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Research

Multiple paths to similar germination behavior in *Arabidopsis thaliana*

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Summary

- Germination timing influences plant fitness, and its sensitivity to temperature may cause it to change as climate shifts. These changes are likely to be complex because temperatures that occur during seed maturation and temperatures that occur post-dispersal interact to define germination timing.
- We used the model organism *Arabidopsis thaliana* to determine how flowering time (which defines seed-maturation temperature) and post-dispersal temperature influence germination and the expression of genetic variation for germination.
- Germination responses to temperature (germination envelopes) changed as seeds aged, or after-ripened, and these germination trajectories depended on seed-maturation temperature and genotype. Different combinations of genotype, seed-maturation temperature, and after-ripening produced similar germination envelopes. Likewise, different genotypes and seed-maturation temperatures combined to produce similar germination trajectories. Differences between genotypes were most likely to be observed at high and low germination temperatures.
- The germination behavior of some genotypes responds weakly to maternal temperature but others are highly plastic. We hypothesize that weak dormancy induction could synchronize germination of seeds dispersed at different times. By contrast, we hypothesize that strongly responsive genotypes may spread offspring germination over several possible germination windows. Considering germination responses to temperature is important for predicting phenology expression and evolution in future climates.

Introduction

Phenology transitions, or the timing of developmental events, are important for determining organismal fitness (Wilczek *et al.*, 2009; Munguía-Rosas *et al.*, 2011; Wolkovich *et al.*, 2013) and are changing, sometimes in unpredictable ways, as climate shifts (Parmesan, 2006; Willis *et al.*, 2008; Roberts *et al.*, 2015). While progress is being made towards understanding how a changing climate may influence the timing of flowering and budburst (Chuine, 2000; Morin *et al.*, 2007), less work has focused on the potential for climate change to alter germination timing (Walck *et al.*, 2011). This is a major knowledge gap because proper germination timing has been shown to be vital for species persistence (Donohue *et al.*, 2010; Kimball *et al.*, 2010). At the same time, it is becoming increasingly clear that phenological transitions such as germination cannot be considered in isolation from other transitions such as flowering – they can have cascading effects on each other (Post *et al.*, 2008) which can have consequences for life-cycle expression (Burghardt *et al.*, 2015; Springthorpe & Penfield, 2015) and potentially adaptation (Chiang *et al.* 2013).

For many species, temperature can play a dual role in controlling the timing of germination (Fenner & Thompson, 2005; Baskin & Baskin, 2014). Temperatures experienced by the maternal plant during seed development can influence germination timing by setting dormancy level (Galloway & Etterson, 2007; Donohue, 2009). Dormancy level in turn defines the range of post-dispersal temperatures that elicit germination (Donohue *et al.*, 2005a; Baskin & Baskin, 2014). How seed-maturation temperature interacts with post-dispersal temperature to influence the probability of germination will determine the timing of germination under seasonally varying conditions (see Fig. 1 for a schematic). Here, we take a step towards understanding this interaction by considering that flowering time (which determines seed-maturation conditions) alters dormancy induction and germination behavior. We do so by empirically assessing germination behavior over time for genetic variants of the model organism *Arabidopsis thaliana*.

In seasonal environments, seed dormancy is a major mechanism used by plants to control germination and is often thought to prevent germination until times of year at which seedling survival is high (Donohue *et al.*, 2010). Dormancy is defined as the failure of seeds to germinate under environmental conditions that would permit germination in nondormant seeds. It has been frequently shown that, as seeds lose dormancy, the range of

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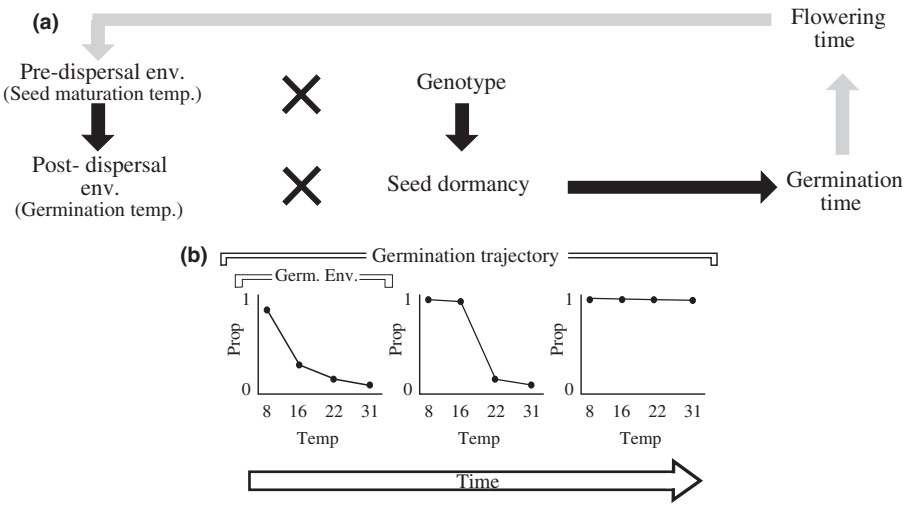

Fig. 1 Schematic of multiple factors known to influence germination timing in *Arabidopsis thaliana*. (a) The relationships between these factors that are explored in this paper. (b) Visualizations of seed dormancy. Here, we define seed dormancy levels via assessment of germination envelopes and trajectories. Germination envelope refers to temperature-dependent germination proportions at a given time after seed shed. Germination trajectory refers to changes in the germination envelope over time after seed shed, as a result of loss of dormancy with after-ripening or gain of secondary dormancy. Temperatures are given in °C.

temperatures under which they can germinate widens. One can therefore characterize seed dormancy by assessing temperature-dependent germination behavior (Finch-Savage & Footitt, 2012). The temperatures that elicit germination define the germination envelope (Fig. 1b).

For many species, including *A. thaliana*, dry seeds lose dormancy over time in a process called after-ripening, which is also characterized by a widening of the germination envelope over time (Finch-Savage & Leubner-Metzger, 2006). For example, in fall-germinating *A. thaliana* populations, seeds are dispersed in the spring and are often dormant at high temperatures soon after dispersal, but gradually gain the ability to germinate at progressively higher temperatures over the summer (Baskin & Baskin, 1972, 1983; Footitt *et al.*, 2011). We refer to these dynamic changes in germination envelopes with after-ripening as the germination trajectory (Fig. 1b). These dynamics have been noted in many species, so characterizing these changes is necessary to understand when seeds will germinate in environments where temperatures vary seasonally.

