dormant (Chehab *et al.*,
118 2011; Dave *et al.*, 2011; Dave *et al.*, 2016). These observations led us to conclude that OPDA
119 specifically acts as a dormancy-promoting factor. In the present work we investigated the role of OPDA
120 in the FR triggered repression of germination of after-ripened seeds. We show that endogenous OPDA
121 in seeds plays a key role alongside ABA to repress germination under shade conditions through an MFT
122 modulated process.

123

124 **Material and methods**

125 **Growth conditions and biological materials.** Plants were grown in a greenhouse supplemented with
126 artificial light to give a photoperiod of 16 h light at a temperature of 20-22 °C. Seeds were harvested
127 when plants stopped flowering and siliques started to dehisce. In all experiments, except for that
128 involving extended after-ripening shown in Figure 6, seeds were after-ripened for no longer than 8
129 weeks. All mutant lines used in this study were described previously: *aos* (Park *et al.*, 2002); *opr3-1*
130 (Stintzi and Browse, 2000); *opr3-3* (Chini *et al.*, 2018); *aba2-1* (Leon-Kloosterziel *et al.*, 1996); *mft-2*
131 (Xi *et al.*, 2010); *rgl1-1 rgl2-2 gai-6 rga-2 (della4)* (Cao *et al.*, 2005); *cyp20-3* (Park *et al.*, 2013).

132

133 **Germination assays.** Seeds were vapour-phase sterilized by exposure to chlorine gas in a sealed glass
134 container to at least three hours, the gas having been produced by mixing 100 ml of bleach with 3 ml of
135 concentrated HCl. Sterilized seeds were plated on water agar (0.9 % w/v) and allowed to imbibe under
136 low light for 4 hours and then LED- irradiated with FR ($4.5 \mu\text{mol m}^{-2}.\text{s}^{-1}$) and R ($20 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) as
137 indicated in Figure 1A. After FR/R, FR and FR48 treatments plates were wrapped in foil and kept at 20
138 °C. Germination was scored on the basis of radical emergence of 50-100 seeds per replica. In
139 experiments where germination assays were conducted with ABA (Sigma-Aldrich), OPDA (Larodan),
140 paclobutrazol (Sigma-Aldrich) or norflurazon (Sigma-Aldrich) the appropriate amounts of these
141 compounds were included in the water agar media.

142

143 **Phytohormone analyses.** At least four biological replicates of 100 mg of seeds were imbibed and light-
144 treated as depicted in Fig. 1A, all imbibed seeds were collected and phytohormones extracted as
145 described previously (Dave *et al.*, 2011). Extracts were resuspended in methanol and 2 μL injected and
146 analyzed on an ultraperformance liquid chromatography (UPLC)-MS system consisting of an Acquity

147 UPLC I-Class system (Waters) coupled to a TSQ Endura triple quadrupole mass spectrometer (Thermo
148 Scientific). Chromatographic separation of phytohormones was performed at 40°C on a Waters Acquity
149 C18 BEH column (50 mm x 2.1 mm x 1.7µm particle size), using a binary gradient of mobile phases
150 with A = water + 0.1% (v/v) acetic acid and B = acetonitrile + 0.1% (v/v) acetic acid. The gradient
151 elution program was as follows: 0 - 0.61 min isocratic 10% B; 0.61 - 2.34 min to 100% B; 2.34 - 2.82
152 min isocratic 100% B; 2.82 - 2.83 min to 10% B; 2.83 - 3.30 min isocratic 10% B. Eluted compounds
153 were ionized on the mass spectrometer using a heated electrospray (HESI) source from 0.2 - 2.5 min, in
154 negative ion mode (spray 2500 V; sheath N2 gas 60 units, Aux N2 gas 20 units, sweep N2 gas 2 units,
155 vaporizer 400 °C, ion transfer tube 380°C). Precursor and product ions were filtered through Q1 and Q3
156 respectively at a mass resolution of 1.2 Da and at a fixed dwell time of 35 ms per transition. The
157 following precursor - product ion transitions were programmed using Thermo Xcalibur software in SRM
158 mode: ABA 263.2 - 153.2; d6-ABA 269.2 - 159.2; JA 209.2 - 59.4; GA4 331.2 - 287.1; JA-Ileu 322.2 -
159 130.2; prostaglandin A1 317.2 - 273.2; OPDA 291.2 - 165.2. For ABA and GA4, product ion peak area
160 ratios relative to their respective deuterated analogs added as internal standards were used to construct
161 calibration curves and calculate concentrations. For all other compounds, prostaglandin A1 was used as
162 the reference internal standard. All standards were obtained as described in Dave et al (2011).

