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Dormancy cycling with mutants

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1 **A laboratory simulation of Arabidopsis seed dormancy cycling provides new insight into its**
2 **regulation by clock genes and the dormancy-related genes *DOG1*, *MFT*, *CIPK23* and *PHYA***

3

4 **Running title:** Dormancy cycling with mutants

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17 **Abstract**

18 Environmental signals drive seed dormancy cycling in the soil to synchronise germination
19 with the optimal time of year; a process essential for species fitness and survival. Previous
20 correlation of transcription profiles in exhumed seeds with annual environmental signals
21 revealed the coordination of dormancy regulating mechanisms with the soil environment.
22 Here, we developed a rapid and robust laboratory dormancy cycling simulation. The utility
23 of this simulation was tested in two ways. Firstly using mutants in known dormancy-related
24 genes (*DELAY OF GERMINATION 1 (DOG1)*, *MOTHER OF FLOWERING TIME (MFT)*, *CBL-*
25 *INTERACTING PROTEIN KINASE 23 (CIPK23)* and *PHYTOCHROME A (PHYA)*). Secondly, using
26 further mutants we test the hypothesis that components of the circadian clock are involved
27 in coordination of the annual seed dormancy cycle. The rate of dormancy induction and
28 relief differed in all lines tested. In the mutants, *dog1-2* and *mft2*, dormancy induction was
29 reduced but not absent. *DOG1* is not absolutely required for dormancy. In *cipk23* and *phyA*
30 dormancy induction was accelerated. Involvement of the clock in dormancy cycling was
31 clear when mutants in the morning and evening loops of the clock were compared.
32 Dormancy induction was faster when the morning loop was compromised and delayed
33 when the evening loop was compromised.

34

35 **Key words:** *Arabidopsis*, circadian clock, circannual rhythm, *DOG1*, dormancy cycling,
36 germination, seed dormancy, thermal time.

37

38 **Introduction**

39 Seeds are highly efficient sensors and interpreters of the prevailing environment and their
40 environmental history. Seeds first sense the maternal environment to set the depth of
41 primary dormancy at maturity (e.g. temperature) (Kendall *et al.* 2011; Penfield and
42 Springthorpe, 2012; He *et al.* 2014; Huang *et al.* 2014 & 2015; Chen *et al.* 2015). Seeds that
43 do not germinate immediately upon shedding enter the soil seed bank and respond to the
44 soil environment by continually adjusting depth of dormancy to time the eventual
45 completion of germination (Footitt *et al.* 2011, 2013, 2014; 2015; Finch-Savage and Footitt,
46 2012, 2017; Penfield and Springthorpe, 2012). When depth of dormancy is low seeds are
47 sensitive to signals that inform of the spatial environment (e.g. light, nitrate and
48 temperature). If these signals are not received to remove the final layer of dormancy then
49 seeds enter secondary dormancy (Finch-Savage and Footitt 2017). In this way seeds
50 determine the time and place of plant establishment to synchronise their life cycle with
51 favourable environments (Finch-Savage and Leubner-Metzger, 2006; Burghardt *et al.* 2016;
52 Springthorpe and Penfield 2015). Recent correlations of annual gene expression patterns in
53 exhumed seeds with environmental signals in the field provided the first insight into the
54 temporal integration of the molecular regulation of dormancy cycling (Footitt *et al.* 2011,
55 2013, 2014).

56

57 However, studying dormancy cycling in the field is a long-term undertaking and ethical and
58 regulatory reasons can preclude the use of seeds from genetically modified plants to dissect
59 the role of individual genes; progress in understanding has therefore been slow. To address
60 this in the work presented we used our field and laboratory observations (Cadman *et al.*

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61 2006; Footitt *et al.* 2011; Huang *et al.* 2015) to develop a representative, but rapid and
62 simple laboratory simulation of dormancy cycling. It enables a full dormancy cycle to be
63 completed in *c.* eight weeks. The method was developed using the deeply dormant winter
64 annual ecotype Cvi then applied to the Col-0 and Ler ecotypes to facilitate mutant analyses.
65 Here we explore the utility of this laboratory simulation in two ways.

66

67 Firstly, we use the simulation to confirm the involvement of genes previously identified in
68 correlative gene expression studies as central to the regulation of seed dormancy cycling
69 (Footitt *et al.*, 2011, 2013). These studies suggest that by influencing the central integrating
70 hormonal balance (Abscisic acid (ABA)/Gibberellins (GA)) DELAY OF GERMINATION 1 (DOG1)
71 and MOTHER OF FLOWERING TIME (MFT) play key roles in the response to temporal signals
72 (e.g. temperature) that regulate dormancy cycling (Finch-Savage and Footitt, 2017). This is
73 linked to temporal changes in the expression of PHYTOCHROME A (PHYA) and CBL-
74 INTERACTING PROTEIN KINASE 23 (CIPK23) that alter sensitivity to signals indicating
75 suitability for germination completion (spatial signals: light and nitrate respectively). Once
76 sensitised, seeds respond to these signals through the ABA/GA balance to bring about the
77 completion of germination when conditions are optimal (Finch-Savage and Footitt, 2017).

78 We confirm the involvement of these genes in ABA sensitivity and dormancy cycling using
79 mutant lines (*dog1-2*, *mft2*, *cipk23* and *phyA*). In the field, dormancy induction and relief
80 during cycling were shown to progress in thermal time (Footitt *et al.* 2011) and we use this
81 approach to analyse data in the present work. Thermal time is quantified as the amount by
82 which temperature exceeds a minimum temperature or threshold for the process in
83 question. When this value is summed over days to give degree days (°C days) thermal time

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84 can be used to measure progress towards the completion of that process (Finch-Savage and
85 Luebner-Metzger, 2006).

86

87 Secondly, we use the simulation to test the previously unstudied hypothesis that
88 components of the circadian clock may be involved in coordination of the annual seed
89 dormancy cycle. The regulation of daily circadian rhythms has been extensively studied in
90 plants (e.g. Salome and McClung 2005; Seung *et al.* 2012; Seo and Mas 2015; and Atamian
91 and Harmer 2016). In a 24 hour cycle, interlocking morning and evening feedback loops
92 control the period and phases of the circadian clock. The morning loop in Arabidopsis
93 contains the MYB-related transcription factors *LATE ELONGATED HYPOCOTYL (LHY)* and
94 *CIRCADIAN CLOCK ASSOCIATED1 (CCA1)* whose increased expression represses the evening
95 loop gene *TIMING OF CAB EXPRESSION (TOC1)*. LHY/CCA1 also induce the sequential
96 expression of the *PSEUDO RESPONSE REGULATOR* genes *PRR9*, *PRR7*, and *PRR5*; which
97 feedback during the day to progressively repress *LHY/CCA1* and therefore relieve repression
98 of *TOC1*. The latter protein, which induces *LHY/CCA1* expression (as reviewed in Hsu and
99 Harmer, 2014) is targeted for degradation by GIGANTEA (GI) in conjunction with ZEITLUPE
100 (ZTL). GI then appears to be repressed by EARLY FLOWERING 3 (ELF3) a member of the
101 evening complex (Mishra and Panigrahi, 2015). The evening complex is formed by the
102 proteins ELF3, ELF4 and LUX ARRHYTHMO (LUX) and this represses the expression of the day-
103 phased clock gene *PRR9* (Hsu and Harmer, 2014).

104

105 Some of these genes are known to influence seed dormancy. For example, *LHY* and *CCA1*
106 mutants were more sensitive to dormancy relieving low temperature stratification and the
107 *GI* mutant less sensitive (Penfield and Hall, 2009). They also alter the hormone balance in

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108 seeds. For example, *GI* and *TOC1* mutants influence ABA and GA sensitivity and the
109 expression of ABA and GA related genes (Penfield and Hall, 2009). The clock was also a key
110 regulator of physiological activity when dormancy of imbibed *Euphorbia esula* seeds was
111 relieved by alternating temperature in the dark (Foley *et al.* 2010). This indicates that the
112 clock could respond to temperature signals in the dark conditions experienced in the soil
113 seedbank; furthermore temperature alternations of 4°C are known to entrain the clock
114 (Salome and McClung, 2005). However, in seeds it is the rhythm of the seasonal
115 temperature cycle that provides a temporal signal indicating time of year (Probert, 2000). In
116 other tissues, components of the circadian clock can respond to seasonal changes in day
117 length and associated alternation of light and temperature to coordinate tree bud dormancy
118 with the time of year (Cooke *et al.* 2012). A similar role for the clock in seeds is largely
119 unexplored. To address this we use a targeted selection of mutant lines of clock genes to
120 determine their contribution to the dormancy cycle and associated ABA sensitivity. We
121 complement this by analysing the annual transcription profiles of these genes during
122 dormancy cycling in the field.