Maternal temperature effects on seed dormancy operate through the seasonal timing of flowering. *Arabidopsis thaliana* has a broad geographic range and displays a range of life cycles that mature their seeds at multiple times of year. Many populations behave as strict winter annuals, with all plants germinating in the fall and flowering in the spring, while others have both fall- and spring-germinating cohorts that flower in the spring. Still other populations have both fall- and summer-germinating cohorts that flower at different times and therefore mature seeds at different temperatures (Pigliucci, 1998; Pico, 2012; Burghardt *et al.*, 2015).

In *A. thaliana*, the temperature experienced during seed maturation has been shown to have a strong influence on dormancy and germination. Decreasing the temperature during seed development results in increased levels of dormancy (i.e. decreased probability of germinating; Donohue *et al.*, 2005b; Kendall *et al.*, 2011; Penfield & Springthorpe, 2012; Huang *et al.*, 2015). The changes in dormancy are mediated by altered gene expression (Kendall *et al.*, 2011), seed provisioning, (Finch-Savage & Leubner-Metzger, 2006), and seed coat properties (MacGregor *et al.*, 2015). Additional factors such as maternal photoperiod can also influence germination of *A. thaliana*, but have a much smaller effect than temperature (Munir *et al.*, 2001; Donohue, 2005).

In addition to maturation-temperature effects, genetic differences among populations collected across Europe can also lead to differential responses to post-dispersal temperatures (Chiang *et al.*, 2009; Atwell *et al.*, 2010). Some genes responsible for these differences in germination behavior between populations have been identified. The *DELAY OF GERMINATION 1* (*DOG1*) locus from the Cape Verde Island (*Cvi*) accession strongly increases dormancy compared with the Landsberg *erecta* (L*er*) allele (Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2006). Further, Graeber *et al.* (2014) recently showed that this gene is involved in defining the range of temperatures over which germination can occur. Another naturally variable locus, *FLOWERING LOCUS C* (*FLC*), which was originally implicated in flowering time, has now been shown to influence dormancy as well (Chiang *et al.*, 2009; Zhao, 2015). Both genes have been shown to be under selection via their effects on germination timing in the field in the USA (Chiang *et al.*, 2009, 2011).

Quantifying how seed-maturation temperatures and post-dispersal temperatures lead to phenotypic differentiation of these naturally occurring allelic variants will clarify their influence on phenology across the variable environments the species inhabits. It has long been known that environmental factors can strongly influence the expression of genetic variation (Fig. 1). Some environmental contexts magnify phenotypic differences between allelic variants, while others can reduce, or canalize, that variation (Falconer & Mackay, 1996; Roff, 1997; Byers, 2005). Defining the environmental contingency of the expression of genetic variation is important because selection among genotypes occurs only under conditions in which phenotypic differences among genotypes are expressed.

In this study, we explore the effect of seed-maturation conditions on post-dispersal germination behavior and ask if different combinations of genotypes and temperatures can produce similar germination behaviors. To do this, we tested how the germination behavior of four genotypes known to vary in dormancy level responded to three seed-maturation temperatures and a range of

post-dispersal temperatures as seeds after-ripened. Specifically, we asked: do temperature ranges for germination change with seed-maturation temperature and after-ripening; how do seed-maturation and post-dispersal temperatures influence the expression of genetic differences; can many combinations of seed-maturation and after-ripening times lead to similar germination behavior? We found that temperature contributed strongly to germination dynamics in *A. thaliana* and that there are many routes to the expression of similar phenotypes. Further, by examining how different dormancy behaviors, represented by different natural dormancy alleles, may influence germination behavior, we characterized in what environments we would expect these genotypes to behave differently from each other.

Materials and Methods

Genetic material and seed-production conditions

We used four genotypes of *Arabidopsis thaliana* (L.) Heynh. known to differ in germination. First, we compared the behaviors of the two standard laboratory accessions: Landsberg *erecta* (L*er*) and Columbia (Col). L*er* was originally collected in Germany and Col probably also originates from that area. Second, to isolate the effect of particular alleles on dormancy and to provide a precise examination of how dormancy *per se* can influence germination dynamics, we used two near-isogenic lines (NILs) that contain the *FLC* or *DOG1* allele from the highly dormant Cape Verde Island (Cvi) accession introgressed into the L*er* background (Ler_{FLC} and Ler_{DOG1}, respectively; Alonso-Blanco *et al.*, 2003; Chiang *et al.*, 2009). These NILs were obtained from Maarten Koorneef. Based on modeling work and empirical observations, we expect L*er* and Col to both be capable of cycling through both winter-annual and summer-annual life cycles in central Europe (Wilczek *et al.*, 2010; Burghardt *et al.*, 2015; Springthorpe & Penfield, 2015). In addition, one of those models (Burghardt *et al.*, 2015) and additional empirical work (Chiang *et al.*, 2011) led to the expectation that the higher dormancy level conferred by the *DOG1* allele from Cvi would result in delayed germination, and, depending on the degree of delay, a winter- or spring-annual life cycle. By contrast, we would expect the lower dormancy of the *FLC* allele from Cvi to result in even faster germination subsequent to dispersal. In addition to their variation in dormancy characteristics, these genotypes were chosen because they are in standard backgrounds for which models of other phenology transitions have been developed.

To test how the seasonal timing of flowering can influence germination behavior, we used three different seed-maturation temperatures for seed production. Growing conditions were selected to generate a range of dormancy levels and represent the breadth of conditions experienced by reproductive *A. thaliana* both within and between maturation seasons across the native European range (14 and 20°C) and the invaded range of the southern USA (25°C). Plants were grown under three constant temperature regimes (hot, 25°C; warm, 20°C; and cool, 14°C) with a 12-h photoperiod in environmental growth chambers (EGC) Model GC8-2 Plant Growth Chambers (Chagrin Falls, OH, USA). Twelve plants per genotype were grown at each temperature. Replicate plants were randomly distributed over three replicate chambers.

Seed sowing was staggered in order to synchronize the seed harvest. After 7 d of dark stratification at 4°C, seeds were sown into pots filled with Metromix 360 (Scotts Sierra, Marysville, OH, USA). Seeds were then germinated under full-spectrum light at 20°C with a 12-h photoperiod. After 10 d, seedlings were transferred to vernalization (*c.* 5°C; 10-h photoperiod) for 28 d before being randomized and placed in their respective growing temperatures. Seedlings were fertilized on two occasions before bolting (Peter's Professional 20 : 20 : 20 fertilizer; The Scotts Company, Marysville, OH, USA). Plants were watered as needed and pot positions were rotated on a weekly basis within each chamber. As siliques approached 70–90% maturity, water was withheld for 2 wk and plants in all temperature treatments were harvested on a common date. Seeds from each plant were collected into Eppendorf tubes and both dried and stored at low relative humidity and 22°C in Secador® 4.0 Auto-Dessicator Cabinets (Bel-Art Products, Pequannock, NJ, USA) until used for germination assays.