163

164 **Gene expression analysis.** RNA extractions were performed as described previously (Vaistij *et al.*,
165 2013). Standard protocols were used for RQ1 RNase-Free DNase treatments (Promega), cDNA
166 synthesis (SuperScript®II, Invitrogen) and qPCRs (iTaQ™ Universal Syber® Green, Bio-Rad).
167 Transcript abundance of a stable endogenous control (*UBQ11*; see Sup. Fig. 1) was used for
168 normalization and gene expression was expressed as a fold change relative to the control sample. Primer
169 sequences for the qPCRs are described in Table S1.

170

171 **Results and discussion**

172 **Differential expression of *AOS* and *OPR3* does not result in OPDA accumulation in FR-treated**
173 **seeds.** In a recent RNAseq-based transcriptomic analysis, we observed that *AOS* expression is FR
174 induced (Vaistij *et al.*, 2018; Sup. Fig. 1). This suggested that, as for ABA, OPDA biosynthesis is
175 induced by FR light. In order to validate the transcriptomic data, we performed RT-qPCR to quantify
176 transcript abundance of *AOS* and *OPR3* in wild-type (Col) after-ripened seeds treated with two
177 consecutive short pulses of FR and R light (FR/R) or a single FR pulse (Fig. 1A) in order to activate and

178 deactivate phyB, respectively. Consistent with the RNAseq data, we found that *AOS* expression was
179 approximately 4 to 5-fold higher in FR-treated seeds compared to FR/R controls at 12 and 24 hours-
180 after-imbibition (hai) (Fig. 1B). In contrast, the *OPR3* transcript abundance was unaffected at 12 hai and
181 was less than 2-fold lower in FR-treated seeds compared to the FR/R treatment at 24 hai (Fig. 1C).
182 These results at the level of gene expression prompted us to assess OPDA levels in FR/R and FR-treated
183 wild-type seeds. We also measured ABA levels and analysed FR-treated *mft-2* seeds. As previously
184 reported, ABA levels were increased in FR-treated wild-type seeds compared to FR/R-treated seeds, and
185 in *mft-2* seeds compared to wild-type seeds under FR light conditions (Seo *et al.*, 2006; Vaistij *et al.*
186 2018; Fig. 1D). Surprisingly, despite the differential *AOS* expression, we detected no significant changes
187 in OPDA accumulation in wild-type and *mft-2* seeds under FR light conditions compared to the
188 respective controls (Fig. 1E). We also measured JA and JA-Ile levels in wild-type seeds upon FR/R and
189 FR treatments (24 hai) but found no significant changes in their accumulation (Sup. Fig. 2). Thus, our
190 findings indicate that total OPDA, JA and JA-Ile accumulation in seeds is not regulated by light quality.
191 However, we cannot rule out the possibility that light may affect oxylipin accumulation in a localised
192 cell specific manner, which would not be detected by the whole seed phytohormone extraction
193 methodology we have available. Future work could explore detection methods that allow more localised
194 mapping of phytohormones within seed tissues.

195

196 **OPDA acts in addition to ABA to repress seed germination in the shade.** Although we could not
197 detect changes in OPDA levels in FR-treated seeds we were curious as to whether oxylipins played a
198 role in regulating germination under FR light conditions. Hence, we analysed germination of after-
199 ripened *aos* mutant seeds, which are deficient in OPDA, JA and JA-Ile (Dave *et al.*, 2011). We observed
200 that, as expected, wild-type and *aos* seeds germinate at high rates upon FR/R treatment and at very low
201 rates (routinely less than 10 %) under FR conditions (Fig. 2A). Presumably, the well documented ABA
202 inhibition of germination in response to FR light causes inhibition of germination in these oxylipin
203 mutants as well as in wild-type seeds. We then used the ABA biosynthesis inhibitor norflurazon to
204 further investigate a possible interaction between OPDA and ABA. We found that blocking ABA
205 biosynthesis in the wild-type background does not rescue the FR block on wild-type seed germination
206 (Fig. 2A), which is consistent with a previous report by Lee *et al.* (2012). This implies that something
207 else is blocking germination in response to FR treatment when ABA biosynthesis is impaired.
208 Interestingly, we found that germination of FR-treated *aos* seeds in the presence of norflurazon

