1 **Altitudinal and climatic associations of seed dormancy and flowering traits evidence**
2 **adaptation of annual life cycle timing in *Arabidopsis thaliana***
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ABSTRACT

The temporal control or timing of the life cycle of annual plants is presumed to provide adaptive strategies to escape harsh environments for survival and reproduction. This is mainly determined by the timing of germination, which is controlled by the level of seed dormancy, and of flowering initiation. However, the environmental factors driving the evolution of plant life cycles remain largely unknown. To address this question we have analysed nine quantitative life history traits, in a native regional collection of 300 wild accessions of *Arabidopsis thaliana*. Seed dormancy and flowering time were negatively correlated, indicating that these traits have coevolved. In addition, environmental-phenotypic analyses detected strong altitudinal and climatic clines for most life history traits. Overall, accessions showing life cycles with early flowering, small seeds, high seed dormancy and slow germination rate were associated with locations exposed to high temperature, low summer precipitation and high radiation. Furthermore, we analysed the expression level of the positive regulator of seed dormancy *DELAY OF GERMINATION 1* (*DOG1*), finding similar but weaker altitudinal and climatic patterns than seed dormancy. Therefore, *DOG1* regulatory mutations are likely to provide a quantitative molecular mechanism for the adaptation of *A. thaliana* life cycle to altitude and climate.

Keywords:

Arabidopsis, natural variation, life cycle, seed dormancy, flowering time, *DELAY OF GERMINATION 1* (*DOG1*), adaptation, climate, *cis*-regulation

Introduction

The life cycle of annual plants is characterised by three major developmental phases: a vegetative growth period that begins with seed germination, a reproductive growth phase that starts with flowering initiation, and a growth arrest period corresponding to the dormant seed bank. The phenology or temporal control of the life cycle is presumed to provide adaptive strategies to avoid harsh environments for seedling establishment or seed development (Chiang *et al.* 2013; Krämer, 2015). This has been supported by the occurrence of both active growth phases during the seasons that provide the most suited environmental conditions for plant survival and reproduction. In addition, substantial natural variation has been described for the timing of the life cycle in natural populations of most annual plants (Montesinos *et al.* 2009; Toorop *et al.* 2012). The evolutionary and molecular bases of the genetic diversity for annual life cycles has begun to be elucidated in the model and wild plant *Arabidopsis thaliana*, whose populations have been classified as winter or spring annuals (Donohue, 2002; Alonso-Blanco *et al.* 2009; Picó, 2012). Plants with winter life cycle germinate in autumn, overwinter as seedlings or rosettes, and flower and disperse seeds in next spring. In contrast, plants with spring life cycle germinate, grow to maturity, flower, set seed and disperse their seeds in the same spring or summer season (Donohue, 2002). Consequently, annual life cycles are mostly determined by the timing of germination under natural conditions, which is controlled by the level of seed dormancy, and by the timing of flowering initiation. Both temporal life history traits show a large amount of quantitative variation among wild accessions of *A. thaliana* analysed under natural or laboratory conditions (Donohue *et al.* 2005a; Lempe *et al.* 2005; Montesinos *et al.* 2009; Kronholm *et al.* 2012; Manzano-Piedras *et al.* 2014). This genetic variation is presumed to reflect adaptations of the life cycle to the broad environmental and ecological diversity spanned by *A. thaliana* in Eurasia (Hoffmann, 2002, Krämer, 2015).

Seed dormancy and flowering time appear as complex plastic traits that have been acquired during the evolution of many plant species, to provide developmental and physiological mechanisms for adaptation to the environment. Seed dormancy contributes to

77 plant adaptation by preventing germination under occasional favourable conditions for
78 seedling establishment, and by extending germination over time (Baskin and Baskin, 1998;
79 Donohue *et al.* 2005b; Fenner & Thompson, 2006). The timing of flowering initiation
80 determines the individual reproductive success (Roux *et al.* 2006). These two life history
81 traits control the time invested in vegetative growth and the time to reproduction, which is
82 achieved by integrating the information from multiple environmental signals, including
83 temperature, light photoperiod and intensity, or nutrient availability (Andrés & Coupland
84 2012; Bewley *et al.* 2013). Overall, seed dormancy and flowering time are affected by
85 environmental and genetic factors, and genotype by environment interactions reflect the
86 genetic variation for their phenotypic plasticity across different environments (Footitt *et al.*
87 2014; He *et al.* 2014; Mendez-Vigo *et al.* 2015). Therefore, understanding the genetic and
88 ecological mechanisms that drive the evolution of life cycles in annual plants requires the
89 simultaneous analysis of both temporal life history traits (Donohue, 2009; Toorop *et al.* 2012;
90 Chiang *et al.* 2013; Debieu *et al.* 2013; Burghardt *et al.* 2015; Springthorpe and Penfield,
91 2015). However, most current studies have been focused mainly on the analysis of flowering
92 time variation due to its easier technical amenability (Alonso-Blanco *et al.* 2009; Weigel,
93 2012).