Germination assays and measures

To determine the range of temperatures under which germination can occur, we assayed seeds in four constant temperatures that span those experienced throughout the life cycle (8, 16, 22 and 31°C) and in a constant 12-h photoperiod. The use of constant temperatures allows these results to be used to build germination models (Alvarado & Bradford, 2002; Bradford, 2002); however, temperatures in natural environments do fluctuate over the day, which has been shown to influence phenological traits such germination (Liu *et al.*, 2013; Fernandez-Pascual *et al.*, 2015) and flowering time (Thingnaes *et al.*, 2003; L. T. Burghardt *et al.*, unpublished) of some species and genotypes but not others. To our knowledge, an examination comparing germination in fluctuating and constant environments has not been performed in *A. thaliana* so it is hard to predict the importance of this factor. However, some experiments have been performed in only fluctuating day–night conditions (Baskin & Baskin, 1983) and these assessments are loosely consistent with those reported here.

Germination assays were conducted on seeds that had after-ripened for 3, 7, 19 and 48 wk. The first two assessments were close together to capture fast changes in dormancy in genotypes that are only weakly dormant. Germination was assessed in controlled, Percival Model GR41LX incubation chambers (Percival Scientific Inc., Perry, IA, USA) and new light bulbs were installed at the beginning of the experiment. For these assays, 12 seeds per genotype were sown onto 35-mm Petri plates containing Whatman P5 filter paper saturated with sterile, double-distilled water. For each genotype × maturation treatment combination, we used 12 independent (biological) replicate plates in every germination temperature treatment. Thus, Petri plates represent our unit of analysis. Petri plates were randomized on trays and the trays rotated within chambers every other day. This set-up resulted in

a total of 2304 plates (4 after-ripening × 4 genotypes × 3 maturation × 4 germination temperatures × 12 replicates). Plates were assessed for germination proportion on days 3, 5, 8 and 14. Germination had reached a clear plateau in all temperatures by day 14 with the exception of 8°C, where we saw high proportions but did not know for sure that germination had plateaued. Dead seeds, though rare, were excluded when they occurred. We calculated final germination proportion for each plate as the number of germinants on day 14 divided by the total number of viable seeds.

Analysis of data

We compared germination proportions among genotypes and treatments using logistic regression (GLM package in R with a binomial link). Genotype (Geno), maturation temperature (Mat), seed age or after-ripening time (AR), and germination temperature (Temp) were treated as predictors in the model, and interactions were evaluated as described in the last three paragraphs of this section. The relationship between each predictor and germination probability was nonlinear, so predictors were modeled as categorical factors. Separation (and quasi-separation) can occur in generalized linear models with a binomial link when combinations of predictor variables lead to an all or nothing response (cases in which all or no seeds germinate). While separation should not influence Akaike information criterion (AIC) values or likelihood ratio tests, it can bias estimates of model coefficients. Therefore, we confirmed our inferences from GLM using Firth's penalized likelihood (BRGLM package in R). This method corrects for biases in maximum likelihood estimation due to small sample bias and has the added benefit of producing finite, consistent parameter estimates (Heinze & Schemper, 2002). Here, we report GLM results except in the cases we note where accurate coefficient estimation was necessary, in which case we report coefficients from BRGLM.

For all models, we used two different methods to assess the importance of individual model terms and interactions: via a likelihood ratio test and via differences in AICc. AICc is a measure of the relative quality of statistical models and is similar to AIC but has a larger penalty for an increased number of parameters and is recommended when the number of data points divided by the number of parameters is <30 (Burnham & Anderson, 2010). When comparing models using AICc, terms that are considered important in the model will result in negative AICc differences, and generally difference values below -2 suggest that including a factor improves model performance. Both methods lead to similar inferences although the AIC tests are perhaps more conservative.

First, to test for a significant four-way interaction among treatment variables, we used a full model of all four predictors. The four-way interaction was significant, so we next tested the importance of the four-way interaction separately for each of the other three genotypes compared with the reference genotype L*er*. All *P*-values were corrected for multiple comparisons using sequential Bonferroni. When the four-way interaction was not highly important ($P>0.001$ or AICc was positive), we proceeded to test the importance of each three-way interaction in a model that included all three-way interactions. To test whether germination envelopes (see Fig. 1b) changed with after-ripening in a genotype- or maturation-specific manner, we removed each three-way interaction and compared the fit of the model to one where all three-way interactions were included. By removing the Geno × AR × Temp interaction, we tested whether genotypes differed in their germination trajectories (see Fig. 1b). Similarly, by removing the Mat × AR × Temp interaction, we tested if the seed-maturation temperature altered germination trajectories.

Because many of the three-way interactions were important, we further separated the data into subsets to see if germination envelopes changed over time. We analyzed each combination of genotype and maturation treatment separately and tested for a significant AR × Temp interaction. We used likelihood ratio tests with corrected *P*-values via sequential Bonferroni to determine whether model fit was significantly improved by the inclusion of this interaction.

Lastly, in order to determine which genotypes were the most divergent across all after-ripening periods and maturation temperatures assessed, we tested for differences in behavior across germination temperatures between genotype pairs (L*er* vs $L{er}_{DOG1}$, L*er* vs Col, and L*er* vs $L{er}_{FLC}$) that were the same age and matured at the same temperature. For all 12 combinations at each temperature, coefficients indicating the strength of the genotypic effect were obtained and likelihood ratio tests were performed to determine significance. We corrected for false discovery rate using the P.ADJUST function in R (Benjamini & Hochberg, 1995). As the accurate estimation of coefficients was important, we used BRGLM (Firth's method outlined earlier) instead of GLM.

Cluster dendrograms

A cluster analysis was used to examine similarities in germination behavior by clustering based on two phenotypes – germination envelopes and germination trajectories. For the first analysis, we compared the germination envelopes of 48 combinations of three factors (Geno(4) × Mat(3) × AR(4)). Combinations of Geno × Mat × AR with similar germination responses to temperature (germination envelopes) were clustered together. In the second cluster analysis, we considered the similarity of germination trajectories of seed cohorts that differed in terms of genotype and maternal conditions (12 combinations: Geno(4) × Mat(3)). Clusters were, therefore, based on the similarity of germination trajectories (how germination envelopes changed with after-ripening). This analysis clusters combinations of genetic and maturation factors together that are more likely to respond similarly to seasonal environmental variation. Cluster dendrograms were drawn using the HEATPLOT.2 function in the R package 'GPLOT' with a prespecified number of clusters (five) using the dist method.