209 germinated at high rates, in a dose dependent manner, suggesting a role for oxylipins in regulating the
210 FR response, at least when ABA biosynthesis is compromised (Fig. 2A). We also assessed germination
211 of two *OPR3* mutant alleles, *opr3-1* and *opr3-3*. Seeds of both *opr3-1* and *opr3-3* accumulate OPDA
212 and ABA at similar levels as wild-type but are both significantly impaired in JA and JA-Ile
213 accumulation (Sup. Fig. 2). The *opr3-1* and *opr3-3* seeds do accumulate very low amounts of JA-Ile,
214 possibly due to the activity of the recently reported OPR3-independent biosynthetic pathway (Chini *et*
215 *al.*, 2018; Wasternack and Hause; 2018). We found that, similar to wild-type, both *opr3-1* and *opr3-3*
216 seeds germinated at high levels under FR/R conditions; and at extremely low levels upon FR-light
217 treatments even in the presence of norflurazon (Fig. 2B). This indicates that OPDA rather than JA/JA-Ile
218 acts to repress germination under FR light, as was found to be the case under white light conditions
219 (Dave *et al.*, 2011; Dave *et al.*, 2016).

220

221 In order to further validate our observations, we also analysed *aos* seeds in the ABA biosynthesis
222 deficient *aba2-1* mutant background (Leon-Kloosterziel *et al.*, 1994). We found that while *aos* and
223 *aba2-1* single mutant seeds do not germinate, *aos aba2-1* double mutant seeds germinate at high rates
224 under the normally repressing FR light conditions (Fig. 2C). These results support the view that blocking
225 the biosynthesis of both OPDA and ABA is required to allow germination of after-ripened seeds under
226 strong phyB-deactivating FR light conditions. Consistent with this, we determined that while application
227 of either OPDA or ABA represses germination of FR-treated *aos aba2-1* seeds (Fig. 2D), exogenous JA
228 has no significant effect on germination of the double mutant (Sup. Fig. 3). Overall, these results
229 demonstrate that the strong repression of germination imposed by the FR light treatment is alleviated
230 when the accumulation of both OPDA and ABA is compromised.

231

232 PhyA-dependent germination of *aos aba2-1* double mutant and control seeds was also assessed after 48
233 h of continuous FR light treatment followed by four days in the dark (FR48; Fig. 1A). Under these
234 conditions phyA and phyB are activated and deactivated respectively. As expected, we observed that
235 FR48-treated wild-type seeds germinated at higher rates than seeds given just a short FR pulse; *aos* and
236 *aba2-1* single mutants germinate at higher levels than the wild-type control seeds; and *aos aba2-1*
237 double mutant seeds germinate at even higher rates than the single mutants (Fig. 2C). There are
238 similarities between the role of ABA in this study and the one described by Lee *et al.* (2012), which
239 showed that attenuation of ABA-dependent responses is required to promote phyA-dependent

240 germination. Overall, these results demonstrate that disruption of either ABA or OPDA biosynthesis
241 results in increased phyA-dependent seed germination.

242

243 To gain insight into the interplay between OPDA, ABA and GA in the control of germination in
244 response to FR light, we analyzed the GA requirement of the *aos aba2-1* double mutant under FR light
245 conditions by treating seeds with the GA-biosynthesis inhibitor paclobutrazol. We observed that under
246 these conditions *aos aba2-1* seeds failed to germinate (Fig. 3A). It is well established that FR conditions
247 are associated with low GA/ABA ratios in seeds (Seo *et al.*, 2006), and that DELLA factors play a
248 critical role in stimulation of ABA biosynthesis under these conditions (Piskurewicz *et al.*, 2009). Thus,
249 we also analysed *rgl1-1 rgl2-2 gai-6 rga-2* quadruple mutant seeds (*della4*) and determined that, as is
250 the case with exogenous ABA, exogenous OPDA represses the high levels of germination that these
251 seeds exhibit under FR light (Fig. 3B). These results suggest that OPDA acts downstream of DELLA
252 factors to repress germination under FR light conditions, which is similar to what has been previously
253 reported for ABA (Piskurewicz *et al.*, 2009). However, it is also possible that OPDA and DELLAs have
254 parallel pathways that repress germination under FR conditions.

