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

Deborah S. Vidigal[1], Alexandre C.S.S. Marques[1], Leo A.J. Willems[1], Gonda Buijs[1], Belén Méndez-Vigo[2], Henk W.M. Hilhorst[1], Leónie Bentsink[1], F. Xavier Picó[3], Carlos Alonso-Blanco[2].

[1] Wageningen Seed Lab, Laboratory of Plant Physiology, Wageningen University, 6708 PB Wageningen, The Netherlands

[2] Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), Madrid-28049, Spain.

[3] Departamento de Ecología Integrativa, Estación Biológica de Doñana (EBD), Consejo Superior de Investigaciones Científicas (CSIC), Sevilla-41092, Spain.

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Corresponding author:
Carlos Alonso-Blanco
Genética Molecular de Plantas
Centro Nacional de Biotecnología (CNB)
Consejo Superior de Investigaciones Científicas (CSIC)
C/Darwin 3, Cantoblanco
Madrid 28049, Spain
e-mail: calonso@cnb.csic.es
Phone # (34) 915854688
Fax # (34) 915854506

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

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

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

2013). In addition, analyses of natural *DOG1* alleles have identified several *cis*-regulatory polymorphisms that affect seed dormancy (Bentsink *et al.* 2006). Nevertheless, *DOG1* haplotypes differentiated by structural polymorphisms have been found to be associated with seed dormancy, thus suggesting that DOG1 protein variation also contributes to local adaptation (Kronholm *et al*. 2012).

Despite the recent progress in understanding the genetic and molecular mechanisms underlying the natural variation for flowering time and seed dormancy in *A. thaliana*, the environmental factors that contribute to maintain this quantitative variation remain largely unknown. To this end, world-wide collections of wild accessions have been used to carry out genetic-environment correlation analyses in sets of populations spanning a wide range of environments. These analyses require geographically-explicit approaches taking into account the spatial autocorrelation patterns that affect the independence among samples (Sokal & Oden, 1978). In this way, significant environmental clines have been detected for multiple flowering traits and genes (Caicedo, *et al*. 2004, Stinchcombe *et al*. 2004; Balasubramanian *et al*. 2006; Hancock *et al*. 2011; Li *et al*. 2014). However, mainly latitudinal associations have been reported for seed dormancy and for *DOG1* expression (Chiang *et al*. 2011; Debieu *et al*. 2013). In general, the low frequency and the strong geographic structure displayed by most natural alleles (Cao *et al*. 2011) has limited the potential of world-wide collections to detect significant genetic and environmental associations (Bergelson & Roux, 2010). To overcome these limitations, several regional collections of accessions have been developed from different world regions (Samis *et al*. 2012; Brachi *et al*. 2013; Long *et al*. 2013). In particular, a set of wild accessions collected from the Iberian Peninsula (Picó *et al*. 2008; Manzano-Piedras *et al.* 2014) has been shown to provide an ideal scenario to study *A. thaliana* adaptation because this region is part of the species native range (Hoffmann, 2002), it spans a large diversity of climates, altitudes (0-2600 m) and ecological habitats (Myers, *et al*. 2000; Ninyerola, Pons & Roure, 2000; Manzano-Piedras *et al.* 2014), and it has been shown to contain the largest amount of genetic variation of *A. thaliana* in Eurasia (Picó *et al.* 2008; Cao *et al.* 2011). This collection

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

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

Materials and Methods

Plant material and growth conditions

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

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

according to their flowering time, late, intermediate or early, which were planted on three different dates and received 8, 4 or 2 weeks of cold treatment (vernalization), respectively.

Seeds were sown on water soaked filter paper in Petri dishes and incubated for four days in a cold room at 4 ºC in the dark to break dormancy (seed stratification). Subsequently, the Petri dishes were transferred to a germination cabinet at 22 ºC (16 hours light per day) for four days before planting. Germinated seedlings were transferred to a greenhouse, placed on 4x4 cm^2 Rockwool plugs and watered with 1 g/l Hyponex fertilizer (NPK = 7:6:19). After three weeks, plants were moved to a climate chamber for vernalization (4°C; 70% RH; 12 h of light per day). Subsequently, plants were transferred to the greenhouse and grown in a complete randomized block design with six replicates. Five or six replicates were harvested for each of 252 accessions, while three or four replicates could be harvested for most of the remaining accessions (Supporting Information Table S1).

Quantitative analysis of life history traits

To measure seed dormancy, germination tests were performed weekly until dormancy had been released from all accessions (> 90% of germination). Germination experiments were performed in plastic trays (15x21 cm^2) containing 47 ml water and two layers of blue filter paper. Six samples of approximately 50–100 seeds were dispersed on the filter paper, using a mask to ensure accurate and reproducible spacing. Trays were kept in an incubator at 22 ºC and constant light, during five days. Photographs were taken once a day and they were analysed by the Germinator software package (Joosen *et al*. 2010) to calculate the maximum percentage of germination (Gmax). Seed dormancy was quantified as DSDS50 (days of seed dry storage required to reach 50% of germination), which was calculated according to He *et al.* (2014). In addition, seed dormancy was also estimated as DSDS10 and DSDS90 (days of seed dry storage required to reach 10% and 90% of germination, respectively) calculated from germination-time fitted curves.

Germination after cold stratification (GAS) was estimated 55 days after harvest (DAH). For that, imbibed seeds were placed for 10 days at 4°C and thereafter they were

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

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

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

Geographical and environmental data

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

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

***DOG1* expression**

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

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

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

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

Data analysis

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

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

Results

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

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

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

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

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

Environmental distribution of traits related to life cycle timing

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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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

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

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

Supporting Information.

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

Figure S2. Development of *DOG1* expression primers.

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

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

Table S3. Correlations among environmental variables.

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