Results

Main effects and overall shapes of germination envelopes

As previously documented, cool seed-maturation temperature imposed higher dormancy (leading to less germination), and

longer durations of after-ripening released dormancy (leading to more germination: Supporting Information Fig. S1; Table 1). Regarding temperature-dependent germination, or 'germination envelopes', germination probability was the highest at 16°C, with slightly lower probability of germination at 8°C and 22°C, and a much lower probability at 31°C (Fig. S1d). Germination envelopes changed dynamically with after-ripening. Fresh seeds germinated primarily at 8°C and 16°C (Fig. 2a1–d1), but the germination envelope widened with after-ripening, and seeds became increasingly more likely to germinate at high temperatures (Fig. 2a4–d4).

For every combination of genotype and seed-maturation temperature, we found a significant germination temperature × after-ripening interaction, but the pattern of response differed among them (discussed later; Table S1). Further, as determined via a likelihood ratio test, all four factors (Geno, Mat, AR, and Temp) interacted to determine germination probability ($df = 54$; $\chi^2 = 142$; $P < 0.001$). However, when we compared each genotype directly to L*er* we found that this significant four-way interaction was dominated by the difference in behavior between L*er* and Ler_{DOG1} (Table S2).

Seed-maturation temperature modified germination trajectories

Changes in temperature-dependent germination over time, or germination trajectories, were modified by seed-maturation temperature, as indicated by significant Mat × AR × Temp interactions for each genotype (Table S3). Cool seed-maturation temperature (14°C) altered germination responses to post-dispersal temperature (Figs S1c, 2) by imposing stronger dormancy, thereby reducing germination proportions and narrowing the germination envelope. The germination envelope expanded more slowly for cool-matured seeds than for seeds matured in hot (25°C) and warm (20°C) temperatures (Fig. 2).

Interestingly, the influence of seed-maturation temperature on germination probability persisted throughout the duration of after-ripening studied here. For most genotypes, seeds matured at cool temperatures had lower germination than those matured at the highest temperature even after 48 wk of after-ripening, and even though the seeds were still viable (Fig. 2).

Table 1 Summary describing general patterns of how the factors investigated here individually influence germination proportions of *Arabidopsis thaliana*

Factor	General trend in germination proportion (Fig. S1)
Genotype (Geno)	Landsberg *erecta* (L*er*) accession = high germination Columbia accession = mid-range germination $Ler_{FLC\text{-}Cvi}$ = high germination $Ler_{DOG1\text{-}Cvi}$ = low germination
Seed maturation temperature (Mat)	Higher temperature = higher germination
Seed age or after-ripening (AR)	Older/more after-ripened seeds = higher germination
Germination temperature (Temp)	8°C = mid-range germination 16°C = maximal germination 22°C = mid-range germination 31°C = minimum germination

Results are summarized from boxplots in Supporting Information Fig. S1.

Genotypes differed in germination trajectories

Genotypes differed in overall germination propensity: L*er* and Ler_{FLC} consistently had the highest germination proportions, whereas Col had intermediate germination proportions and Ler_{DOG1} had the lowest (Fig. S2). All genotypes had a high germination proportion at 16°C and lower germination proportion at 31°C, but genotypes differed in how germination envelopes changed with after-ripening (significant Geno × Temp × AR interaction; Table S4). L*er* and Ler_{FLC} acquired the ability to germinate at higher temperatures as they after-ripened much more than did Ler_{DOG1} and Col (Fig. 2). This pattern was observed for all seed-maturation temperatures. However, both L*er* and Ler_{FLC} genotypes matured in the cold regained some dormancy at 48 wk.

In general, genotypic differences in germination proportion were most apparent at temperature extremes. Differences between seeds of Ler_{DOG1} and L*er* were observed across seed maturation conditions, and the magnitude of divergence between genotypes increased with temperature (Figs 2, S2). By contrast, Ler_{FLC} was surprisingly similar to L*er* at all germination temperatures, with some notable exceptions at cool temperatures. Columbia behaved like L*er* at 16°C, but had less germination at 8 and 22°C and considerably less germination at 31°C. Thus, allelic variation was frequently revealed at temperature extremes, but masked in intermediate conditions.

Highly disparate combinations of factors can produce similar germination envelopes

To determine which combinations of factors – genotype, seed-maturation temperature, and after-ripening duration – produced the most similar germination responses to temperature, we clustered our data based on similarity of germination envelopes (48 different combinations of factors). We identified five major germination responses to temperature; clusters 1–3 had high germination proportions, whereas little germination occurred in clusters 4 and 5 (Fig. 3).

Specifically, in cluster 1 (the largest cluster), seeds germinated to high proportions at all temperatures except 31°C. Some of the recurrent treatment factors found in this cluster included seeds that were after-ripened for mid (7 or 19 wk) or long (48 wk) durations, warm and hot seed-maturation temperatures, and low dormancy genotypes matured in cool temperatures. Cluster 2 germinated to high proportions at all temperatures and included only the least dormant genotypes, L*er* and Ler_{FLC}, and after-ripened seeds that were matured in warm or hot temperatures. Cluster 3 was characterized by high germination at all temperatures except 8°C, and only one treatment combination produced this phenotype (cool-matured L*er* seeds at 19 wk). Germination was restricted to 8 and 16°C in cluster 4, and included factors

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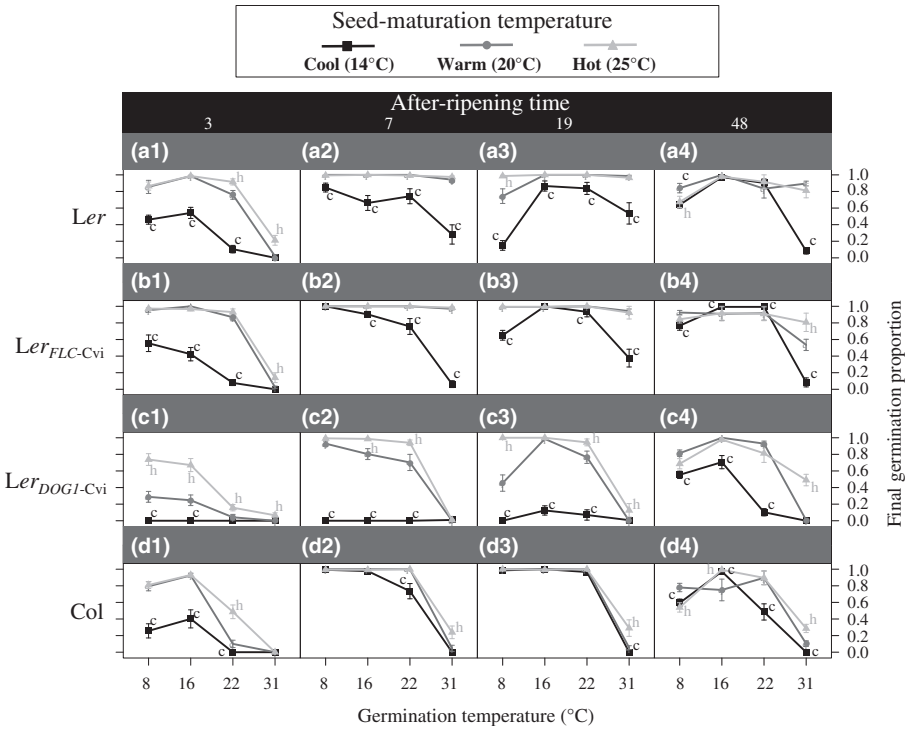