255

256 **OPDA and/or ABA repression of germination correlates with *MFT* expression.** Previously, we
257 established that OPDA has no effect on repressing germination of *mft-2* mutant seeds under white light
258 (Dave *et al.*, 2016); here we show that OPDA also fails to repress *mft-2* germination under FR light
259 conditions (Fig. 3C). This strengthens our view that OPDA acts upstream of MFT. In addition, we
260 previously demonstrated that *MFT* expression is induced in a PIF1- and SOM-dependent manner under
261 FR conditions (Vaistij *et al.*, 2018). Here we show that the transcript levels of *PIF1*, *SOM* and *MFT* are
262 strongly increased upon FR-treatment compared to FR/R in wild-type seeds, and this increase is
263 intermediate under FR48 conditions (Fig. 4A). Comparing germination rates of wild-type (Fig. 2C) with
264 *MFT* expression under FR/R, FR and FR48 (Fig. 4A) reveals a negative correlation. These observations
265 led us to hypothesise that a deficiency in both ABA and OPDA is required to overcome the strong
266 germination inhibitory effects of FR conditions when *MFT* is highly expressed; whereas under phyA-
267 dependent germination conditions (FR48), where *MFT* expression is reduced, the absence of either ABA
268 or OPDA is sufficient to alleviate the block on germination. To test this hypothesis we assessed wild-
269 type, *aos*, *aba2-1* and *aos aba2-1* seeds under FR48 light conditions and determined that *MFT*
270 expression is reduced in the mutant backgrounds, with the strongest effect seen in *aba2-1* and *aos aba2-*

271 *1* (Fig. 4B). Taken together, these observations lead us to conclude that MFT integrates both ABA and
272 OPDA signalling pathways in order to repress germination in the shade: The necessity for just one or
273 both of ABA and OPDA for repression of germination depends on endogenous levels of MFT. It is
274 worth noting however, that although *MFT* expression is at a similar low level in *aba2-1* and *aos aba2-1*
275 (Fig. 4B), the germination rate of the double mutant seeds is higher than the single mutant seeds (Fig.
276 2C) suggesting that factors other than MFT also play a role.

277

278 **OPDA and/or ABA repression of germination correlates with seed age.** Lee *et al.* (2012) observed
279 that blocking ABA biosynthesis by norflurazon treatment of wild-type seeds does not alleviate the
280 repression of germination by FR light, which is in agreement with our observations (Fig. 2A). However,
281 Seo *et al.* (2006) reported that ABA biosynthesis deficient mutant seeds germinate partially under FR
282 conditions, which contrasts with our analyses of *aba2-1* seeds (Fig. 2B). While the report of Seo *et al.*
283 (2006) did not indicate the age of after-ripened seeds used in their germination assays, our study and that
284 of Lee *et al.* (2012) were performed with seeds not older than 8 weeks from the time of
285 maturation/collection. This led us to question whether seed age may influence the sensitivity to OPDA
286 and ABA in terms of germination repression under shade light conditions. In order to address this, seeds
287 after-ripened for more than nine months were treated with FR/R and FR light. Interestingly, germination
288 rates of long-term after-ripened norflurazon-treated wild-type seeds and *aos* seeds (not treated with
289 norflurazon) were 75 % and 40 %, respectively, under FR conditions (Fig. 5A and 5B). These
290 germination rates are much higher than those found routinely for the same treatments of short-term (less
291 than 8 weeks) after-ripened seeds (Fig. 2A). Noteworthy also is the fact that long-term after-ripened wild
292 type seeds are still very responsive to the germination repressing effects of FR light. Taken together
293 these results demonstrate that as seeds age there is a necessity for both ABA and OPDA to block
294 germination under FR light whereas in younger after-ripened seeds either one is sufficient. A possible
295 explanation for this might be that aged seeds are less sensitive to dormancy-promoting factors than
296 younger seeds (Holdsworth *et al.*, 2008; Holman *et al.*, 2009). We have previously shown that MFT is a
297 strong promoter of seed dormancy (Vaistij *et al.*, 2013). Therefore, we hypothesized that MFT may be
298 involved in the age-dependent requirement of OPDA and/or ABA to repress germination. To test this we
299 assessed *MFT* expression in young (less than 8 weeks) and old (more than 9 months) wild-type seeds
300 treated with FR light and found that *MFT* expression is reduced in the older seeds (Fig. 5C). This
301 parallels the negative correlation between *MFT* expression levels and OPDA and/or ABA requirements