123

124 Materials and Methods

125

126 **Seed production:** Seeds of the Arabidopsis Cape Verde Island (Cvi) and Burren (Bur)
127 ecotypes were produced in a heated glasshouse with supplemental lighting in 2007 (Cvi) and
128 2008 (Bur) and were harvested, processed and then stored at -80°C as described elsewhere
129 (Footitt *et al.* 2011, 2013). Seeds of the Arabidopsis mutants' *toc1-101* (Kikis *et al.* 2005),
130 *lhy20 cca1-1* (Yakir *et al.* 2009), *lhy20 cca1-1 toc1-2* (Yamashino *et al.* 2008), *prr5-11 prr7-11*
131 *prr9-10* (Nakamichi *et al.* 2005), *dog1-2* (Nakabayashi *et al.* 2012), *mft2* (Xi *et al.* 2010),

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132 *cipk23* (N503652) and *phyA* (N6223) are in the Col-0 (N1092) genetic background. While the
133 overexpressing lines *LHY-OX* and *CCA1-OX* are in the Ler and Col-0 backgrounds respectively
134 (Green *et al.* 2002). All lines and their wild types were produced in the same growth cabinet
135 (16°/16°C 16h L/8 h D). Following harvesting and processing seeds were stored at -80°C (See
136 supplementary methods for seed production conditions).

137

Dormancy cycling in the laboratory

138 The annual variation in soil temperature and water potential are seen to impact the annual
139 seed dormancy cycle in the field (Footitt *et al.* 2011). These observations were used to
140 develop a protocol for dormancy cycling in the laboratory. Dormancy/germination
141 experimental treatments and procedures used surface sterilised seeds and were all carried
142 out in the dark under a green safe light unless otherwise stated.
143

144

145 **Impact of water potential on dormancy status in Cvi:** Decreasing soil water potential was
146 associated with low temperature induction of dormancy in Cvi in the field (Footitt *et al.*
147 2011). Consequently its role was tested in the laboratory. Dormant seeds were surface
148 sterilised in 2.5% dilution of domestic bleach for 5 minutes and washed three times in
149 water. Seeds were then placed (3 x 40 seeds) into boxes (124 x 88 x 22 mm) (Stewart
150 Plastics Ltd, UK). Each box contained 25 ml of solution set at a range of water potentials (0, -
151 0.4, -0.8, and 1.2 MPa) using PEG 8000. This PEG solution volume represents a solution
152 volume/paper weight ratio of 3.55 that minimises the concentrating effect of filter paper on
153 the solution (Hardegree and Emmerich 1990). This liquid reservoir was accommodated
154 beneath the seeds as follows. In the base of each box was placed a piece of glass-drying mat
155 (Nisbits Ltd, UK). The drying mat was an open lattice 3 mm deep to create space for the PEG

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156 solution. On top of this was placed nylon mesh (1 mm mesh size) (Plastok, UK) to support
157 the single sheet of Whatman 3MM chromatography paper (Camlab, UK) that is then placed
158 on top. Strips of nylon mesh (125 μm mesh size, 45% open mesh) (Plastok, UK) were then
159 laid on the paper and each seed replicate was placed on one of those individual strips (for a
160 visual representation see Fig. S1). Boxes were then sealed inside freezer bags to minimize
161 evaporation and wrapped in two layers of aluminium foil to exclude light and incubated at
162 5°C for up to 14 days. Germination tests were carried out on these seeds after increasing
163 intervals at 5°C. The nylon strips holding the seeds were transferred to new boxes
164 containing 2 sheets of chromatography paper and 8 ml of 50 or 250 μM Gibberellin₄₊₇ in
165 citrate/phosphate buffer (pH 5.0) or a buffer control in the light at 20°C and germination
166 recorded over 28 days (Footitt *et al.* 2011). Gibberellin₄₊₇ was dissolved in 100 μl 0.1 M KOH
167 before preparing stock solution.

168 Seeds incubated on water (0 MPa) were also transferred at intervals to fresh water, or 10
169 mM KNO₃ and incubated at 20°C/light for 28 days to record germination. In all treatments
170 dark germinated seeds were recorded on transfer to the light. Germination was recorded as
171 protrusion of the radicle through the seed coat and micropylar endosperm.

172 **Dormancy cycling in Cvi:** The contrasting impact of winter and summer temperature on the
173 annual dormancy cycle of Cvi was simulated using lower and higher constant temperatures
174 to simulate dormancy cycling in the laboratory. Dormant seeds were plated (3 x 40 seeds)
175 onto nylon mesh strips in boxes containing a -1.2 MPa PEG 8000 solution as above and
176 incubated at 5°C for up to 21 days. At this point seeds were transferred to boxes containing
177 2 sheets of chromatography paper and 8 ml water and incubated at 25°C for 35 days. At
178 each transfer point dark germinated seeds were counted. At intervals, boxes were removed

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9

179 and dormancy tested by transferring seeds to boxes in the light as above containing 50 or
180 250 μ M Gibberellin 4+7 or a buffer control as above.

181 **Dormancy cycling in Col-0 wild type and mutants:** Using the laboratory dormancy cycling
182 simulation, mutants in the Col-0 genetic background were used to test the contribution of
183 selected genes to dormancy cycling. Seeds were plated (3 x 40 seeds) into boxes containing
184 a -1.0 MPa PEG 8000 solution as above. A series of experiments testing a wide range of
185 treatment temperatures and durations were then performed to evaluate the role of
186 temperature in the induction and relief of dormancy. For the impact of cold conditioning on
187 high temperature dormancy induction seeds were incubated at 5°C/ -1 MPa for up to 28
188 days then transferred to germination plates containing 2 sheets of chromatography paper
189 and 8 ml of water and incubated at 20 (clock mutants only), 25 and 30°C for up to 14 days at
190 which point plates were transferred to 5°C for up to 29 days. At intervals during each
191 incubation period boxes were removed for germination testing at 25°C/light for 14 days.
192 Loss of dormancy in the presence of nitrate in Col-0 and *cipk23* seeds subjected to 5°C/ -1
193 MPa followed by 25°C was also tested by transferring seeds to 10 mM KNO₃ at 25°C/light
194 for 14 days. Germination tests were carried out at 25°C as the Col-0 wild type retains
195 greater thermodormancy at this temperature compared to 20°C. Seeds on PEG 8000
196 solution were transferred to water prior to germination testing in the light. Dark
197 germination was recorded at each transfer point and prior to germination testing.

198 **ABA sensitivity:** As changing ABA sensitivity has an integral role in the dormancy continuum
199 the sensitivity of mutants was determined. Seeds were plated on to nylon mesh in boxes
200 containing water as above. They were cold stratified at 5°C/dark for three days then
201 transferred to boxes containing 10 - 250 nM (\pm)-ABA (Sigma, UK) in citrate/phosphate buffer

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202 (pH 5.0) and incubated in the light at 25°C. ABA was dissolved in 100 µl 0.1 M KOH before
203 preparing stock solution.

204 **Dormancy cycling in the field and gene expression analysis:** As seed dormancy cycling
205 displays an annual rhythm in response to seasonal soil temperature patterns we determined
206 the transcriptional profile of seven clock genes in seeds recovered over twelve months from
207 field soil. Experiments on dormancy cycling in the field were performed as described
208 previously (Footitt *et al.* 2011 and 2013). Seeds were recovered from the soil in the morning
209 of the day of harvest. QPCR of circadian clock gene expression was performed using the
210 touchdown PCR thermal cycle: one cycle at 95°C for 10 min followed by 50 cycles at 95 °C
211 for 30s, 70°C (decreasing by 0.2°C/cycle to a target temperature of 67°C) for 30s, and 72 C
212 for 30s. All other details regarding QPCR procedures and analysis were described previously
213 (Footitt *et al.* 2015). Primer sequences are given in Table S2.

214

215 Results

216

217 Dormancy cycling in *Cvi* under laboratory conditions:

218 *Cvi* seeds in field soil are induced into deeper primary dormancy by low temperatures in
219 winter, dormancy then declines to low levels in response to higher temperatures in
220 spring/summer; deeper dormancy (secondary dormancy) is then re-induced by
221 autumn/winter low temperatures (Footitt *et al.* 2011). A series of experiments were
222 conducted to reproduce this behaviour in the laboratory. Primary dormant seeds on water
223 did not germinate in the dark and germination was less than 5% at 20°C/light (Fig. 1(a)).
224 Periods of pre-exposure to low temperature in the dark up to 14 days had a limited effect

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225 on germination on water (Fig. 1a) and the buffer control (Fig. 1(b)). Germination on nitrate
226 marginally increased from 5-17 % in the first 4 days, then declined on further exposure.
227 However, full germination was induced by imbibition on GA (250 μ M GA₄₊₇), which then
228 progressively declined on exposure to low temperature indicating an increasing depth of
229 dormancy not evident on water, or the buffer control (Fig. 1b). Depth of dormancy
230 increased more rapidly on exposure to low temperature when seeds were incubated in
231 negative water potentials (down to -1.2 MPa) consistent with the observations of Auge *et*
232 *al.* (2015).

233

234 A second lot of seeds from the same harvest were exposed to low temperature (5°C/dark)
235 for 21 days with and without water stress at -1.2 MPa and then transferred to water at
236 25°C/dark to simulate a full dormancy cycle (Fig. 2). This second seed lot had been stored at
237 -20°C, which resulted in a lower dormancy level. With these seeds, germination on the
238 buffer control increased to < 40% after 6 days of low temperature indicating this proportion
239 of the population had the lowest level of primary dormancy. In this portion, dormancy could
240 then be relieved by light, with the remainder not yet light sensitive. With continued low
241 temperature exposure deeper dormancy was induced in the population as a whole.
242 Sensitivity to GA₄₊₇ declined (i.e. dormancy deepened) so that no seeds germinated even at
243 250 μ M GA₄₊₇ after exposure to low temperature for 21 days. Depth of dormancy then
244 declined progressively in the subsequent high temperature phase of the cycle. This began
245 after 2 days on GA and then later in the control after 40 days.