Fig. 2 Reaction norms of germination proportion in response to temperature ('germination temperature envelopes') across all after-ripening durations (columns 1–4) for each genotype (rows a–d) of *Arabidopsis thaliana*. Lines within each panel indicate the seed-maturation temperature (black squares, 14°C; dark gray circles, 20°C; light gray triangles, 25°C). Data points depict the mean germination proportion at each temperature for 12 replicates. Error bars indicate ± SE. Letters beside data points (h and c) indicate a significant difference between warm-matured seeds and hot-matured seeds or between warm-matured seeds and cool-matured seeds, respectively. Significance at the $P < 0.05$ level was determined using likelihood ratio tests of GLMS comparing the germination probability of either hot- or cool-matured seeds directly to that of the warm-matured seeds. Significance was corrected for multiple tests using sequential Bonferroni.

associated with high levels of dormancy: the Ler_{DOG1} genotype, cool seed-maturation temperature, and fresh seeds. Little to no germination occurred at any temperature in cluster 5. It primarily included the Ler_{DOG1} genotype, but also 3-wk-old seeds of the Col genotype matured in cool temperatures.

This analysis shows that highly divergent combinations of factors can produce similar germination phenotypes. In particular, environmental factors that increase dormancy (cool seed-maturation temperatures or short after-ripening durations) can cause less dormant genotypes to resemble more dormant genotypes. Likewise, more dormant genotypes that have experienced germination-promoting conditions (warm maturation and long after-ripening) can resemble less dormant genotypes that have been induced into stronger dormancy. For example, fresh seeds of Ler_{FLC} and *Ler* genotypes when matured in cool temperatures were phenotypically similar to fresh seeds of the Ler_{DOG1} genotype matured in hot temperatures or Ler_{DOG1} seeds that were matured in cool temperatures and after-ripened for 48 wk. Combinations of factors can also cancel each other out. For example, the phenotype of Col seeds matured in hot temperatures after 3 wk of after-ripening resembles that of Col seeds matured in cool temperatures after 48 wk of after-ripening. Thus, there are multiple routes by which to achieve similar germination envelopes in *A. thaliana*.

Clustering of germination trajectories reveals strong response to seed-maturation temperature and genetic variation in that response

We next evaluated which combinations of genotypes and seed-maturation temperatures produced the most similar germination trajectories – changes in temperature-dependent germination over the course of after-ripening (Fig. 4). Five clusters were identified. Cluster 4, the least dormant cluster, germinated to high proportions in every germination temperature except when seeds were after-ripened for 3 wk. This cluster included hot- and warm-matured *Ler* and Ler_{FLC}. Cluster 3 was similar, but had reduced germination at 31°C at all after-ripening durations. Factor values in this cluster included hot- and warm-matured Col seeds and hot-matured Ler_{DOG1} seeds. In cluster 1, germination proportions were low until 7 wk of after-ripening, and factor values in this group included cool-matured Ler_{FLC} and Col genotypes as well as warm-matured Ler_{DOG1}. Cluster 5 was highly divergent from the other clusters; germination proportions in this cluster remained low throughout after-ripening and included only Ler_{DOG1} seeds matured in cool temperatures.

Neither genotype nor seed-maturation temperature solely determined how germination envelopes changed with after-ripening; instead, seed-maturation temperature influenced how similar or dissimilar genotypes were in response to after-ripening. For example, while *Ler* and Ler_{FLC} clustered together when seeds were matured in warm and hot temperatures, they diverged when matured in cool temperatures. In fact, cool-matured Ler_{FLC} seeds were more similar to cool-matured Col and warm-matured Ler_{DOG1} seeds. This is because cool-matured *Ler* seeds exhibited a distinctive decline in germination at low temperature with prolonged after-ripening. Moreover, genotypes differed in their responsiveness to seed-maturation temperature. Specifically, Ler_{DOG1} was highly responsive to seed-maturation temperature, and it was distributed over three divergent clusters; by contrast, Col was least responsive to seed-maturation temperature and was restricted to two adjacent clusters (Fig. 4).