302 of young seeds under FR and FR48 conditions (Fig. 2B and 4A). These observations further support our
303 conclusion that MFT integrates both ABA and OPDA signalling pathways in order to repress
304 germination and that both environmental conditions such as light quality or developmental factors such
305 as seed age playing an important role in regulating germination through MFT expression. As seeds age
306 other changes may also occur, such as decrease in phytohormone levels. While we have demonstrated an
307 important role for MFT, we cannot rule out the possibility of other factors also having an effect on the
308 sensitivity to OPDA and ABA under FR light conditions.

309

310 **CYP20-3 is involved in OPDA signalling in seeds.** The crosstalk between ABA and OPDA may
311 influence their abundance as well as their associated signalling pathways. We established previously that
312 both gene expression and protein accumulation of the ABI5 transcription factor are induced by OPDA
313 (Dave *et al.*, 2011; Dave *et al.*, 2016). It has also been shown that the forever-dormant phenotype of the
314 OPDA over-accumulating *ped3-3* mutant is dependent on ABI5 (Kanai *et al.*, 2010), and that ABI5
315 accumulation is induced by FR light (Piskurewicz *et al.*, 2009). However, despite this apparent
316 involvement of ABI5 in signalling both ABA and OPDA, *abi5* mutant seeds fail to germinate under FR
317 light (Lee *et al.*, 2012). This indicates that factors other than ABI5 are involved in signalling the ABA-
318 and OPDA-triggered repression of germination under shade light conditions. Interestingly, it has been
319 shown that, in wounded leaves, CYCLOPHILIN20-3 (CYP20-3) acts as a plastid localised receptor
320 linking OPDA signalling to cellular redox homeostasis in the response to stress in Arabidopsis (Park *et al.*
321 *et al.*, 2013). We tested whether CYP20-3 also plays a role in seed OPDA signalling under different light
322 conditions. To do this we assessed germination of *cyp20-3* knockout mutant seeds under FR and FR48
323 treatments, but observed no significant germination increase, even in the presence of norflurazon (Fig.
324 S4). However, we did find that *cyp20-3* seeds were resistant to the germination repressive effect of
325 exogenously applied OPDA under white light conditions (Fig. S4). These results indicate that CYP20-3
326 is involved in the mechanism by which exogenous OPDA inhibits seed germination, but that CYP20-3 is
327 not required for transducing the OPDA effect under FR light conditions (although we cannot exclude
328 that it may act redundantly with other signalling factors).

329

330 **Conclusions**

331 The integration of the data presented in this and our previous studies allows us to propose a model in
332 which the germination repression effect of OPDA and ABA under shade light conditions is, at least

333 partially, modulated by *MFT* (Fig. 6). We have demonstrated that under FR light conditions that lead to
334 *phyB* deactivation, accumulation of OPDA or ABA are sufficient to repress germination (*i.e.* the
335 presence of either phytohormone is enough for the complete FR-driven repression of germination). In
336 contrast, under FR48 light conditions, when the effect of *phyB* deactivation is partially compensated by
337 *phyA* activation, both OPDA and ABA are required for the complete repression of germination. We
338 show a correlation of this dependence on OPDA and/or ABA to repress germination with the levels of
339 *MFT* expression: When *MFT* is highly expressed (FR light conditions) OPDA and ABA act redundantly
340 whereas when *MFT* is lowly expressed (FR48 light conditions) OPDA and ABA act non-redundantly.
341 Moreover, we also show a correlation of the OPDA and/or ABA requirements of young and old seeds to
342 repress germination under FR light conditions with *MFT* expression: Compared to young seeds, old
343 seeds express *MFT* at a lower level and require both OPDA and ABA to fully repress germination. It is
344 still not obvious why two phytohormone-based repression pathways have evolved to control seed
345 germination. One could argue that, because of the critical importance of germination in the plant life
346 cycle, it has been advantageous to adopt a ‘belt and braces’ approach to its control. The deployment of
347 two repressor systems may also allow a greater flexibility or fine tuning of the different temporal, spatial
348 and physiological factors that could all be influencing when a seed germinates.