94 In the past few years, the genetic architecture of the intraspecific variation for
95 flowering time and seed dormancy of *A. thaliana* has been dissected by quantitative trait
96 locus (QTL) analyses (Alonso-Blanco *et al.* 2009; Weigel, 2012). Thirteen genes have been
97 demonstrated to contribute to the natural variation for flowering time (Alonso-Blanco and
98 Mendez-Vigo, 2014). In contrast, only the seed dormancy locus *DELAY OF GERMINATION*
99 *1* (*DOG1*) encoding a protein of unknown function has been isolated (Bentsink *et al.* 2006,
100 2010). Artificial loss-of-function mutants of *DOG1* show no dormancy after harvest, indicating
101 that this gene is essential to induce seed dormancy. It has been shown that *DOG1* effects
102 are largely mediated by regulation of its gene expression because it is seed specific, it peaks
103 during the last phase of seed development, and maternal temperatures affect both, *DOG1*
104 expression and dormancy (Bentsink *et al.* 2006; Nakabayashi *et al.* 2012; Chiang *et al.*

105 2013). In addition, analyses of natural *DOG1* alleles have identified several *cis*-regulatory
106 polymorphisms that affect seed dormancy (Bentsink *et al.* 2006). Nevertheless, *DOG1*
107 haplotypes differentiated by structural polymorphisms have been found to be associated with
108 seed dormancy, thus suggesting that DOG1 protein variation also contributes to local
109 adaptation (Kronholm *et al.* 2012).
110 Despite the recent progress in understanding the genetic and molecular
111 mechanisms underlying the natural variation for flowering time and seed dormancy in *A.*
112 *thaliana*, the environmental factors that contribute to maintain this quantitative variation
113 remain largely unknown. To this end, world-wide collections of wild accessions have been
114 used to carry out genetic-environment correlation analyses in sets of populations spanning a
115 wide range of environments. These analyses require geographically-explicit approaches
116 taking into account the spatial autocorrelation patterns that affect the independence among
117 samples (Sokal & Oden, 1978). In this way, significant environmental clines have been
118 detected for multiple flowering traits and genes (Caicedo, *et al.* 2004, Stinchcombe *et al.*
119 2004; Balasubramanian *et al.* 2006; Hancock *et al.* 2011; Li *et al.* 2014). However, mainly
120 latitudinal associations have been reported for seed dormancy and for *DOG1* expression
121 (Chiang *et al.* 2011; Debieu *et al.* 2013). In general, the low frequency and the strong
122 geographic structure displayed by most natural alleles (Cao *et al.* 2011) has limited the
123 potential of world-wide collections to detect significant genetic and environmental
124 associations (Bergelson & Roux, 2010). To overcome these limitations, several regional
125 collections of accessions have been developed from different world regions (Samis *et al.*
126 2012; Brachi *et al.* 2013; Long *et al.* 2013). In particular, a set of wild accessions collected
127 from the Iberian Peninsula (Picó *et al.* 2008; Manzano-Piedras *et al.* 2014) has been shown
128 to provide an ideal scenario to study *A. thaliana* adaptation because this region is part of the
129 species native range (Hoffmann, 2002), it spans a large diversity of climates, altitudes (0-
130 2600 m) and ecological habitats (Myers, *et al.* 2000; Ninyerola, Pons & Roure, 2000;
131 Manzano-Piedras *et al.* 2014), and it has been shown to contain the largest amount of
132 genetic variation of *A. thaliana* in Eurasia (Picó *et al.* 2008; Cao *et al.* 2011). This collection

133 appears as a unique resource to uncover complex genetic and environmental associations
134 due to its large size, its ecological unbiased coverage and its precise environmental
135 documentation (Manzano-Piedras et al., 2014). Furthermore, previous analyses of different
136 subsets of this collection have found substantial natural genetic variation for flowering time
137 (Méndez-Vigo *et al.* 2011; Manzano-Piedras *et al.* 2014) and seed dormancy (Kronholm *et*
138 *al.* 2012).

139 In this study we aim to identify environmental factors that might drive the evolution of
140 life cycles in annual plants. To this end, we have analysed *A. thaliana* regional collection
141 from the Iberian Peninsula for the two major components that control the timing of life cycles,
142 seed dormancy and flowering time, as well as for other related life history traits, such as the
143 rate of seed germination and seed size. These quantitative phenotypic data have been used
144 to carry out association analyses with geographic and environmental factors, including
145 climatic, ecological and edaphic variables. In addition, we intend to determine if the
146 regulatory genetic variation affecting *DOG1* expression might provide a molecular
147 mechanism contributing to the adaptation of annual life cycles.

148

149 **Materials and Methods**

150 **Plant material and growth conditions**

151 A regional collection of 300 wild accessions of *A. thaliana* sampled in different local
152 populations from the Iberian Peninsula was analysed (Picó *et al.* 2008; Manzano-Piedras *et*
153 *al.* 2014). Each accession corresponds to the selfed progeny produced by a single random
154 individual collected per population during 2000 and 2010. All accessions are genetically
155 different according to their genotypes for 250 SNP markers (Manzano-Piedras et al., 2014).

156 To obtain the samples of seeds for the analyses of seed traits, all accessions were
157 multiplied in a single experiment containing six replicates per accession and one plant per
158 replicate in a greenhouse at Wageningen University (The Netherlands) in 2013. To
159 synchronise the seed production of all accessions, these were classified in three groups

160 according to their flowering time, late, intermediate or early, which were planted on three
161 different dates and received 8, 4 or 2 weeks of cold treatment (vernalization), respectively.
162 Seeds were sown on water soaked filter paper in Petri dishes and incubated for four
163 days in a cold room at 4 ºC in the dark to break dormancy (seed stratification). Subsequently,
164 the Petri dishes were transferred to a germination cabinet at 22 ºC (16 hours light per day)
165 for four days before planting. Germinated seedlings were transferred to a greenhouse,
166 placed on 4x4 cm^2 Rockwool plugs and watered with 1 g/l Hyponex fertilizer (NPK = 7:6:19).
167 After three weeks, plants were moved to a climate chamber for vernalization (4°C; 70% RH;
168 12 h of light per day). Subsequently, plants were transferred to the greenhouse and grown in
169 a complete randomized block design with six replicates. Five or six replicates were harvested
170 for each of 252 accessions, while three or four replicates could be harvested for most of the
171 remaining accessions (Supporting Information Table S1).
172