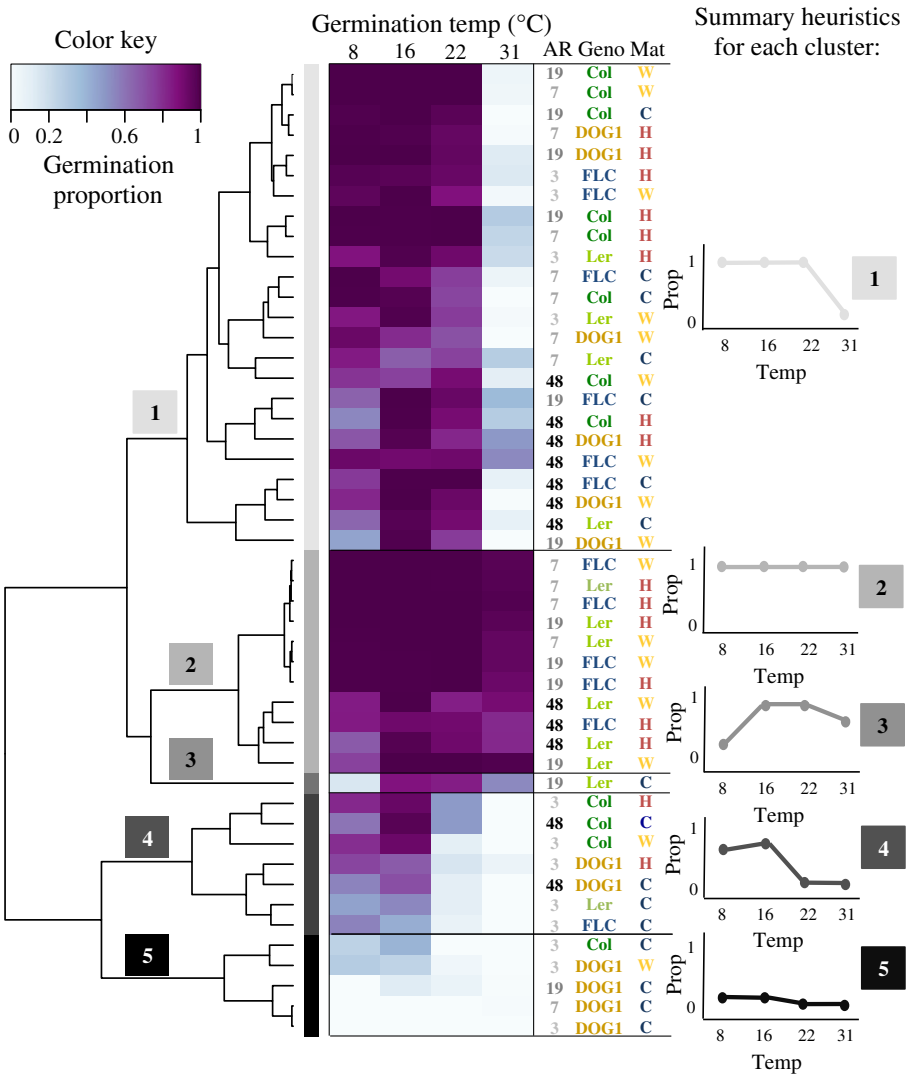

Fig. 3 Cluster dendrogram and heat map of similarity of *Arabidopsis thaliana* germination envelopes for all combinations of seed age (AR), genotype (Geno), and maturation (Mat). The grayscale boxed numbers on branches denote major clusters. The color scale indicates germination proportion, with darker color shades indicating higher germination proportions. On the far right are heuristics describing the representative shape of the germination envelopes for each major cluster. Ler_{DOG1} and Ler_{FLC} are abbreviated DOG1 and FLC, respectively.

Discussion

We found that germination temperature envelopes (Fig. 1b) changed with seed age, or after-ripening, and these germination trajectories depended on seed-maturation temperature and genotype. Further, different combinations of genotype, seed-maturation temperature, and after-ripening can produce similar germination envelopes, and different genotype and seed-maturation temperatures can combine to produce similar germination trajectories over time. Thus, in combination and when exposed to seasonal temperature changes, these interactions result in genetic differences in germination being masked in some temperatures, but revealed in others.

Dual effects of temperature on germination behavior and dependence of genetic variation on environmental factors

Our results clearly show that inputs from maturation and post-dispersal temperature do not have additive effects on germination behavior and are influenced strongly by allelic variation. This is in congruence with previous work showing that natural ecotypes differ in the magnitude of their phenotypic response to seed-maturation temperature (Penfield & Springthorpe, 2012) as well as in their pattern of response to post-dispersal temperatures (Atwell *et al.*, 2010). In general, cool seed-maturation temperature reduced the temperatures under which germination occurred, and this effect persisted for the duration of after-ripening assessed here. Recent work has provided mechanistic insights into these patterns suggesting that cool maturation temperatures cause changes in the seed coat that reduce permeability and decrease germination (MacGregor *et al.*, 2015) and that *DOG1* regulates temperature-dependent endosperm weakening of imbibed seeds through changing gibberellic acid metabolism (Graeber *et al.*, 2014).

The environmental context into which seeds are dispersed will determine the extent to which genetic differences in germination alleles will manifest as phenotypic variation within a seasonal environment. Quantitative trait locus (QTL) mapping and genome-wide association studies (GWASs) in *A. thaliana* and other species have continually emphasized the

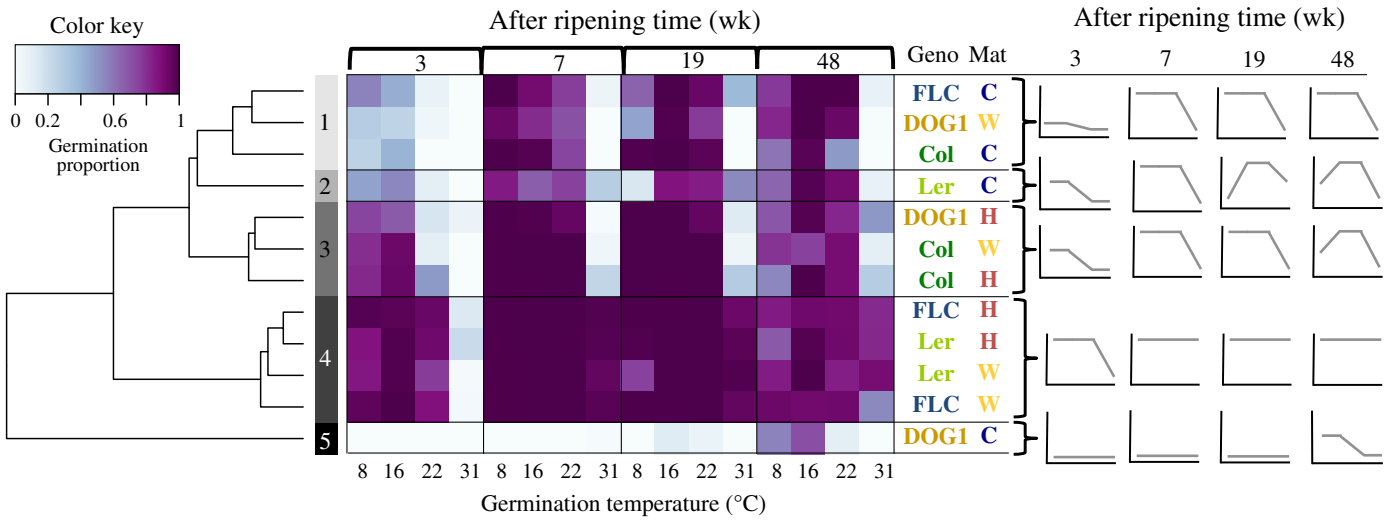

Fig. 4 Cluster dendrograms describing the similarities in germination trajectories of *Arabidopsis thaliana* between genotypes with seeds matured at different temperatures. Clustering is based on the mean response of each genotype × maturation combination to each germination temperature over the course of after-ripening. The major clusters are indicated by grayscale numbers (1–5) at the tips of the dendograms. Germination proportion is indicated by the color key, with darker color shades indicating higher germination proportions. Cartoons on the right depict representative germination trajectories (temperature-dependent germination at four time-points of after-ripening) of each of the five major clusters. Ler_{DOG1} and Ler_{FLC} are abbreviated *DOG1* and *FLC*, respectively.

importance of the environment in shaping which loci are associated with phenotypes and the strength of their effects (see El-Soda *et al.*, 2014 for a review). Gene network structures that lead to environment-dependent allelic effects have been elucidated for flowering time in *A. thaliana* and a few other species (Amasino, 2010; Pajoro *et al.*, 2014; Shrestha *et al.*, 2014), allowing prediction in some cases of environment-specific phenotypes (Wilczek *et al.*, 2009). Interestingly, seed-maturation environments have recently been shown to influence the expression of genetic variation in progeny as well (Postma & Ågren, 2015). Because the mechanistic bases of dormancy and germination are becoming clearer (Graeber *et al.*, 2012; Nonogaki, 2014; Zhao, 2015), interpreting those gene networks in an environmental context should become increasingly possible in the future (Graeber *et al.*, 2014; MacGregor *et al.*, 2015).