246

247 **Dormancy cycling in Col-0 and Ler:**

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248 Col-0 and Ler seeds were produced by maturing them at the relatively low temperature of
249 16°C. This lower temperature increased the level of primary dormancy and prevented dark
250 germination at low temperature. Subsequent imbibition of these seeds at low temperature
251 relieved primary dormancy and high temperature then induced secondary dormancy (Fig.
252 3). Dormancy was initially similar in Col-0 and Ler, but the exposure to low temperature
253 revealed Ler was the more dormant ecotype (Fig 3(a) & (c)).

254

255 To simulate a dormancy cycle in the laboratory, Col-0 and Ler seeds were first subjected to
256 low temperature (5°C/dark) at -1.0 MPa for up to 28 days. On day 28, seeds were
257 transferred to water at 25 °C/dark (Fig. 3; results at 25 and 30 °C are shown in Fig. S2, also
258 20, 25 and 30 °C in Fig S3, & S4), which resulted in the rapid induction of secondary
259 dormancy in all seeds. This was followed by a second low temperature phase to relieve
260 secondary dormancy. At all stages dormancy level was determined by germination following
261 transfer to 25°C/light. At this temperature these ecotypes exhibit high temperature
262 thermodormancy. Seeds from lines with mutated dormancy regulating genes (*dog1-2*, *mft2*,
263 *cipk23* and *phyA*) and mutated clock genes and over expressing lines were also subjected to
264 this simulation.

265

266 To test if incubation of Col-0 seeds beyond 28 days at low temperature (5°C/dark) at -1.0
267 MPa would induce secondary dormancy seeds were incubated for up to 42 days. Although
268 primary dormancy was relieved and germination was 94% after 21 days it only declined to
269 84% after 28 days and 83% at 42 days indicating a slow induction of secondary dormancy
270 that may increase if the treatment was extended further(see Discussion).

271

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272 **Dormancy cycling in mutants of dormancy-related genes:** Primary dormancy in the mutants
273 *mft2*, *cipk23* and *phyA* was similar to Col-0, but *dog1-2* was non-dormant (Day 0; Fig. 3(a)).
274 The response of these mutants to the dormancy cycle simulation differed greatly from Col-0.
275 The initial low temperature phase relieved primary dormancy and then induced secondary
276 dormancy in *cipk23* and *phyA* after 21 days. On transfer to higher temperature (25°C),
277 secondary dormancy induction was complete after only four days in *cipk23* and *phyA*, but
278 seven days in Col-0 (Fig. 3(a)). In contrast, *dog1-2* and *mft2* secondary dormancy induction
279 was slower. Maximum induction was after 14 days in *dog1-2* (germination 48%) and seven
280 days in *mft2* (germination 33%). On transfer to the second low temperature phase,
281 secondary dormancy was broken after two days in *dog1-2* and *mft2*; and after four days in
282 Col-0, *cipk23* (83%) and *phyA* (65%). Secondary dormancy was then re-induced in Col-0,
283 *cipk23* and *phyA*, but not in *dog1-2* and *mft2*. The rate of change was greater when 30°C
284 was used to induce secondary dormancy, but the relative performance of the lines was very
285 similar (Fig. S2). As CIPK23 is involved in the regulation of nitrate transport and signalling the
286 nitrate sensitivity of Col-0 and *cipk23* was tested when secondary dormancy was induced at
287 25°C for 14 days. Germination was 85% and 77% respectively in the presence of 10mM
288 nitrate at 25°C/light.

289

290 To determine the role of the initial cold treatment seeds were exposed directly to high
291 temperature (25 or 30°C) in the dark. Secondary dormancy was not induced in *dog1-2* but
292 was in the wildtype and the other dormancy related mutants (Fig. S5).

293

294 **Selection of lines to test whether clock genes influence seed dormancy cycling:** We

295 subjected seeds from lines with the following clock mutations: *toc1-101*, *lhy20 cca1-1*, *lhy20*

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296 *cca1-1 toc1-2* and *prp5-11 prp7-11 prp9-10* and the overexpressing lines *LHY-OX* and *CCA1-*
297 *OX* to the dormancy cycling simulation. This combination of mutants allowed us to
298 investigate whether altering the balance between the morning and evening loops of the
299 clock would alter the dormancy cycling response under the relatively long-term, but
300 changing, constant temperatures of the simulation in the dark (i.e. in the absence of an
301 imposed external daily rhythm).

302

303 **Dormancy cycling in clock mutant lines:** Primary dormancy of all lines was initially relieved
304 during the low temperature phase, but secondary dormancy induction started between
305 days 21 and 28 except in *prp5-11 prp7-11 prp9-10*; and induction increased in the order *toc1-*
306 *101, lhy20 cca1-1*, and *lhy20 cca1-1 toc1-2* (Fig. 3(b)). On transfer to higher temperature
307 (25°C) at 28 days, secondary dormancy was completely induced after a further seven days
308 and was slowest in *prp5-11 prp7-11 prp9-10*. On transfer back to low temperature secondary
309 dormancy was rapidly relieved and then re-induced in all lines (Fig. 3(b)). The impact of the
310 high temperature phase on rate of dormancy induction and its subsequent relief differed
311 with temperature (20, 25, & 30°C, Fig. S3)

312

313 The overexpressing lines behaved differently from their respective wild type comparisons. In
314 the *CCA1-OX* (Col-0 background) overexpressing line secondary dormancy was more rapidly
315 induced during the first low temperature phase than in Col-0 and was complete after only
316 four days on transfer to 25°C compared to 7 in Col-0 (Fig. 3(c)). Whereas, the *LHY-OX* (Ler
317 background) overexpressing line was more dormant than Ler and the first low temperature
318 phase did not relieve dormancy. Dormancy increased on transfer to 25°C (Fig. 3(c)). In the
319 second low temperature phase, secondary dormancy was relieved, but rapidly re-induced

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320 only in *CCA1-OX*. The response to the second low temperature phase was dependent on the
321 previous temperature. For example, in contrast to that shown on transfer from 25°C (Fig.
322 3(b) & S4(b)), on transfer from 20°C secondary dormancy was not relieved by low
323 temperature in *CCA1-OX* (Fig. S4(a)). Furthermore, on transfer from 30°C to low
324 temperature secondary dormancy was relieved, but only re-induced with prolonged
325 incubation (Fig. S4(c)).

326

327 **Response of clock genes to temporal signals in the field:** To further understand the
328 response of clock mutants we analysed the transcription profiles of selected clock genes in
329 seeds of the deeply dormant winter annual ecotype *Cvi* and the shallow dormant summer
330 annual ecotype *Bur* during dormancy cycling in the field (Fig. 4). *Bur* is a summer annual
331 ecotype whose dormancy cycling behaviour is highly characterised (Footitt *et al.* 2013;
332 2015). As such it is used here as a model for the summer annual behaviour of the ecotype
333 *Col-0* the genetic background of the clock mutants used in the laboratory simulation. In both
334 *Cvi* and *Bur* ecotypes there were clear annual transcript profiles, however the profiles of the
335 morning genes *CCA1* and *LHY* differed between ecotypes (Fig. 4 (b) & (f)). In *Cvi*, the
336 transcription profiles of *LHY* and *TOC1* were similar; but opposite to the soil temperature
337 profile. Whereas in *Bur*, *CCA1* and *TOC1* transcript profiles are similar, but have little
338 relationship with the temperature profile (see Table S1). In *Cvi* and *Bur*, *GI*, *PRR7* and *ELF3*
339 transcription profiles are the same and inversely tracked soil temperature and in the case of
340 *Cvi* also tracked dormancy (Fig. 4(c), (d), (g) & (h)). Of the evening complex genes examined,
341 *LUX* transcription had no obvious pattern in contrast to *ELF3* (Fig. 4(d) & (h)).

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343 Significant correlations occurred between the transcription-profiles of the clock and
344 dormancy related genes and the annual soil temperature cycle in both ecotypes (Table S1).
345 In particular there were strong correlations between the evening genes *ELF3* and *GI* and
346 chromatin remodelling genes involved in gene activation/repression such as *HUB1* and
347 *OTLD1* and silencing (*KYP/ROS1*) (Footitt *et al.* 2015) (Table S1).

348

349 **Dormancy induction and thermal time:** In the field, dormancy induction and relief during
350 cycling were shown to progress in thermal time (Footitt *et al.* 2011). We therefore used this
351 approach to analyse data in the laboratory simulation during dormancy induction at 20, 25
352 and 30°C (Fig. 5 & S6). Induction of secondary dormancy in Col-0, *mft2* and the clock
353 mutants followed an exponential decay response with thermal time. In the clock mutants
354 the thermal time required to induce secondary dormancy in 50% of the population
355 decreased in the order *prp5 11 prr7-11 prr9-10* (153 °C days) > Col-0 (105 °C days) > *toc1-101*
356 (82 °C days) > *lhy20 cca1-1* (60 °C days) > *lhy20 cca1-1 toc1-2* (54 °C days). In *dog1-2*
357 induction of secondary dormancy had a linear response (see Fig. S6 for regression
358 equations).