173 **Quantitative analysis of life history traits**

174 To measure seed dormancy, germination tests were performed weekly until dormancy had
175 been released from all accessions (> 90% of germination). Germination experiments were
176 performed in plastic trays (15x21 cm^2) containing 47 ml water and two layers of blue filter
177 paper. Six samples of approximately 50–100 seeds were dispersed on the filter paper, using
178 a mask to ensure accurate and reproducible spacing. Trays were kept in an incubator at 22
179 ºC and constant light, during five days. Photographs were taken once a day and they were
180 analysed by the Germinator software package (Joosen *et al.* 2010) to calculate the maximum
181 percentage of germination (Gmax). Seed dormancy was quantified as DSDS50 (days of
182 seed dry storage required to reach 50% of germination), which was calculated according to
183 He *et al.* (2014). In addition, seed dormancy was also estimated as DSDS10 and DSDS90
184 (days of seed dry storage required to reach 10% and 90% of germination, respectively)
185 calculated from germination-time fitted curves.
186 Germination after cold stratification (GAS) was estimated 55 days after harvest
187 (DAH). For that, imbibed seeds were placed for 10 days at 4°C and thereafter they were

188 transferred to an incubator at 22°C and constant light. Photographs were taken three times a
189 day and Gmax was estimated as described above. In addition, these pictures were used to
190 measure the rate of germination with three different variables that were calculated with the
191 Germinator package: the time required for 10% and 50% germination of the non-dormant
192 seeds, referred to as t10 and t50 respectively; and the uniformity of germination, U8416,
193 defined as the time interval between 84% and 16% of viable seeds to germinate (Joosen *et*
194 *al.* 2010).

195 Seed size was analysed by image analysis from photographs of imbibed seeds on
196 blue filter paper using a Nikon D80 camera fixed to a repro stand with a 60 mm macro
197 objective. Photographs were analysed using ImageJ (http://rsbweb.nih.gov/ij/) by combining
198 colour thresholds ($Y_{0\text{-}255}U_{0\text{-}255}V_{140\text{-}255}$) with particle analysis.

199 Flowering time (FT) was measured as the number of days from the planting date
200 until the anthesis of the first flower. For this, plants were grown at the CNB-CSIC (Spain) in a
201 growth chamber at 21°C with a long-day photoperiod (16 hours light: 8 hours darkness), as
202 previously described (Méndez-Vigo *et al.* 2011). All accessions were grown simultaneously in
203 a single experiment organised in a three-complete-blocks design, which included six plants
204 per accession in each pot and block. The experiment was finished after 220 days, and this
205 FT value was given to accessions that had not flowered at that time. These non-flowering
206 accessions correspond to about 20% of genotypes from the Iberian Peninsula, which have
207 been previously shown to have an obligate vernalization requirement (Méndez-Vigo *et al.*
208 2011).

209

210 **Geographical and environmental data**

211 The 300 local populations of *A. thaliana* covered a region of around 800 x 700 km^2 (Figure 1)
212 and were *in situ* geo-referenced for their latitude, longitude, and altitude with a global
213 positioning system receiver (Supporting Information Table S1). They were spaced at an
214 average distance of 357 ± 202 km, with a minimum and maximum of 1 and 1042 km
215 respectively. Altitudes ranged from 0 to 2600 m above sea level. Environmental information

216 (including climate, landscape use, and soil pH) was collected as described previously
217 (Méndez-Vigo *et al.* 2011; Manzano-Piedras *et al.* 2014). Briefly, climatic data of each
218 population location were obtained from the Digital Climatic Atlas of the Iberian Peninsula
219 (http://www.opengis.uab.es/ wms/iberia/index.htm) developed at a 200-m resolution following
220 the climatic models described by Ninyerola *et al.* (2000). Models were based on
221 meteorological records of 15 to 50 years, for the period 1950 to 1999, from 2285
222 meteorological stations located across the Iberian Peninsula. The following climatic variables
223 were obtained for each location: mean monthly and mean annual temperature, mean
224 minimum and maximum monthly and annual temperature, total monthly and total annual
225 precipitation, and mean monthly and mean annual solar radiation. Population habitats were
226 quantified as the proportions of anthropic and natural types of vegetation cover in each
227 location, which were estimated from the CORINE Land Cover Map (http://www.idee.es). The
228 land cover in a 78-ha circular area around the global positioning system coordinates of each
229 location was classified as the proportion of the following categories: urban, crops, bushes,
230 and woods. Anthropic and natural land cover was estimated by summing the proportional
231 cover of urban and crops, and bushes and woods, respectively. Soil pH was obtained from
232 the Soil Geographical Database of Eurasia v.4 (http://eusoils.jrc.ec.europa.eu).
233

234 ***DOG1* expression**

235 To analyse *DOG1* gene expression, 100 accessions covering the whole dormancy range
236 were selected (Supporting Information Figure S1). Three biological replicates per accession
237 were used to quantify cDNA amplification of *DOG1* (RT-qPCR) using the iQ SYBR green
238 supermix (Bio-rad). RNA was isolated from fresh dormant seeds that were stored at -80 °C,
239 using the Nucleospin RNA plant kit (Macherey-Nagel) according to the manufacturer's
240 protocol but adding Plant RNA Isolation Aid (Life technologies). cDNA was synthesized using
241 the iScript cDNA Synthesis Kit (Bio-Rad).