349

350 **Supplementary data**

351 Figure S1. Relative *AOS*, *OPR3* and *UBQ11* gene expression.

352 Figure S2. OPDA, JA and JA-Ile accumulation in Col, *opr3-1* and *opr3-3*.

353 Figure S3. Effect of JA-treatment on germination of *aos aba2-1* and *della* quadruple mutant seeds.

354 Figure S4. Analysis of *cyp20-3* seed germination.

355 Table S1. Sequence of primers used in this study

356

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366

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509

510 **Figure Legends**

511 **Fig. 1. Analyses of gene expression and accumulation of OPDA and ABA.** (A) Scheme of the
512 experimental design: after-ripened seeds were imbibed for 4 hours under white-light (WL) and then
513 treated with (i) two consecutive 5 minutes FR and R pulses (FR/R); (ii) only one FR pulse (FR); or (iii)
514 48 hours of continuous FR irradiation (FR48). Seeds were kept in the dark after light treatments.
515 Samples were collected for analyses at 12 and 24 hours-after-imbibition (hai) as stated in the figure. (B-

516 C) Relative *AOS* and *OPR3* expression. (D-E) ABA and OPDA levels in FR/R and FR-treated wild-type
517 (Col) and FR-treated *mft-2* seeds. Data are means of three and four biological replicates for gene
518 expression and germination assays, respectively, and error bars represent standard deviation. Asterisks
519 (*) denote statistical significant difference compared to the respective controls as determined by
520 Student's t-test ($P < 0.05$).

521

522 **Fig. 2. Germination assays of OPDA and ABA deficient seeds.** (A) Wild-type (Col) and *aos* seeds on
523 control and norflurazon (Norf; 50 and 100 μ M) supplemented plates under FR/R and FR light. (B) Col,
524 *opr3-1* and *opr3-3* seeds on control and norflurazon (Norf; 50 and 100 μ M) supplemented plates under
525 FR/R and FR light. (C) Col, *aos* and *aba2-1* single, and *aos aba2-1* double mutant seeds under FR/R, FR
526 and FR-FR treatments. (D) Germination of *aos aba2-1* seeds treated with OPDA or ABA (1 and 10 μ M)
527 under FR light conditions. Germination was assessed 144 hai (seeds had been after-ripened for not
528 longer than 8 weeks). Data are means of four biological replicates and error bars represent standard
529 deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as
530 determined by Student's t-test ($P < 0.05$).

531

532 **Fig. 3. Effect of GA, OPDA or ABA on germination of mutant seeds under FR light conditions.** (A)
533 *aos aba2-1* double mutant seeds on control and Paclobutrazol (PAC; 5 μ M) supplemented plates. (B)
534 *rgl1-1 rgl2-2 gai-6 rga-2* quadruple (*della4*) mutant seeds on control, OPDA and ABA (1 and 5 μ M)
535 supplemented plates. (C) *mft-2* mutant seeds on control and OPDA (10 μ M) supplemented plates.
536 Germination was assessed 144 hai. Data are means of four biological replicates and error bars represent
537 standard deviation. Asterisks (*) denote statistical significant difference compared to the respective
538 controls as determined by Student's t-test ($P < 0.05$).

539

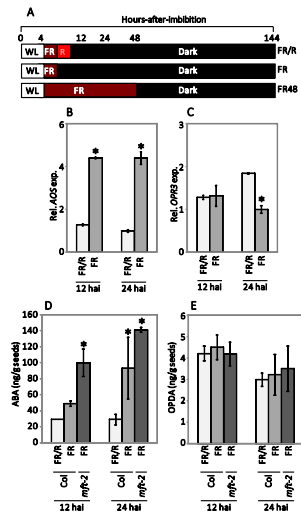
540 **Fig. 4. *PIF1*, *SOM* and *MFT* gene expression.** (A) Relative gene expression in after-ripened wild-type
541 (Col) seeds under FR/R, FR and FR48 light treatments (48 hai). (B) Relative MFT expression in after-
542 ripened Col, *aos*, *aba2-1* and *aos aba2-1* seeds under FR48 light treatment (48 hai). Data presented are
543 the means of three biological replicates and error bars represent standard deviation. Asterisks (*) denote
544 statistical significant difference compared to the respective controls as determined by Student's t-test (P
545 < 0.05).