Using genotypes known to differ in germination behavior, we found that genetic differences in germination behavior between Col/L*er* and Ler_{FLC}/L*er* are most likely to be expressed when seeds experience high or low, but not intermediate, temperatures. This implies that selection can only distinguish genotypes at temperature extremes, as would occur early and late in the growing season or at the northern or southern edges of the range. By contrast, the *DOG1* allele from Cvi reduced germination proportions compared with the L*er* allele at nearly every temperature and for all maturation combinations tested and would therefore have a phenotypic effect in most seasonal environments. This allele comes from the Cape Verdi Islands, but other populations in the native range, particularly in southern Europe, also display strong dormancy (Kronholm *et al.*, 2012). It would be intriguing to compare the temperature-dependent effects of these unique *DOG1* haplotypes.

Potential effects of seed-maturation temperature on germination timing

The adaptive rationale for the existence of maternal effects on seed dormancy has not been firmly established for *A. thaliana*. However, strong selection for germination timing within a season has been documented for many species (Donohue *et al.*, 2010). One hypothesis is that the environment experienced during seed maturation provides information about the post-dispersal environment that enables germination to occur at a suitable time of year (Galloway & Etterson, 2007; Wagmann *et al.*, 2012). This process could operate either for seeds matured on the same plant across the dispersal season or for different plants that flower at different times within a population.

One possibility regarding maternal-temperature effects is that seeds matured and dispersed early in the season may need to be more dormant, whereas seeds dispersed later may need to be less dormant in order to achieve similar germination schedules (Fig. 5a). Alternatively, maternal effects could distribute germination across multiple times of year (Fig. 5b) and act as a bet-hedging strategy against unpredictable abiotic conditions or competition with others (Brown & Venable, 1986; Montesinos-Navarro *et al.*, 2011; Metcalf *et al.*, 2015). Germination has been documented to occur in autumn, spring, and summer, and some populations exhibit more than one germination cohort (Wilczek *et al.*, 2010; Pico, 2012; L. Burghardt, pers. obs.). Our results suggest that maternal temperature effects may contribute to such observed variation in germination time.

To interpret how variation in germination behavior might change phenology in seasonal environments, we used the observed germination proportions in each temperature at each point in time after seed shed to interpolate the estimated

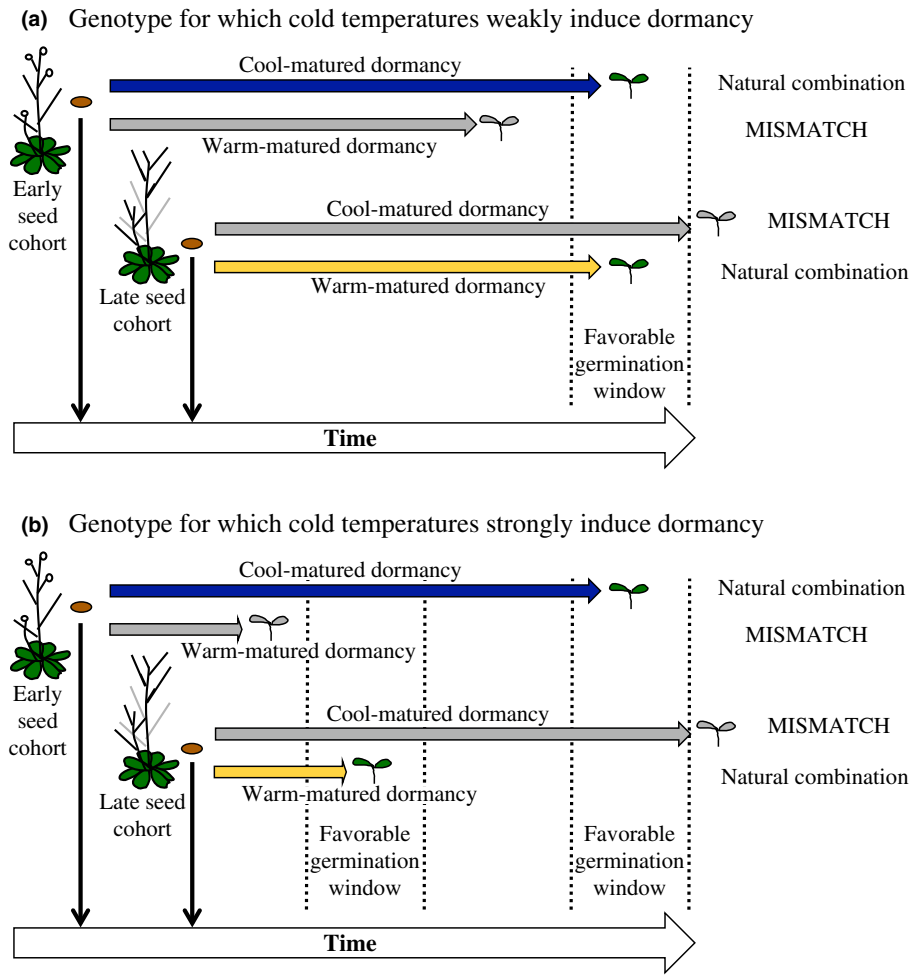

Fig. 5 Schematic illustrating two hypotheses derived from our empirical data concerning how dispersal timing and seed-maturation temperatures may interact to influence germination timing of *Arabidopsis thaliana*. In a natural seasonal environment, early seed cohorts would be matured in cooler conditions leading to higher dormancy (blue arrows), and later seed cohorts would be matured in warmer conditions leading to lower seed dormancy (yellow arrows). The combinations of seed maturation and dispersal timing that do not occur in nature are shown in gray and illustrate what would happen if dormancy were not sensitive to seed-maturation temperature. Maturation temperature differences could occur as a result of seeds maturing early and late on a single plant or seeds maturing on separate early- and late-flowering plants in a population. (a) Illustration of how maternal effects on dormancy may compensate for differences in dispersal timing to synchronize germination timing in a single germination window. This requires a small change in dormancy between seed cohorts dispersed early and late in the dispersal season (as with Col, L*er*, and L*er*$_{FLC}$), such that seeds matured and dispersed later germinate after less time than those matured and dispersed earlier, leading to synchronization. (b) Illustration of how a strong maternal effect on dormancy levels (as occurs for the L*er*$_{DOG1}$ genotype) may cause the two different cohorts to germinate during two different favorable windows for germination.

germination fraction that would occur for warm- and cool-matured seeds over the course of a year, assuming seasonal variation in temperature (Methods S1; Fig. S3a). For these interpolations, we used climate data from Halle, Germany – a location for which we have an estimate of dispersal timing in natural populations that is also near where both laboratory accessions used here are thought to originate.