242 To develop *DOG1* primers that do not contain polymorphisms segregating among
243 accessions, which could interfere with amplification, we sequenced the coding region in 19

244 accessions distributed all over Iberia. Two sets of primer pairs were designed in gene
245 regions that are conserved among all accessions (Supporting Information Figure S2).
246 GenBank accession numbers of DNA sequences generated in this work are KU052185-
247 KU052202.

248 Expression was calculated using qbasePLUS software (Hellemans *et al.* 2007;
249 www.biogazelle.com). *DOG1* expression was normalized by the expression of At4g12590
250 and At4g34270 control genes that are constantly expressed in dry seeds (Dekkers *et al.*
251 2012). Three replicates of pooled RNA from all samples were included in each plate
252 containing the primers of At4g12590 reference gene to correct for potential amplification
253 variation among plates (Hellemans *et al.* 2007). Since results from the two sets of *DOG1*
254 primers were highly correlated (R^2=0.96), statistical analyses from only of one them are
255 presented.

256

257 **Data analysis**

258 Broad sense heritabilities (h^2_b) were estimated as the variance component among
259 accessions derived from type III ANOVAs. Correlations among life history traits were
260 estimated using Dutilleul's modified *t* test, which corrects the variance of the statistical test
261 and the degrees of freedom according to the extent of spatial autocorrelation of each variable
262 (Dutilleul, 1993; Manzano-Piedras *et al.* 2014). The relationship between life history traits
263 and environmental variables or altitude were tested with simultaneous autoregressive models
264 (SAR), a regression technique based on generalised least squares (GLS) that estimates
265 regression parameters taking into account the spatial patterns of data by including the
266 autocorrelation matrix of the errors (Beale *et al.* 2010). Dutilleul's *t*-test and SAR were
267 conducted using SAM software (Rangel, Diniz-Filho & Bini, 2010). The spatial autocorrelation
268 patterns of life history traits and environmental variables were analysed according to
269 Manzano-Piedras *et al.* (2014), using the software PASSaGE v.2 (Rosenberg & Anderson,
270 2011). Briefly, for each environmental variable and trait, Moran's *I* autocorrelation coefficients
271 were computed (Moran, 1950) and their significance estimated from 1000 permutations.

272 Additional canonical correlation analyses (CCA) that included simultaneously a set of
273 selected environmental variables and the set of phenotypic traits, were conducted using
274 SYSTAT v.13, as previously described (Manzano-Piedras *et al.* 2014).

275

276 **Results**

277 **Natural variation for traits related to life cycle timing in *A. thaliana***

278 In order to determine the quantitative genetic diversity for the timing of life cycle in *A. thaliana*
279 we grew 300 Iberian accessions under controlled laboratory conditions and estimated their
280 seed dormancy levels (DSDS10, DSDS50 and DSDS90) and flowering times (FT) (Figures 1
281 and 2). Forty days after harvest (DAH) only 39 accessions (13%) germinated completely and
282 the average germination was 25%. To test if this low germination was due to overall high
283 levels of seed dormancy or to low seed viability, seeds were stratified for 10 days at 4°C at
284 55 DAH, and we measured the amount of germination (GAS), the rate of germination (t10,
285 t50 and U8416) and seed size (SS). Most accessions (74%) showed GAS higher than 90%
286 (Figure 2) indicating that dormancy was broken by the cold treatment and that their seeds
287 responded strongly to stratification. However, 30 accessions showed GAS values lower than
288 50%, indicating that 10% of the Iberian accessions responded weakly to cold stratification
289 (Supporting Information Table S1). Dormancy release of all accessions was monitored by
290 monthly germination tests for nearly two years (559 days) to quantify seed dormancy levels
291 as DSDS10, DSDS50 and DSDS90. Correlation analyses among seed germination traits
292 showed high values between t10 and t50, as well as among DSDS10, DSDS50 and DSDS90
293 (Table 1). Therefore only DSDS50, GAS, t50, U8416, SS and FT were used for further
294 analyses.

295 As displayed in Figure 2, all traits showed substantial genetic variation among
296 accessions. Overall, t50, U8416 and SS presented normal distribution patterns, with a two- to
297 five-fold variation, whereas the distribution of GAS was skewed towards 100% because most
298 of the lines fully germinated after the stratification treatment (Figure 2). In contrast, DSDS50
299 showed a tri-modal frequency distribution, with groups of accessions corresponding to low,

intermediate and high dormancy around values of 100, 300 and 550 days. In addition, FT showed a bi-modal distribution (Figure 2), with numerous accessions flowering around 70 days after planting or at the end of the experiment. Sixty-two accessions (21%) did not flower after 220 days, indicating that they have an obligate vernalization requirement, in agreement with previous observations (Méndez-Vigo *et al.* 2011).

To determine the genetic relationships among the different traits, we carried out correlation analyses (Table 1). Interestingly, DSDS50 and FT showed a significant negative correlation indicating that both temporal life cycle traits are not independent. This relationship was also detected when classifying *A. thaliana* accessions in three flowering and dormancy categories (Figure 1), since most very dormant accessions (DSDS50>400) also flowered early (FT<100), whereas most accessions with low dormancy (DSDS50<200) showed middle or late flowering initiation. In addition, DSDS50 and FT showed positive and negative correlations, respectively, with t50, and opposite correlations with SS (Table 1). Furthermore, GAS showed negative correlations with the rate of germination traits and SS, whereas t50 correlated positively with U8416 (Table 1). Together, these results indicate that on average, under our laboratory conditions, *A. thaliana* accessions flowering early produce more dormant seeds that are also smaller and that germinate more slowly after cold treatment.