359

360 **ABA sensitivity of Col-0, dormancy and clock mutants:** Due to the role of ABA in the
361 induction of dormancy we investigated ABA sensitivity of both groups of mutants. Dormancy
362 mutants showed large differences in ABA sensitivity. With the exception of *dog1-2* final
363 germination was similar in all lines (Fig. 6(a)). However, the speed of germination
364 represented by the time to 50% germination (T50) (a measure of germination velocity)
365 revealed that ABA sensitivity increased in the order *dog1-2* > *mft2* > Col-0 > *phyA* > *cipk23*
366 (Fig. 6(b)) similar to that seen for the induction of secondary dormancy in thermal time (Fig.

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367 5(a) & S6(a)). The response of dormancy mutants to 100 nM ABA (Fig. 6(c)) illustrates
368 further their different ABA sensitivities. ABA sensitivity in *dog1-2* was greatly reduced in
369 agreement with that reported for *dog1-1* (Ler background) (Bentsink *et al.* 2006). The onset
370 of low ABA sensitivity was delayed in *mft2* potentially indicating delayed ABA catabolism as
371 an uplift in germination occurred at the same time in all lines (Fig. 6(c)).

372

373 Clock mutants also exhibit different ABA sensitivities (Fig 6(d)). The time to 50% germination
374 (T50) revealed that ABA sensitivity increased in the order *prp5 11 prp7-11 prp9-10 > toc1-101*
375 *> Col-0 > lhy20 cca1-1 > lhy20 cca1-1 toc1-2* (Fig. 6(e)) again similar to secondary dormancy
376 induction in thermal time (Fig. 5(b)). The response to 50 nM ABA indicates these differences
377 are constant during germination (Fig. 6(f)). The overexpressing lines have similar ABA
378 sensitivity to their parental wild types (Fig. S7).

379

380 **Germination in the dark:** Germination is reported following transfer to the light including
381 the limited dark germination. Dark germination was also recorded to determine if
382 temperature manipulation replaced dormancy relief by light. There was no dark germination
383 in the first low temperature phase. In the high temperature phase dark germination in the
384 dormancy mutants peaked at 25% in *dog1-2* and 32% in *mft2* at 25°C; and 9% and 2% at
385 30°C (Fig. S8). In the clock mutants and over expressers, dark germination was 5% or less at
386 20 and 25°C (Fig. S9) with none at 30°C. In Col-0, maximum dark germination at high
387 temperature was 11% (Fig. S8 & S9). In the second low temperature phase, dark
388 germination shows little response. In *cipk23* and *phyA*, maximum dark germination was 2%.

389

390 **Discussion**

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391 Gene expression patterns during annual dormancy cycling in the field can differ from those
392 anticipated from more static laboratory dormancy studies (Footitt *et al*, 2011, 2013; Finch-
393 Savage and Footitt, 2017). We therefore established a robust laboratory simulation of
394 dormancy cycling in different *Arabidopsis* ecotypes by manipulating temperature and water
395 potential. The central role of temperature in dormancy cycling is well known (Probert 2000);
396 and the role of low water potential on the induction of secondary dormancy in the dark was
397 originally shown by Khan and Karssen (1980). Furthermore, primary dormancy status upon
398 shedding is known to influence subsequent cycling, for example, it can impact on the
399 induction of secondary dormancy by low water potential in Col-0 (Auge *et al.*, 2015). In the
400 experiments presented, ecotypic differences in the relief and induction of dormancy by the
401 temperatures used in the simulation were consistent with those previously shown for Bur,
402 Col-0, Ler, and Cvi (Cone and Spruit, 1983; Huang *et al.* 2015, Springthorpe and Penfield
403 2012, 2015). These differences presumably arose during adaptation to their specific climates
404 from a common underlying species response. This adaptation occurs in the initial depth of
405 primary dormancy and the subsequent balance of induction and relief so that cycling
406 behaviour may differ within and between ecotypes if the environment changes.

407

408 Initial depth of dormancy is determined by both genetics and environmental exposure pre-
409 and post-shedding (Finch-Savage and Footitt, 2017). The effect of the latter is illustrated
410 here in data from seeds of the winter annual ecotype Cvi from the same harvest, but with
411 different depths of dormancy resulting from post-harvest conditions. Seeds with greater
412 depth of dormancy (Fig. 1) did not become light sensitive upon exposure to low
413 temperature, but became more dormant. Whereas, a proportion of seeds in a less dormant
414 seed lot (Fig. 2) became light sensitive before the whole seed population subsequently

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415 became more dormant on continued exposure. Cycling behaviour therefore differed
416 depending on the environmentally determined initial depth of dormancy. In both seed lots
417 dormancy in Cvi was then relieved by exposure to higher temperatures (Footitt *et al.* 2011;
418 Huang *et al.* 2015). This is apparently in direct contrast to the results with the summer
419 annual ecotypes Bur, Col-0 and Ler in which increasing temperature accelerated the
420 induction of secondary dormancy (Figure 3; Cone and Spruit, 1983; Huang *et al.* 2015,
421 Springthorpe and Penfield, 2015). However, Col-0 can behave as both a winter and summer
422 annual in the field (Springthorpe and Penfield, 2015) suggesting secondary dormancy may
423 also be relieved by high temperature as seen in Cvi depending on the environment before
424 and after shedding. Thus in Col-0, further induction of secondary dormancy by low
425 temperature may be required before a change to high temperature results in relief.
426 However, this intriguing aspect of dormancy cycling in Col-0 is yet to be demonstrated.

427

428 The above results raise the question of how dormancy cycling is driven by temperature and
429 time (thermal time) to alter the balance between induction and relief of dormancy as part of
430 a dormancy continuum. In this continuum, as primary dormancy in the dispersed seed is
431 relieved, in response to the prevailing environmental conditions (predominantly
432 temperature), the same conditions will start to induce secondary dormancy if the
433 environmental signals required to remove the final layer of dormancy are not received. This
434 behaviour is consistent with the hypothesis that temperature impacts the rate of dormancy
435 induction and relief independently, but importantly that these processes may occur
436 simultaneously (Totterdell and Roberts, 1979; Battla *et al.* 2009). These opposing processes
437 are largely governed by the environmental sensitivity of the ABA/GA hormone balance
438 (Finch-Savage and Leuber-Metzger, 20016; Finch-Savage and Footitt, 2017). Initial primary

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20

439 dormancy level determines the temperature sensitivity of both induction and relief via
440 changes in this balance. The hypothesis implies that the terms primary and secondary
441 dormancy are only descriptive of sequences in the cycle with no physiological relevance as
442 dormancy is a continuum, and only the level changes.

443

444 The protocol presented could be used to test this hypothesis and further develop our
445 understanding of dormancy cycling by evaluating responses of different ecotypes to
446 temperature and water potential. The ecotypes used in the present work included Col-0 as
447 the common genetic background for 1,000's of mutant lines (<http://arabidopsis.info/>) to
448 facilitate genetic dissection of dormancy cycling. We discuss below how the protocol also
449 has great potential as an investigative tool in advancing our understanding of the role of
450 genes in dormancy regulation.

451

452 Regulation of dormancy cycling.

453 How ABA and GA -signalling pathways are co-ordinated during dormancy cycling by
454 temperature and water potential is not fully understood (Finch-Savage and Footitt, 2017). It
455 was argued previously (Footitt *et al.* 2013) that changing temporal signals linked to the
456 transcription of *DOG1*, *MFT*, *PHYA* and *CIPK23* drives regulation of dormancy cycling. *DOG1*
457 and *MFT* expression contributes to thermal time sensing linked to changes in *CIPK23* and
458 *PHYA* expression that result in altered sensitivity to spatial signals (nitrate and light
459 respectively) indicating suitability for germination. The data presented for mutants of these
460 genes subjected to the laboratory simulation of dormancy cycling (Fig. 3) supports the
461 correlative observations made in the field. Thermal time analysis showed that dormancy
462 induction in the absence of *DOG1* (*dog1-2*) was linear with thermal time and exponential in

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463 its presence (Fig. 5). This adds to the contention that *DOG1* is part of a thermal mechanism
464 sensing an annual seasonal pattern (circannual rhythm) and may amplify thermal signals by
465 increasing ABA sensitivity. Mutants in *CIPK23* and *PHYA* show an increased induction of
466 secondary dormancy. Both *PHYA* and *CIPK23* also influence hormone signalling consistent
467 with the importance of the dynamic ABA/GA balance determining dormancy levels in
468 response to environmental signals (Finch-Savage and Footitt, 2017).