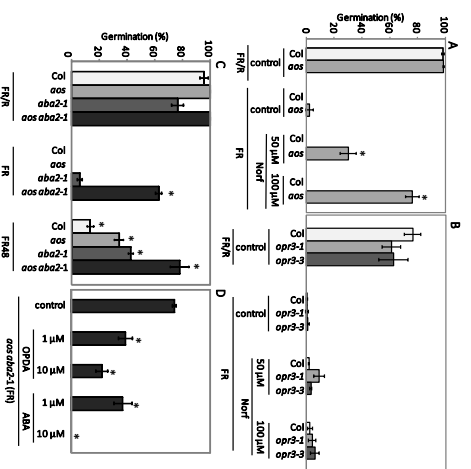
546

547 **Fig. 5. Analyses of long-term after-ripened seeds.** (A) Germination (144 hai) of wild-type (Col) seeds
548 upon FR/R and FR treatments on control and norflurazon (Norf; 100 μ M) supplemented plates. (B)
549 Germination (144 hai) of Col and *aos* seeds upon FR/R and FR treatments. All seeds were after-ripened
550 for at least nine months before conducting germination assays. (C) Relative *MFT* expression in young
551 and old (8-weeks and 9-months after-ripened, respectively) FR-treated seeds. Data are means of four
552 (for germination) and three (for gene expression) biological replicates and error bars represent standard
553 deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as
554 determined by Student's t-test ($P < 0.05$).

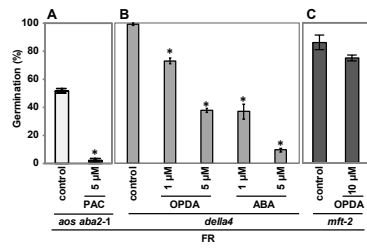
555
556 **Fig. 6. Model of interaction between OPDA, ABA and MFT to repress germination.** Treatment with
557 FR light early after seed imbibition deactivates phyB but has no effect on phyA as it has not yet
558 accumulated. Under these conditions endogenous OPDA and ABA fully repress seed germination and
559 promote expression of the germination repressor MFT (A). In the absence of either OPDA and ABA, the
560 action of the remaining phytohormone and the reduced level of MFT is sufficient to fully repress
561 germination (B and C). When both phytohormones are absent, the low level of MFT expression is not
562 sufficient to repress germination (D). This model also explains the partial germination of OPDA or ABA
563 deficient seeds following a FR48 treatment, which activates phyA and deactivates phyB leading to a
564 reduction of *MFT* expression (compare Figs 2 and 4). Similarly, the model explains the partial
565 germination in response to FR treatment of old seeds deficient in ABA or OPDA.

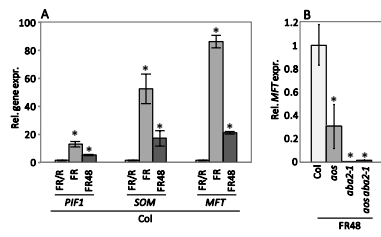
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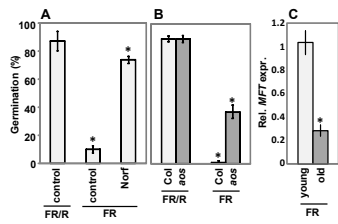