Geographical distribution of traits related to life cycle timing

To test if the natural variation for the timing of life cycle might be involved in adaptation to different environments we first analysed the spatial autocorrelation pattern of the traits by Moran's *I* test. DSDS50 (Moran's I = 0.19) and FT (Moran's I = 0.18), as well as t50 (Moran's I = 0.13) showed significant spatial autocorrelations (P<0.05) indicating that populations located geographically closer are genetically more similar for these traits. As shown in Figure 1, accessions with high seed dormancy and early flowering clustered in the south-west, whereas those displaying low dormancy and late flowering occurred mostly in the north-east of Iberia. The largest geographical distance between accession pairs with significant autocorrelation was about 280 km for FT, 210 km for DSDS50 and 180 km for t50. Therefore,

328 these traits are not randomly distributed across the Iberian geography, suggesting that their
329 variation might be shaped by environmental factors showing similar patterns of spatial
330 autocorrelation, such as climatic parameters.

331 Since *A. thaliana* accessions are distributed across an altitudinal range of more than
332 2000 m (Figure 1), we next analysed their altitudinal distribution as a geographical proxy for
333 climatic variation. The variation for DSDS50, FT and SS displayed strong altitudinal clines,
334 indicating that the higher the altitude, the lower the seed dormancy level, the later the
335 flowering time and the larger the seed size (Figures 3A and B). These altitudinal clines
336 accounted for 38.0, 37.8 and 21.2% of the phenotypic variance for DSDS50, FT and SS,
337 respectively. Interestingly, nearly all accessions with high seed dormancy (DSDS50> 400)
338 and extremely early flowering (FT<50 days) appeared distributed below 1000 m (Figures 3C
339 and 3E) indicating that such behaviours are not maintained at high altitude. In contrast,
340 accessions with very low seed dormancy (DSDS50<100), or late flowering time (FT>200),
341 were found along the complete altitudinal range, supporting that the life cycles determined by
342 these behaviours are adapted to a wider environmental range.

343

344 **Environmental distribution of traits related to life cycle timing**

345 To dissect the geographical patterns into environmental clines we first analysed the
346 correlations between life history traits and climatic factors, the anthropic or natural habitat of
347 the populations, and the pH of the soil (Supporting Information Table S2). DSDS50 and GAS
348 correlated positively with the percentage of anthropic habitat and negatively with soil pH. In
349 contrast, FT and SS were negatively correlated with humanised habitat and positively with
350 pH. Analyses of the annual climatic variables detected stronger clines, especially for
351 DSDS50, FT and SS. The most significant clines were found with mean annual temperature,
352 which correlated positively with DSDS50 (r=0.538; P <0.001) and t50 (r=0.218; P=0.003), but
353 negatively with FT (r=-0.555; P<0.0006) and SS (r=-0.446; P<0.0006). These correlations
354 explained between 11.5% (for t50) and 45.9% (for DSDS50) of the phenotypic variance.
355 Overall, accessions from populations exposed to warmer mean annual temperature flowered

356 earlier and produced smaller and more dormant seeds (Figures 3E and 3F). All the extremely
357 dormant and very early flowering accessions came from locations with mean annual
358 temperature higher than 11°C or 8.8 °C, respectively. On the contrary, accessions with very
359 low seed dormancy or very late flowering time span almost the complete range of variation
360 for mean annual temperatures (4.9°C to 15.5°C), in agreement with the observed altitudinal
361 clines (Figures 3E and 3F).

362 To analyse in detail the relationships between life history traits and climate we
363 applied simultaneous autoregressive models (SAR) to monthly climatic variables over the
364 year (Figure 4, Supporting Information Table S2). Seed dormancy showed significant
365 correlations with all climatic parameters. In particular, DSDS50 showed strong positive
366 correlations with minimum and maximum temperatures over the year, but negative
367 correlations with precipitations in spring and summer seasons (Figure 4). In addition,
368 DSDS50 displayed weak positive correlations with potential solar radiation from April to
369 September. In contrast, GAS only correlated weakly with precipitation during August and
370 September (Supporting Information Table S2). The rate of germination measured as t50
371 showed similar but weaker correlations than seed dormancy for maximum and minimum
372 temperatures, as well as for precipitations along the year (Figure 4). However, as expected
373 from the negative correlation between DSDS50 and SS described above, SS showed similar
374 climatic correlation patterns over the year, but with opposite sign, for temperature,
375 precipitation and solar radiation (Figure 4). Furthermore, FT correlated negatively with
376 minimum and maximum temperatures, as well as with fall and winter precipitations, whereas
377 it showed positive correlations with summer precipitation (Figure 4).