469

470 **DOG1 and MFT:** During seed development *DOG1* is absolutely required for the induction of
471 dormancy (Dekkers *et al.* 2016). However, in *dog1-1* (Ler background) low dark germination
472 was seen in fresh seeds that could be removed by low temperature indicating a low level of
473 primary dormancy was present at maturity (Bentsink *et al.* 2006). In the dormancy
474 simulation, high temperature alone did not induce secondary dormancy in *dog1-2* as it had
475 high levels of dark germination followed by full germination on transfer to light (Fig. S5).
476 However, cold preconditioning at -1 MPa induced a low level of secondary dormancy at the
477 end of the initial cold phase. Light was increasingly unable to remove the final layer of
478 dormancy in Col-0 but not *dog1-2* (Fig. 2(a) days 21 to 28). This small loss of sensitivity to
479 light indicates that in the Col-0 genetic background secondary dormancy induction was
480 starting to dominate its relief. On transfer to the higher temperature this level of secondary
481 dormancy was sufficient to prevent dark germination in *dog1-2* as well as in Col-0 while
482 dormancy induction increased to the point where seeds were no longer light sensitive (Fig.
483 3). On the basis that any environmental signal that widens the conditions required for
484 germination is in effect altering dormancy (Finch-Savage and Footitt, 2012) we conclude
485 that the induction of a light requirement and the decreasing sensitivity to light with
486 increasing thermal time is evidence for the induction of secondary dormancy in *dog1-2*. This

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487 indicates that these conditions allow other factors to impose secondary dormancy in the
488 absence of *DOG1*. One potential candidate is *MFT*.

489

490 Induction of secondary dormancy in thermal time was slower in *dog1-2* than in *mft2*
491 indicating the primacy of *DOG1* over *MFT* during dormancy induction. Further research is
492 required to confirm a role for *MFT* in thermal sensing. The greatly reduced induction of
493 dormancy in *dog1-2* is consistent with *DOG1* amplifying thermal signals via increased
494 sensitivity to ABA. The dramatically lower ABA sensitivity of *dog1-2* reported here supports
495 this (Fig. 6(a-c)).

496

497 The loss of ABA sensitivity in *mft2* shows *MFT* contributes positively to ABA signalling (Fig.
498 6). This is via the oxylipin, 12-oxo-phytodienoic acid (OPDA), which acts through *MFT* to
499 induce ABA biosynthesis and sensitivity (Dave *et al.* 2016). Then *MFT* and ABA via a feedback
500 loop enhance OPDA levels further contributing to *DOG1* germination repression (Dave *et al.*
501 2016) explaining the ABA hypersensitive germination of *MFT* overexpressing lines (Hu *et al.*
502 2016). The delayed response to ABA compared to *dog1-2* may reflect declining ABA levels
503 when the OPDA pathway is blocked. In contrast, fully after-ripened *mft2* seeds are ABA
504 hypersensitive (Xi *et al.* 2010). This may reflect a changing temporal sensing role for *MFT*
505 dependent on ecotype and the seasonal onset of the dormancy cycle as reflected in altered
506 timing of *MFT* transcription in the field (Footitt *et al.* 2013, 2014). This role for *MFT* in
507 shallow dormancy when *DOG1* levels are low is discussed elsewhere (Finch-Savage and
508 Footitt, 2017).

509

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510 During the final low temperature phase secondary dormancy is broken faster in *dog1-2* and
511 *mft2*, than in the wild type. Low temperature treatment then re-induced secondary
512 dormancy in the wild type but not in these mutants showing that dormancy cycling at low
513 temperature is compromised.

514

515 **PHYA and CIPK23:** Unlike *mft2* and *dog1-2* secondary dormancy was induced in *phyA* and
516 *cipk23* by low temperature, which then accelerated on transfer to higher temperatures (Fig.
517 3(a) & S2). This induction of secondary dormancy at high temperature, its relief and re-
518 induction in the second low temperature phase is consistent with increased ABA sensitivity
519 compared to Col-0. This is supported by the ABA hypersensitivity of *cipk23* (Fig. 5(a-c)). The
520 limited ABA response of *phyA* reflects the increased contribution of other negative
521 regulators of germination potential in this mutant (Ibarra *et al.* 2013).

522

523 **PHYA:** PHYA is responsible for the Very Low Fluence Response whereby the final layer of
524 dormancy is removed by brief exposure to light during soil disturbance (Battla and Benech-
525 Arnold, 2014). The increased sensitivity of *phyA* seeds to temperature and water stress is
526 consistent with enhanced ABA sensitivity (Fig 3a and Fig 6(a-c)). Transcriptome comparisons
527 between wildtype and *phyA* seeds support this with 11% of the expressed transcriptome
528 significantly regulated by PHYA (Ibarra *et al.* 2013). Of those significantly up regulated by
529 PHYA, 7% are transcription factors linked with auxin and GA responses; and ABA catabolism.
530 While down regulated genes contain representatives of the ABA signalling pathways and
531 DELLA genes that relieve repression of GA signalling (Ibarra *et al.* 2013). So in *phyA* seeds
532 the balance of the ABA/GA signalling pathways favours ABA amplifying the response to
533 dormancy inducing temporal signals.

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534

535 **CIPK23:** This protein forms a calcium sensing complex with CBL1 or CBL9 (CALCINEURIN B-
536 LIKE PROTEIN), which is involved in iron, nitrate and potassium transport and sensing (Leran
537 *et al.* 2015; Manik *et al.* 2015; Tian *et al.* 2016). Its role in regulating nitrate transport and
538 signalling by the NITRATE TRANSPORTER1.1 (NRT1.1) transceptor (dual nutrient
539 transport/signalling function) and the crucial role this plays in the regulation of ABA levels in
540 seeds is well characterised (reviewed in Finch-Savage and Footitt, 2017).

541

542 The ABA hypersensitivity of *cipk23* seeds (Fig 6(a-c)) indicates ABA signalling is enhanced in
543 the absence of CIPK23. In the field, low dormancy is coincident with increased nitrate
544 sensing, which is preceded by enhanced NRT1.1 expression and reduced CIPK23 expression.
545 The subsequent onset of secondary dormancy induction appears to reduce nitrate signalling
546 below threshold levels both by reducing the amount of NRT1.1 and its phosphorylation via
547 CIPK23-CBL1/9 (reviewed in Finch-Savage and Footitt, 2017; Footitt *et al.* 2011; 2013, 2014).
548 Here Col-0 and *cipk23* lose light but not nitrate sensitivity during induction of secondary
549 dormancy suggesting loss of nitrate sensitivity is related to NRT1.1 protein levels and the
550 action of factors such as DOG1 that regulate deep dormancy (reviewed in Finch-Savage and
551 Footitt, 2017). CIPK23-CBL complexes also have other functions, for example as nutrient
552 sensors to monitor mineral homeostasis in general (Tian *et al.* 2016). Further work is
553 therefore needed to fully understand the role of CIPK23 in dormancy regulation.

554

Dormancy regulation and clock genes:

556 The annual seasonal rhythm of soil temperature (Fig. 6a and b) was correlated with
557 transcriptional responses of the dormancy related genes discussed above (see Table S1).

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25

558 During seasonal bud dormancy regulation in trees, perception of seasonal temperature
559 signals involves components of the circadian clock (Cooke *et al.* 2012). We tested if this
560 could also be occurring in seeds by analysis of clock mutants in the laboratory simulation
561 and by measuring clock gene expression over an annual cycle in the field. The results
562 obtained were consistent with the balance between the evening and morning phases of the
563 clock contributing to the interpretation of temperature signals (thermal time) that
564 determine cycles of dormancy induction and relief.

565

566 **Clock mutants in the laboratory simulation:** In this series of laboratory simulations
567 successive relatively long-term incubations at constant temperatures in the dark show the
568 clock has an impact on dormancy status without an imposed external daily rhythm (Fig. 3,
569 S2, S3, & S4). In the parental wild type (Col-0) secondary dormancy was induced on transfer
570 to high temperature and increased further as temperature was raised (20 > 25 > 30°C). Lines
571 with mutations in the morning genes *LHY* and *CCA1* (*lhy20 cca1-1* and *lhy20 cca1-1 toc1-2*)
572 had the highest ABA sensitivity and the most rapid induction of secondary dormancy.
573 Whereas, the triple mutant *prp5 11 prp7-11 prp9-10* had the lowest ABA sensitivity and
574 slowest induction (Fig. 6 (d-f)). This disruption of the morning loop by mutations in *LHY* and
575 *CCA1* would reduce repression of the evening loop genes *TOC1*, *Gi* and the evening complex
576 genes *LUX*, *ELF3* and *ELF4* (Pokhiloko *et al.* 2013). The *prp5 11 prp7-11 prp9-10* mutant would
577 reduce repression of *LHY* and *CCA1*. Therefore, this result indicates a critical balance
578 between the morning and evening signalling components of the clock influences the
579 induction of dormancy. It further implies that in the absence of a fully functioning morning
580 loop repression of *TOC1*, *Gi* and the evening complex genes is incomplete. This is consistent
581 with observations of delayed bud burst (loss of dormancy) in *Populus* *LHY* mutants (Ibanez

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582 *et al.* 2010). These data are also consistent with clock gene transcription recorded during
583 the annual soil temperature cycle in the field (Fig. 4) and are discussed below. This field data
584 indicates the annual seasonal cycle is analogous to an extended diurnal cycle with low
585 winter temperatures representing the evening phase, and summer temperatures the
586 morning phase (circannual dormancy rhythm). Thermal time analysis (Fig. 5) shows that
587 dormancy cycling responds to the strength of the inductive thermal time signal generated
588 by the clock.