In accordance with our general expectations, the lower dormancy genotypes were expected to germinate in the summer while the higher dormancy genotype germinated in the early fall and spring (Fig. S3b,c). Moreover, maternal effects synchronized germination timing and/or allocated germination timing to occur in multiple seasons (Fig. S4). The first scenario, that maternal effects may synchronize germination time, was observed in our interpolations of low-dormancy genotypes (L*er*, L*er*$_{FLC}$, and Col). Seeds matured early in cool temperatures were slightly more dormant than seeds matured later in warm temperatures, which caused later germination and more similar germination times than if the maternal effect did not exist. Thus, delayed dispersal timing can be compensated for by lower dormancy and faster germination of later dispersed seed cohorts (Fig. S4). By contrast, for the L*er*$_{DOG1}$ genotype, maternal temperature effects determined which of three germination windows was utilized – warm seed-maturation temperature led to summer germination, whereas cooler temperatures led to late fall/spring germination (Fig. S4). Thus, our results suggest that maternal temperature effects reduce variation in germination time in low-dormancy genotypes that respond weakly to seed maturation temperature, but increase it the high-dormancy genotype that is strongly responsive. While field data from across Europe in natural environments would provide the ultimate test of the hypotheses generated here, we do think these results and projections provide a

relevant context to stimulate thought about the impact of maternal effects and changes in temperature envelopes on life cycles.

These interpolations also illustrate how correlations between conditions before and after seed dispersal limit the range of environmental combinations and thus the range of phenotypic variation that occurs in nature. Thus, when measuring genetic effects, care must be taken in matching seed-maturation conditions with post-dispersal conditions that actually occur in nature. In practice, laboratory seed maturation conditions tend to be much warmer than those that natural populations would experience (Burghardt *et al.*, 2015; L. T. Burghardt *et al.,* unpublished; Springthorpe & Penfield, 2015).

Germination modeling in *A. thaliana*

Here, we demonstrate that a process that is generally overlooked in hydrothermal germination models is important for *A. thaliana*: temperatures ranges for germination are dynamic and thus model parameters such as optimal and supra-optimal temperatures are not static (Batlla & Benech-Arnold, 2015). These temperature ranges change during dry after-ripening and are altered by the seed-maturation environment. Other work has shown that temperature ranges can also be shifted by another key process, secondary dormancy cycling (Footitt *et al.*, 2011, 2014; Auge *et al.*, 2015; Springthorpe & Penfield, 2015; L. T. Burghardt, unpublished). To move toward truly predictive models of germination timing in *A. thaliana* will require extending the empirical data outlined here to include additional components involved in regulating germination phenology such as hydrotime parameters, secondary dormancy cycling, and seed bank dynamics.

These data also highlight the importance of knowing seed-maturation temperatures and dispersal timing of natural *A. thaliana* populations. Without knowledge of phenology, it is impossible to make predictions about germination timing because both the dormancy level of the seed and the sequence of post-dispersal temperatures it experiences are unknown. Both observational studies of phenology in natural populations and field experiments that measure germination timing of seeds of known genotypes matured in known environments will be necessary to understand germination dynamics and corroborate the hypotheses proposed here.

Conclusions

As the climate changes, there are multiple scenarios whereby pre- and post-dispersal environments could alter germination phenology. As the length of the unfavorable season for growth (summer) is projected to lengthen, higher dormancy levels may be essential to prevent seeds from germinating at the wrong time. How climate change shifts the conditions experienced during seed maturation then becomes critical: if the temperature during seed maturation increases, dormancy levels will decrease – the opposite of what is advantageous given longer unfavorable seasons. However, it is not necessarily so that climate change will result in higher seed-maturation temperatures, as flowering time is also extremely labile (Wilczek *et al.*, 2009, 2010) and, if shifted, could result in lower or higher temperatures during seed maturation.

Natural variation in both flowering time (i.e. the environment during seed maturation) and germination is ubiquitous in this species, and as climates change, the expression of genetic variation in these traits will also change. For instance, if warming temperatures lead to less winter chilling, flowering times may become more variable for genotypes with a chilling requirement. For germination, our results suggest that warmer temperatures during seed maturation and after dispersal will have a stronger effect on germination timing for some genotypes than others. These results suggest that maturing and germinating plants in conditions that are similar to the seasonal conditions to which they have adapted may be important for discovering ecologically relevant natural variation in traits across the life cycle. Continued exploration of how multiple environmental factors shape the expression of allelic variation, as we have carried out here, is fundamental to understanding the capacity of organisms to evolve in response to changing climates.

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Author contributions

L.T.B. and K.D. planned and designed the research, L.T.B. and B.R.E. performed the experiment and collected the data, and L.T.B., B.R.E., and K.D. analyzed the data and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Summary boxplots describing how experimental factors influenced germination proportion, pooled over the other treatments.

Fig. S2 Boxplots summarizing the extent to which the behavior of Ler_{DOG1}, Col, and Ler_{FLC} diverged from that of L*er* at each of the germination temperatures.

Fig. S3 Schematic of germination projections, and projections of naturally occurring combinations of seed-maturation temperature and dispersal timing for each genotype using climate data from Halle, Germany.

Fig. S4 Germination projections of all combinations of pre- and post-dispersal environments for each genotype using climate data from Halle, Germany.

Table S1 Likelihood ratio tests to identify significant Temp × AR interactions for each combination of genotype and seed-maturation temperature

Table S2 Likelihood ratio tests and AICc differences testing for significant Geno × Mat × Temp × AR interactions for germination probability

Table S3 Likelihood ratio tests and AICc differences testing for significant Mat × Temp × AR interactions for germination probability of each genotype

Table S4 Likelihood ratio tests and AICc differences testing for significant Geno × Temp × AR interactions for germination probability of each seed-maturation treatment

Methods S1 Description of germination interpolations.

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