378 To test further the robustness of the environmental patterns obtained with SAR
379 models, we also conducted a complementary approach by performing canonical correlation
380 analyses (CCA). These tests included multiple environmental variables and phenotypic traits
381 simultaneously (Supporting Information Tables S3 and S4). CCA generated four significant
382 canonical correlation variates (Supporting Information Table S4), but the first variate was the
383 most important since it was almost two-fold higher than the others (1st coefficient = 0.78; 2nd

384 coefficient = 0.43). The strongest correlations with the first canonical variate were found with
385 annual mean temperature, flowering time and seed dormancy, which showed coefficients of -
386 0.90, 0.87 and -0.78, respectively (Supporting Information Table S4). Therefore, annual
387 mean temperature also correlated positively with seed dormancy and negatively with
388 flowering time, when both temporal life cycle traits were analysed simultaneously.
389

390 **Associations between *DOG1* expression and life cycle traits or environmental factors**

391 Since *DOG1* is the main gene accounting for the natural variation for seed dormancy in *A.*
392 *thaliana*, and *DOG1 cis*-regulatory polymorphisms contribute to this variation (Bentsink *et al.*
393 2006), we aim to determine if genetic modifications of *DOG1* expression might provide one of
394 the molecular mechanisms that underlie life cycle adaptation to different environments. To
395 test this, we quantified *DOG1* expression as a molecular trait in 100 Iberian accessions
396 covering the seed dormancy range (Figure 5A and Supporting Information Figure S1). *DOG1*
397 expression showed six-fold variation, although nearly half of the accessions displayed rather
398 low expression. In addition, two dormant accessions displayed very high expression levels
399 outside *DOG1* variation range (Figure 5A). *DOG1* expression correlated significantly with
400 DSDS50 and FT (r=0.261 and r=-0.321, respectively; Table 1), which is in agreement with
401 *DOG1* function as a dormancy promoter and with the negative correlation between FT and
402 DSDS50. These correlations were also significant when removing the two outlier accessions
403 (r=0.253 and r=-0.315, respectively; P<0.05).

404 *DOG1* expression did not correlate with the geographic distance among populations
405 indicating a random spatial distribution of this trait. However, the genetic variation for *DOG1*
406 expression correlated weakly but significantly with altitude and mean annual temperature (-
407 0.219<r<0.278, P<0.05; Supporting Information Table S2), high expression appearing
408 associated with low altitude and with high temperature. In addition, *DOG1* expression
409 showed positive correlations with maximum and minimum temperatures during winter and
410 spring seasons (Figure 5B). These geographical and environmental associations of *DOG1*
411 expression were similar but weaker than those detected for DSDS50. Therefore, mutations

412 affecting the regulation of *DOG1* expression likely contribute to the quantitative variation for
413 seed dormancy and, subsequently, to adaptation to altitude and temperature.
414
415 **Discussion**

416 Understanding the evolutionary mechanisms of plant adaptation requires the identification of
417 the environmental factors that contribute to maintain phenotypic variation in nature (He *et al.*
418 2014, Manzano-Piedras *et al.* 2014; Krämer, 2015). The systematic analysis of 300 *A.*
419 *thaliana* accessions from a native region carried out in this study, identified altitude and
420 temperature as the major geographical and climatic factors associated with the temporal
421 control of germination and flowering, the two main developmental transitions of the life cycle
422 of annual plants. In particular, several results support the involvement of the natural genetic
423 variation for seed dormancy and flowering time in altitudinal and climatic adaptations, in a
424 non-independent manner. First, both traits are negatively correlated and displayed strong
425 spatial autocorrelation, in agreement with correlations previously described for 112
426 accessions across Europe (Debieu *et al.* 2013). Second, the natural variation for seed
427 dormancy and flowering time shows strong altitudinal and temperature clines, in agreement
428 with latitudinal clines described along Europe (Debieu *et al.* 2013) and with previous results
429 from regional studies of flowering time (Manzano-Piedras *et al.* 2014, Méndez-Vigo *et al.*
430 2011). Third, analysis of the environmental distribution of the natural variation for the life
431 cycle shows that *A. thaliana* accessions displaying extreme dormant phenotypes and early
432 flowering come from populations distributed exclusively below 1200 m altitude and from
433 locations with a mean annual temperature higher than 9 °C. Thus, natural selection is
434 probably acting outside these environmental ranges, against the life cycle that is determined
435 by such genetic combination. On the contrary, the broad altitudinal and climatic distribution of
436 accessions with low dormancy and late flowering indicates that the corresponding life cycles
437 are more common and adapted to a wider range of environments. Consequently, both
438 temporal life cycle traits are likely to share adaptive coevolution, which is not determined by a
439 major trade–off relationship since their genetic bases are mostly independent (Alonso-Blanco

440 *et al.* 2009). In fact, only the natural variation at *FLC* gene has been shown to affect
441 pleiotropically both traits under some laboratory conditions (Chiang *et al.*, 2009).

442 In addition, we found significant altitudinal and climatic clines for seed size, another
443 important evolutionary and ecological trait that has coevolved linked to other life history traits
444 (Moles *et al.* 2005). Small seeds have been associated with the persistence of seeds in the
445 soil for several species (Bakker *et al.* 1996; Bekker *et al.* 1998), although some studies failed
446 to find this association (Leishman *et al.* 2000). Moreover, it is often assumed that seed
447 dormancy and persistence of the seed bank are synonymous (Anderson, 1990; Rees, 1996;
448 Baskin & Baskin, 1998), but this association has not always been reported (Thompson *et al.*
449 2003). In concordance with these relationships, our *A. thaliana* study shows strong negative
450 or positive correlations between seed size and seed dormancy or flowering time,
451 respectively. These patterns indicate that *A. thaliana* populations from low altitude or warm
452 areas flower earlier, and produce more dormant and smaller seeds than populations from
453 high or cold locations.