589

590 **Annual clock gene expression in the field:** We followed gene expression in the contrasting
591 ecotypes Bur (summer annual) and Cvi (winter annual). The transcript profiles of evening
592 genes increased with falling temperature and therefore in general were negatively
593 correlated to the annual soil temperature cycle in both ecotypes (Table S1). Surprisingly, the
594 morning genes *LHY* (in Cvi) and *CCA1* (in Bur) have the same transcript profiles as *TOC1*.
595 While only *LHY* transcription in Bur correlates positively with temperature. This contrasts
596 the general situation in the clock where the transcript profile of *TOC1* is in the opposite
597 phase to both *LHY* and *CCA1* (Salome and McClung, 2005; Gould *et al.* 2006). However, it is
598 consistent with high transcription of *TOC1* and *LHY* in Chestnut internodes during winter
599 when the clock becomes arrhythmic (Ibanez *et al.* 2008). Notably *LHY* (in Cvi) and *CCA1* (in
600 Bur) transcription do not return to the opposite phase of *TOC1* in the warm summer
601 months. It is also notable that in Bur *TOC1* transcription also increases with summer
602 temperature and at that point is similar to both *LHY* and *CCA1*. This suggests that adaptation
603 of dormancy cycling to the environment may involve allelic variation in clock genes as seen
604 in *Drosophila* (Yamada and Yamamoto, 2011).

605

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606 Components of the clock will alter the central integrating ABA/GA balance controlling
607 dormancy cycling. *TOC1* and the clock are involved in the gating of ABA responses (Seung *et*
608 *al.* 2012). *TOC1* is induced by ABA and interacts with genes involved in ABA signalling
609 responses (Seung *et al.* 2012). In addition it interacts with the positive regulator of
610 dormancy *ABA INSENSITIVE3 (ABI3)* (Kurup *et al.* 2000). *ABI3* mutants also exhibit altered
611 circadian rhythms (Pearce, 2003). The consequence of increased *TOC1* transcription
612 therefore appears to be an upregulation in ABA signalling. Gibberelin biosynthesis is
613 repressed by the evening loop with increased expression of the GA biosynthesis gene
614 *GA20OX2* found in *toc1*; and increased levels of bioactive Gibberelins and *GA20OX2* found
615 in *elf3* (Atamian and Harmer, 2016). This again indicates evening loop involvement in
616 dormancy cycling.

617

618 Dormancy and ABA levels initially increase together, but a point is reached where dormancy
619 increases are ascribed to increasing ABA sensitivity via *DOG1* (Footitt *et al.* 2011).
620 Interestingly circadian rhythm micro array data from Col-0 seedlings (Edwards and Millar
621 2007) shows rhythmic *DOG1* transcription (See: [http://bar.utoronto.ca/efp/cgi-](http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Light_Series)
622 [bin/efpWeb.cgi?dataSource=Light_Series](http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Light_Series)) (Fig. S10). In contrast, morning gene transcription
623 is more positively correlated with genes up-regulated in the spatial sensing phase of the
624 dormancy cycle (Table S1).

625

626 Circannual dormancy rhythm:

627 Annual cycling of the depth of dormancy is well documented (Baskin and Baskin, 1998) and
628 understanding of how this is regulated by a range of dormancy mechanisms in response to
629 environmental signals is developing (Finch-Savage and Footitt, 2017). These mechanisms

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630 operate via a central integrating ABA/GA balance to time germination completion in the
631 optimum season and habitat. Here we confirm the key involvement of *DOG1*, *MFT*, *CIPK23*
632 and *PHYA* in regulating the depth of dormancy. Furthermore we show based on mutant
633 analyses and transcript profiles that the balance between the evening and morning phases
634 of the clock also reflects this circannual dormancy rhythm. Based on the thermal time and
635 ABA sensitivity data, dormancy cycling appears to respond to the strength of the inductive
636 thermal time signal generated by the clock. Further directed research is required to test
637 these hypotheses and provide detail of the clocks involvement. Nevertheless, circannual
638 rhythms for germination timing are seen in seeds of the desert annual *Mesembryanthemum*
639 *nodiflorum* and in cysts of the marine dinoflagellate *Alexandrium* in constant conditions over
640 several years (Gutterman and Gender 2005; Matrai *et al.* 2015) and may be part of a bet
641 hedging strategy. How a circannual clock contributes to and maintains annual rhythms over
642 several years is unclear.

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650 experiments. S.F and A.J.H. performed all other experiments. All authors analysed data. S.F
651 and W.E.F-S wrote the manuscript.

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820 Supporting information

821

822 Text S1. Materials and methods

823

824 Table S1. Correlation table

825

826 Table S2. Primers

827

828 Figure S1. Box layout for incubation of seeds at reduced water potential.

829

830 Figure S2. Simulated dormancy cycling in dormancy related mutants at 25°C and 30°C.

831

832 Figure S3. Simulated dormancy cycling in clock mutants at 20°C, 25°C and 30°C.

833

**834 Figure S4. Simulated dormancy cycling in *CCA1* and *LHY* overexpressing lines at 20°C, 25°C
835 and 30°C.**

836

837 Figure S5. Response of dormancy related mutants when placed directly in high**838 temperature without cold conditioning at low water potential.**

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840 **Figure S6. Thermal time analysis of dormancy induction at high temperature following low**
841 **temperature conditioning of the dormancy mutants' *dog1-2* and *mft2* and clock mutants.**

842

843 **Figure S7. ABA sensitivity of Col-0 and Ler wild types and *CCA1* and *LHY* overexpressing**
844 **lines.**

845

846 **Figure S8. Dark germination of Col-0, and the dormancy mutants *dog1-2* and *mft2*.**

847

848 **Figure S9. Dark germination of Col-0, clock mutants and *CCA1* and *LHY* overexpressing**
849 **lines.**

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851 **Figure S10. *DOG1* transcript level in Col-0 seedlings entrained to a light/dark cycle.**

852

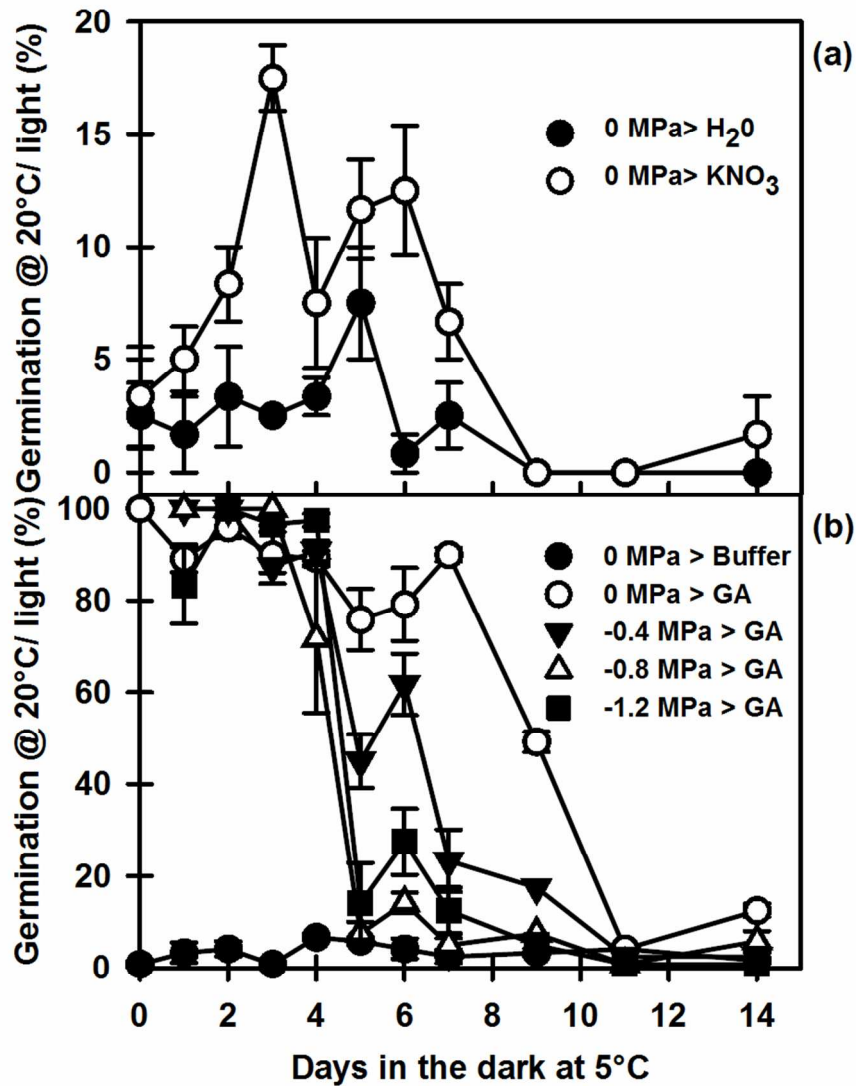


Figure 1. Induction of secondary dormancy in Cvi in response to cold stratification and decreasing water potential. Primary dormant Cvi seeds were incubated at 5°C/dark on water or a range of water potentials from -0.4 to -1.2 MPa. At increasing periods of time dormancy status was determined by measuring germination following transfer of seeds to (a) water or 10 mM KNO₃, or (b) a buffer control or 250 μM GA₄₊₇ buffered at pH 5.0 at 20°C/light for 28 days. Data are mean ± SE (n = 3). Absence of error bars indicates SE is smaller than the symbol.