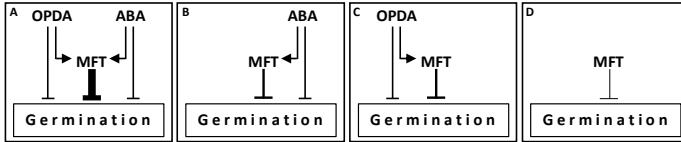


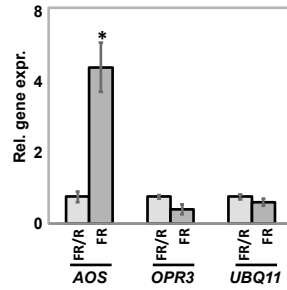
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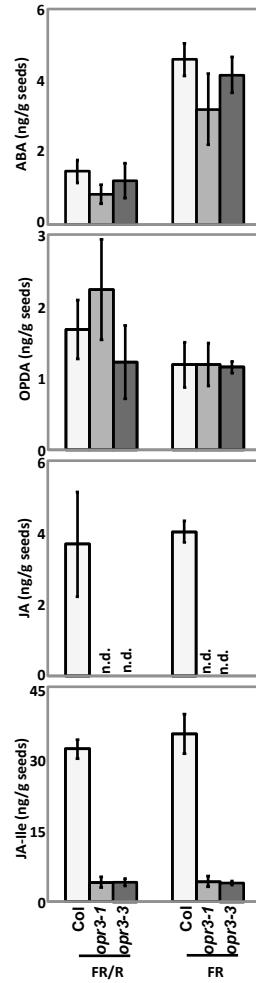




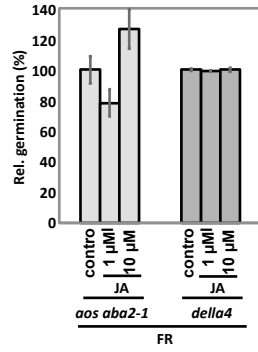


Sup. Fig. 1 Relative AOS, OPR3 and UBQ11 gene expression. Data extracted from an RNAseq-based transcriptomic analysis performed previously (Vaistij *et al.*, 2016) of FR/R- and FR-treated Col seeds (24 hai). Data presented are the means of three biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

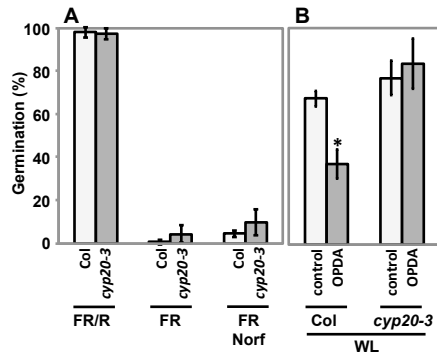
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Sup. Fig. 2 OPDA, JA and JA-Ile accumulation in Col, *opr3-1* and *opr3-3* seeds. Seeds were FR/R and FR treated and material collected 24 hai. Data presented are the means of four biological replicates and error bars represent standard deviation. (n.d.: not detected.)



Sup. Fig. 3 Effect of JA-treatment on germination of *aos aba2-1* double and *della* quadruple mutant seeds. A-B Relative germination (144 hai; FR-treated) of *aos ab2-1* double (A) and *rgl1-1 rgl2-2 gai-6 rga-2* quadruple (*della4*; B) mutant seeds on control and JA (1 and 10 μM) supplemented plates. Data presented are the means of four biological replicates and error bars represent standard deviation.



Sup. Fig. 4 Analysis of *cyp20-3* seed germination. **A** Germination (144 hai) of Col and *cyp20-3* seeds treated with FR/R, FR and FR supplemented with 100 μ M norflurazon (Norf). **B** Germination (144 hai) of Col and *cyp20-3* seeds under white light (WL) on control and OPDA (10 μ M) supplemented plates. Data presented are the means of four biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

587
588
589

Table S1 | Sequence of primers used in this study for RT-qPCR in gene expression analyses

Oligo name	Forward	Reverse
AOS	AAGTCAAAGCCGGTCAAAT	CTTACCGGCGCATTGTTTAT
OPR3	TGGACGCAACTGATTCTGAC	CTCATCACTCCCTTGCCTTC
PIF1	TGTCAATGGGATGTGGAATGA	CATCGCCATATGAGGCATGTA
SOM	TCCGGATGTTTCAATTCAAGAT	GCAAAAGGACAATCAGTCCAATC
MFT	ATCACTAACGGCTGCGAGAT	CGGGAATATCCACGACAATC
UBQ11	TTCATTTGGTCTTGCCTCTG	GAAGATGAGACGCTGCTGGT

590