454 Despite the fact that several life history traits appeared associated with the same
455 mean annual climatic variables, their genetic variation seems affected differentially by
456 climatic factors, since all traits differ in the precise climatic patterns along the year. In
457 particular, seed dormancy and seed size were the only life history traits associated
458 significantly with all climatic parameters, including solar radiation, with DSDS50 showing the
459 strongest associations. This result suggests that the natural variation for seed dormancy is
460 more sensitive to climatic factors than the variation for the remaining traits analysed, in
461 agreement with the strong plasticity of seed dormancy to numerous environmental factors
462 (Munir *et al.* 2001; Kendall *et al.* 2011; Penfield & Springthorpe, 2012; He *et al.* 2014; Huang
463 *et al.* 2014; Postma & Ågren, 2015). The distribution of the genetic variation for seed
464 dormancy seems to be affected by maximum and minimum temperatures over the year, high
465 temperatures favouring dormant genotypes. In addition, high dormancy is also associated
466 with low summer precipitation and high summer solar radiation. A similar climatic pattern is
467 found for the rate of germination, although the latter showed lower strength, in agreement

468 with the weak correlation between DSDS50 and t50. Furthermore, seed size displayed
469 climatic patterns opposite to seed dormancy, but high precipitation appeared associated
470 significantly with larger seed size over the whole year. In contrast, flowering time showed
471 several specific climatic associations, supporting that climate also acts independently on this
472 trait. Again, temperature presented the strongest flowering associations, with accessions
473 from warmer locations flowering earlier. Moreover, precipitation in winter and summer
474 seasons showed opposite effects, accessions from populations with high winter precipitation
475 or with low summer precipitation flowering early, in accordance with previous field and
476 laboratory observations (Manzano-Piedras *et al.* 2014, Méndez-Vigo *et al.* 2011). Overall,
477 locations with high temperature, low summer precipitation and high radiation, appear as
478 selecting, directly or indirectly, for life cycles with early flowering, small seeds, high seed
479 dormancy and slow germination rate. These results indicate that temperature largely drives
480 the adaptation of the two temporal traits controlling the timing of annual life cycles in *A.*
481 *thaliana*. However, also precipitation and solar radiation likely contribute to shape the
482 geographical distribution of each life history trait specifically.

483 In the past few years, numerous studies have shown that the natural variation for
484 flowering time and seed dormancy of *A. thaliana* is determined by a high number of genes
485 (reviewed in Alonso-Blanco *et al.* 2009). In addition, large allelic series have been
486 demonstrated for some of the flowering genes (Alonso-Blanco and Mendez-Vigo, 2014) and
487 for *DOG1* (Bentsink *et al.* 2006; Kronholm *et al.* 20012). This allelic heterogeneity has limited
488 the detection of these loci contributing to the natural variation for life history traits by
489 genome–wide association studies (GWAS) (Atwell *et al.* 2010; Bergelson & Roux, 2010).
490 However, the phenotypic and environmental correlations found in this study for *DOG1*
491 expression show that *trans*- and/or *cis*-regulatory mutations affecting *DOG1* expression
492 probably contribute, weakly but significantly, to the adaptation of *A. thaliana* life cycle through
493 their quantitative effects on seed dormancy. This conclusion is supported, first, by the
494 positive correlation detected between *DOG1* expression and seed dormancy, which is in
495 agreement with *DOG1* function as a positive regulator of seed dormancy induction. Second,

496 *DOG1* and seed dormancy showed similar association patterns with altitude and temperature
497 parameters. Interestingly, *DOG1* expression also correlated with flowering time, which might
498 suggest that *DOG1* also affects pleiotropically this trait, as proposed by the detection of
499 *DOG1* in GWAS of flowering time (Atwell *et al.* 2010). However, previous analyses of *DOG1*
500 mutants and introgression lines, under similar environmental conditions than those used in
501 this study, did not detect any pleiotropic flowering effect (Bentsink et *al.*, 2006). Therefore,
502 the reported associations between flowering time and *DOG1* are likely to be consequence of
503 the aforementioned correlation and coevolution between seed dormancy and flowering time.
504 In addition, it can be expected that structural mutations in *DOG1* will also contribute to life
505 cycle adaptation (Kronholm *et al*, 2012) together with regulatory and structural mutations in
506 many other genes. Our analysis shows the usefulness of expression data, as molecular
507 quantitative traits that are caused by multiple regulatory mutations, to carry out
508 environmental association studies. Such analyses provide an alternative and complementary
509 approach that overcomes some limitations of GWAS for the detection of genes contributing
510 to plant adaptation.

511 The analyses presented here show that the extant natural variation for the timing of
512 annual life cycles is likely involved in complex adaptations to altitude and climate. However, it
513 remains unknown how the climatic clines displayed by the genetic variation detected for life
514 history traits under our laboratory conditions, relate to adaptions of the life cycle of *A.*
515 *thaliana* to different natural environments. Taking into account the behaviour of winter and
516 spring annual cohorts of natural populations (Donohue, 2009; Montesinos *et al.*, 2009; Picó,
517 2012), it can be speculated that the strong seed dormancy and very early flowering observed
518 mainly in accessions from low altitudes and warm areas will determine a spring annual life
519 cycle with a very short growing period adapted to these locations. However, climatic factors
520 like ambient temperature, affect not only flowering time, seed dormancy and germination
521 (Fenner, 1991; Probert, 2000; Finch-Savage & Leubner-Metzger, 2006; Chiang *et al.*, 2011;
522 Verhage, *et al.* 2014), but also other related traits like the induction of secondary dormancy
523 (Penfield & Springthorpe, 2012). Moreover, several recent studies have revealed that under