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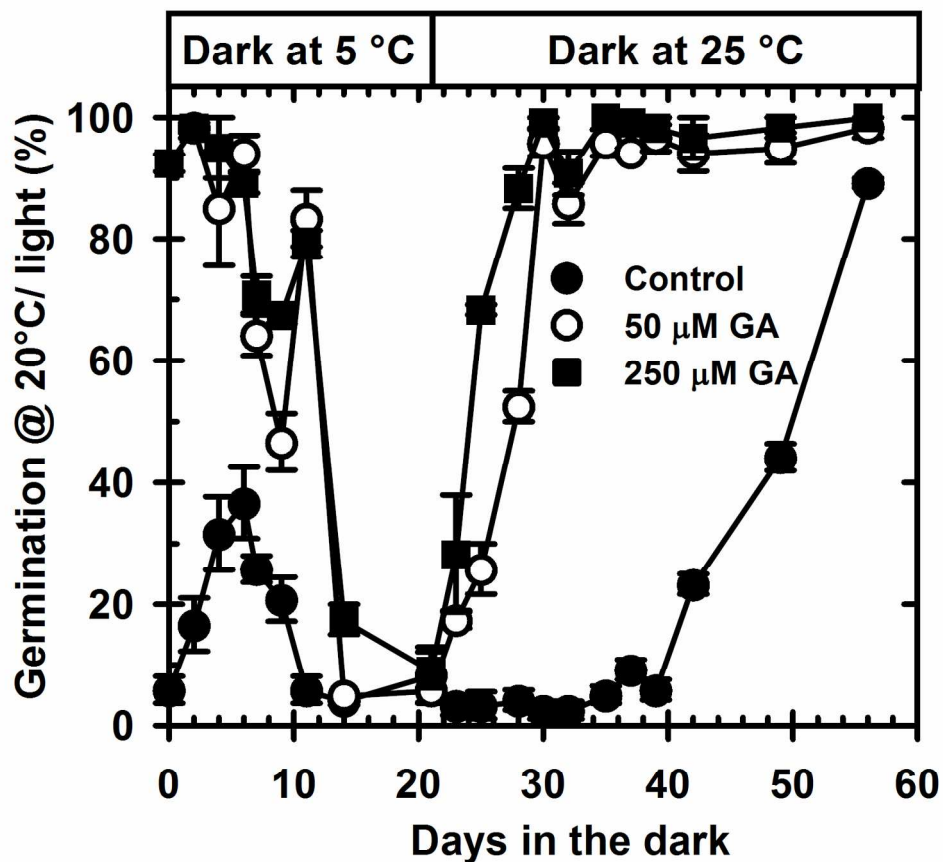


Figure 2. Simulated dormancy cycling in *Cvi*: Seeds were incubated at 5°C/dark at -1.2 MPa for up to 21 days before being transferred to water at 25°C/dark. At increasing periods of time dormancy status was determined by measuring germination following transfer of seeds to a buffer control, 50 or 250 μM GA4+7 buffered at pH 5.0 at 20°C/light for 28 days. Data are mean ± SE (n = 3). Absence of error bars indicates SE is smaller than the symbol.

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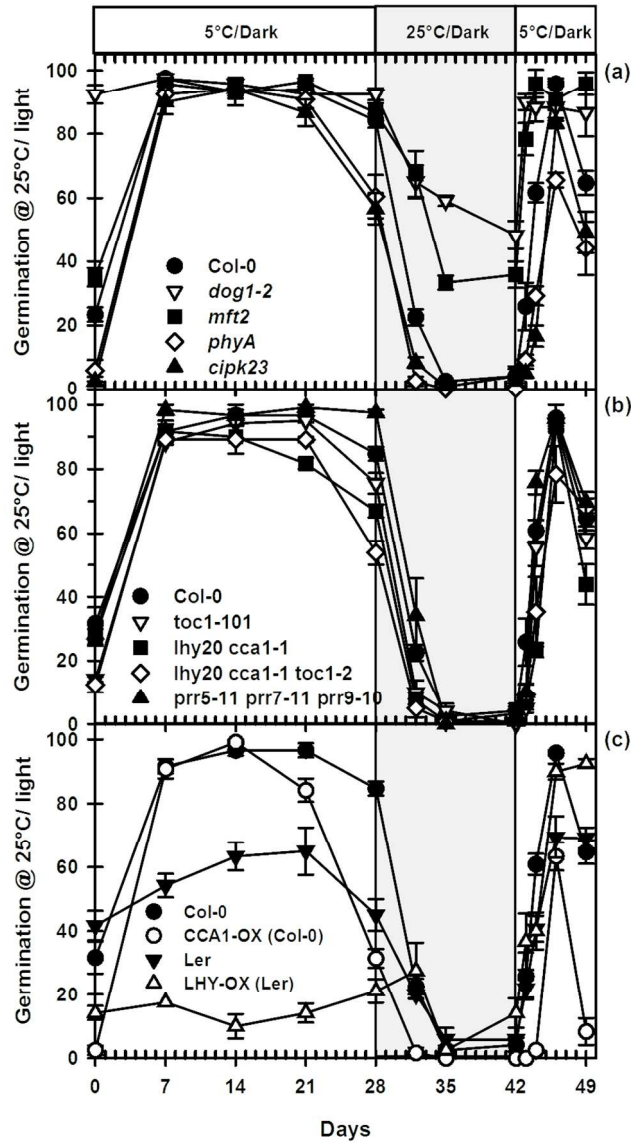


Figure 3. Simulated dormancy cycling in Col-0, Ler and mutants in dormancy related and clock genes. Following 5°C/dark at -1.0 MPa for 28 days seeds were transferred to water and incubated in the dark at 25°C for 14 days before transferring to 5°C/dark. At increasing intervals dormancy status was determined by measuring germination on water at 25°C/light for 14 days. (a) Dormancy related mutants. (b) Circadian clock mutants. (c) CCA1 and LHY overexpressing lines. Data are mean \pm SE ($n = 3$). Absence of error bars indicates SE is smaller than the symbol.

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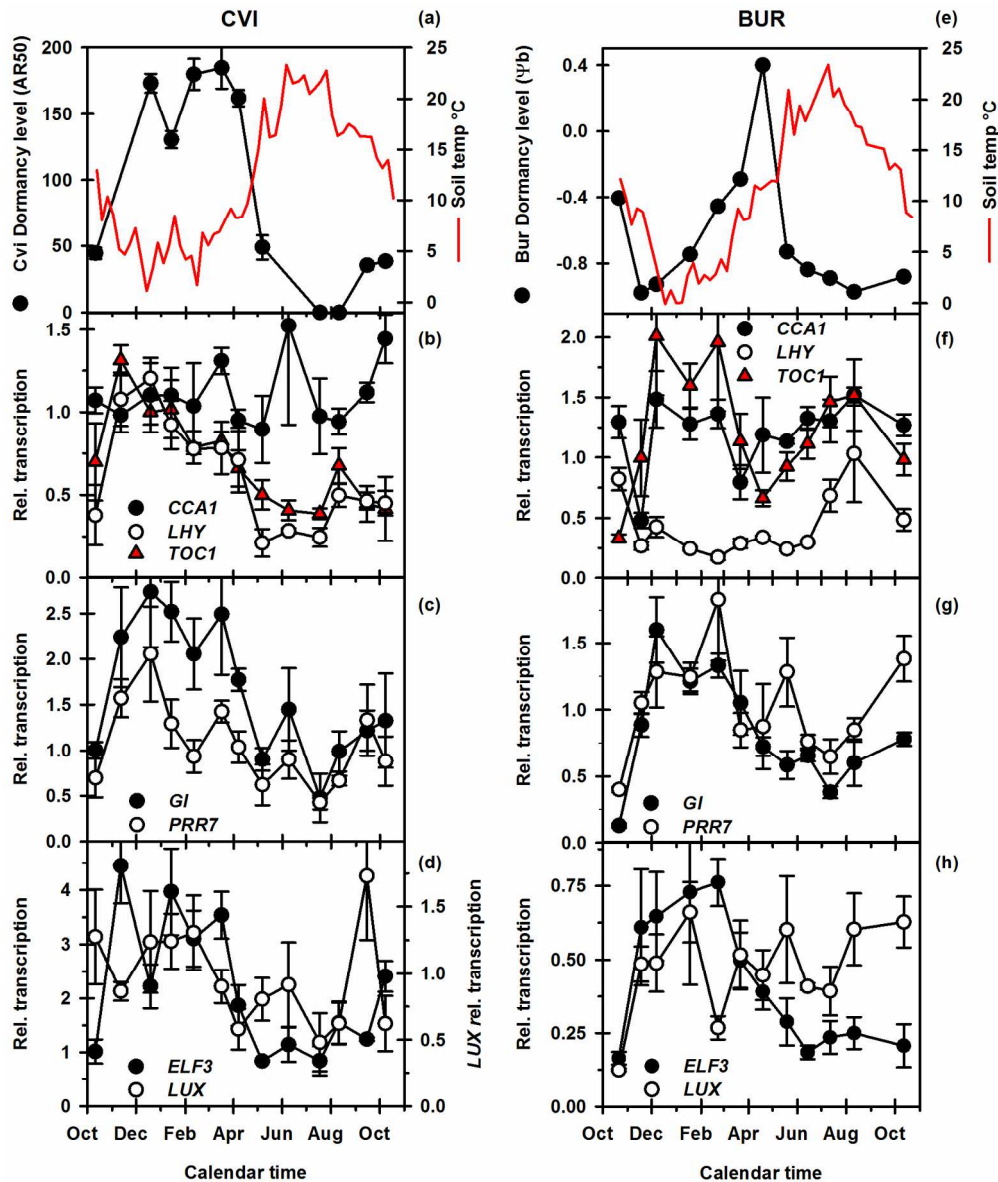


Figure 4. Seasonal coordination of clock gene transcription in winter (Cvi) and summer annual (Bur) ecotypes. Depth of dormancy in (a) Cvi (time to 50% after-ripening (AR50)) and (e) Bur (base water potential (Ψ_b)) with soil temperature at seed depth (Data from Footitt et al. 2011 and 2013). Transcription profiles of the morning genes *CCA1*, *LHY*, and the evening gene *TOC1* in (b) Cvi and (f) Bur. Transcription profiles of *GI* and *PRR7* in (c) Cvi and (g) Bur. Transcription profiles of evening complex genes *ELF3* and *LUX* in (d) Cvi and (h) Bur.

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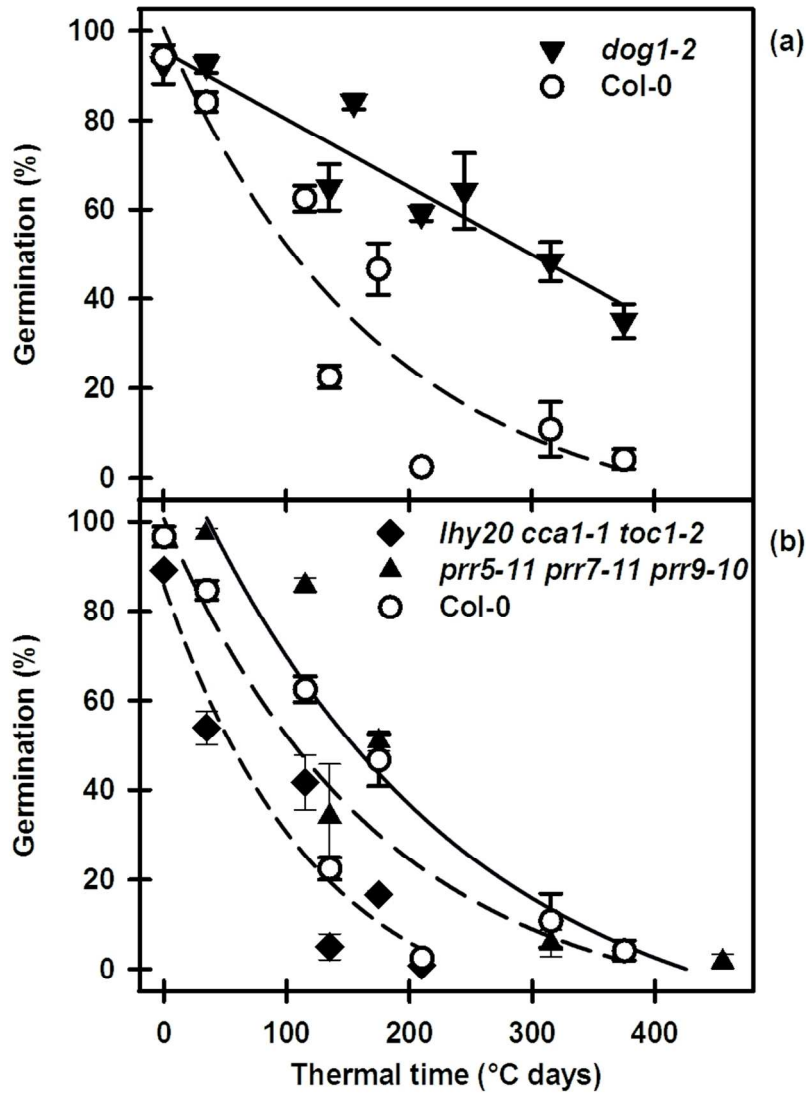


Figure 5. Thermal time analysis of dormancy induction at high temperature following low temperature conditioning. (a) Wild type (Col-0) and the dormancy mutant, *dog1-2*. (b) Wild type (Col-0) and the circadian clock mutants, *lhy20 cca1-1 toc1-2* and *prp5-11 prp7-11 prp9-10*. Data from Fig. 3, S1 and S2 are replotted against thermal time (sum of temperature above 0°C) for secondary dormancy induction at 20, 25 and 30°C. The response to thermal time fits the following relationships: Exponential decay (3 parameter) regressions describe Col-0 ($R^2 = 0.972$), *lhy20 cca1-1 toc1-2* ($R^2 = 0.897$) and *prp5-11 prp7-11 prp9-10* ($R^2 = 0.860$); while a linear regression describes *dog1-2* ($R = 0.928$). The same data for Col-0 appears in (a) and (b).

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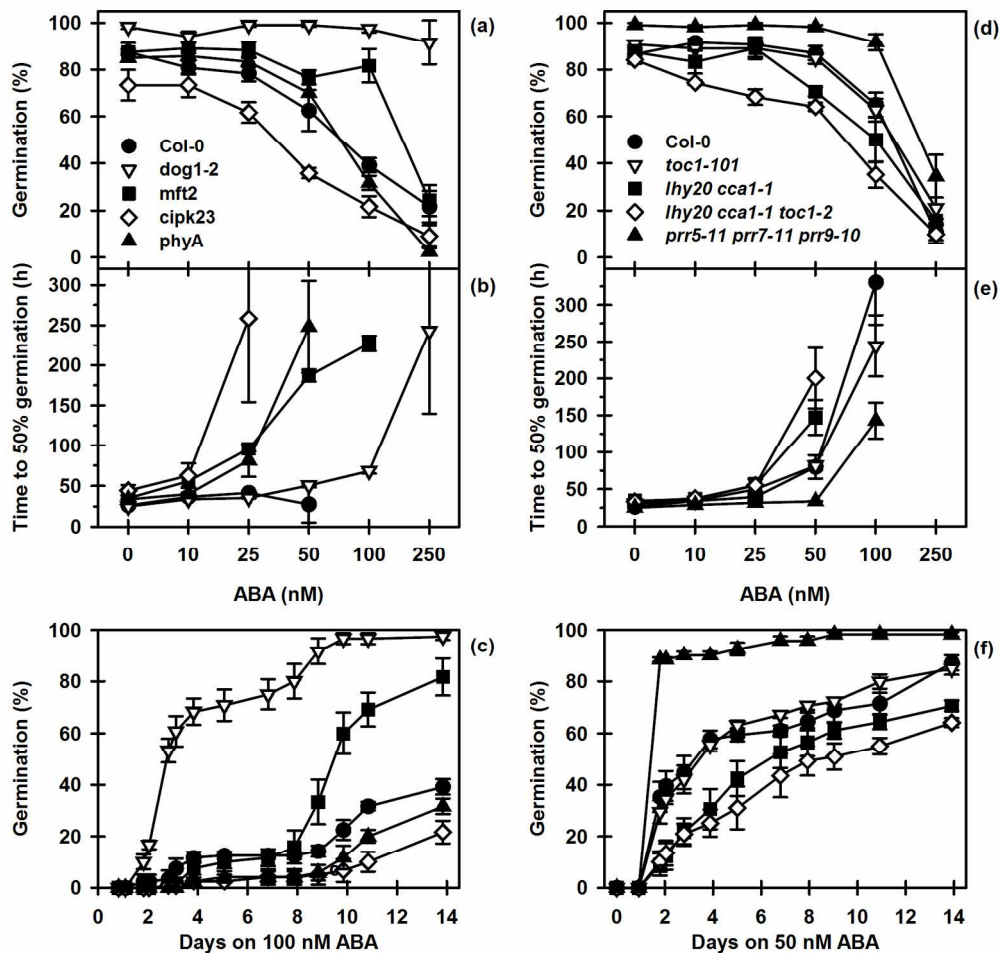


Figure 6. ABA sensitivity of dormancy and clock mutants. Following three days at 5°C/dark on water seeds were transferred to ABA (10 – 250 nM) in buffer at pH 5.0 and cumulative germination recorded during incubation at 25°C/light over 14 days. Final germination at each concentration after 14 days (a) and (d).

The time to 50% germination (b) and (e) in hours (h) of data in (a) and (d) respectively. Cumulative germination of dormancy mutants in the presence of 100 nM ABA (c). Cumulative germination of clock mutants in the presence of 50 nM ABA (f). Data are mean \pm SE ($n = 3$). Absence of error bars indicates SE is smaller than the symbol.

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