524 natural seasonal environments, the same wild accessions of *A. thaliana* might adopt winter
525 annual, summer annual or rapid cycling life strategies depending on the environment
526 (Wilczek *et al*. 2009; Chiang *et al*. 2013; Springthorpe & Penfield, 2015). Under such natural
527 conditions, *DOG1* has been shown to affect the season of germination and the subsequent
528 environment experienced by plants during vegetative growth, which regulates flowering
529 initiation. Thus, in nature, *DOG1* affects flowering time by changing the postgermination
530 environment, an effect that has been referred to as environmentally induced pleiotropy
531 (Chiang *et al*. 2013). In addition, it has been recently proposed that in *A. thaliana* accessions
532 with a winter annual life cycle, the temperature mediated control of flowering time has
533 evolved to constraint the maternal environment for setting seeds into a specific temperature
534 window that ensures the production of a mixture of dormant and non-dormant seeds
535 (Springthorpe & Penfield, 2015). Therefore, further analyses under natural conditions are
536 required to get a deeper evolutionary understanding about the genetic covariation between
537 the temporal traits that control the annual life cycles of *A. thaliana*.
538

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547

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757

Tables

Table 1. Dutilleul's correlations between life history traits. DSDS10, DSDS5 and DSDS90: days of seed dry storage required to reach 10, 50 and 90 % germination, respectively; GAS: germination after stratification; t10 and t50: time required for 10 and 50 % of viable seeds to germinate, respectively; U8416: uniformity of germination measured as the time interval between 84% and 16% of viable seeds to germinate; SS: seed size; FT: flowering time; DOG1: *DOG1* expression. Statistical significance: **, *P* <0.001; *, *P* <0.05.

	DSDS10	**DSDS50**	**DSDS90**	**GAS**	**t10**	**t50**	**U8416**	**SS**	**FT**
DSDS10									
DSDS50	0.896**								
DSDS90	0.743**	0.904**							
GAS	-0.029	-0.074	-0.114						
t10	0.252*	0.362**	0.397**	-0.348**					
t50	0.22*	0.318*	0.351**	-0.427**	0.901**				
U8416	0.016	0.019	-0.014	-0.262**	-0.015	0.417**			
SS	-0.36**	-0.36**	-0.361**	-0.187*	-0.107	-0.097	0.001		
FT	-0.371*	-0.433**	-0.438**	-0.021	-0.299*	-0.227*	0.1	0.409**	
DOG1	0.162	0.261*	0.277*	0.066	0.166	0.065	-0.219*	-0.18	-0.321**

Figure legends

Figure 1. Geographical distribution of *A. thaliana* variation for the timing of life cycle in the Iberian Peninsula. Each map shows the distribution of accessions classified as low (left map), moderate (middle map) or high (right map) seed dormancy based on DSDS50 values. Accessions within each map are classified in early, middle and late flowering based on FT values. The number of accessions of each class is included in the legends.

Figure 2. Frequency distributions of life history traits in *A. thaliana*. (a) Seed dormancy (DSDS50: days of seed dry storage required to reach 50% of germination); (b) Germination after stratification; (c) t50 (time required for 50% of viable seeds to germinate); (d) U8416 (uniformity of germination measured as the time interval between 84% and 16% of viable seeds to germinate); (e) Seed size; (f) Flowering time. The number of accessions analysed, the population mean, the minimum and maximum accession means and the broad sense heritabilities are indicated inside each panel.

Figure 3. Altitudinal and climatic clines of traits related with life cycle timing. (a) Average seed dormancy (DSDS50) and seed size, or (b) flowering time, in six different altitudinal ranges. (c) Seed dormancy or (d) flowering time distributions across the altitudinal range. (e) Seed dormancy or (f) flowering time distributions across the mean annual temperature range. In a-b, data points are means ± SE. DSDS50: days of seed dry storage required to reach 50% of germination.

Figure 4. Climatic associations of life history traits along the year. Each panel shows the correlation coefficients between the phenotypic traits indicated and monthly minimum temperature, maximum temperature, precipitation and potential solar radiation. Months on the x-axis are indicated with the first letter of the month. Black and grey filled colours indicate significant correlations with $P<0.006$ or $P<0.05$, respectively, whereas no colour depicts non-significant coefficients tested by SAR models. DSDS50: days of seed dry storage required to

796 reach 50% of germination; SS: seed size; t50: time required for 50% of viable seeds to
797 germinate; and FT: flowering time.

798

799 **Figure 5.** Climatic associations of *DOG1* expression. (a) Frequency distribution of *DOG1*
800 expression in *A. thaliana* accessions. (b) Correlation coefficients between *DOG1* expression
801 and monthly climatic variables throughout the year. Months on the x-axis are indicated with
802 the first letter of the month. Grey filled colours indicate significant correlations (P<0.05) and
803 no colour depicts non-significant coefficients tested by SAR models. The number of
804 accessions analysed, the population mean, the minimum and maximum accession means
805 and the broad sense heritability are indicated inside panel (a). The analyses presented in (b)
806 do not included the two outlier accessions shown in (a).

807

808

809 **Supporting Information.**

810 **Figure S1.** Seed dormancy of accessions analysed for *DOG1* expression.

811 **Figure S2.** Development of *DOG1* expression primers.

812 **Table S1.** Geographic and phenotypic information of Iberian *A. thaliana* accessions.

813 **Table S2.** Correlations between life history traits and geographical or environmental
814 variables.

815 **Table S3.** Correlations among environmental variables.

816 **Table S4.** Canonical correlations between life history traits and environmental variables.