Seed after-ripening and dormancy determine adult life history independently of germination timing

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Summary

- Seed dormancy can affect life history through its effects on germination time. Here, we investigate its influence on life history beyond the timing of germination.
- We used the response of *Arabidopsis thaliana* to chilling at the germination and flowering stages to test the following: how seed dormancy affects germination responses to the environment; whether variation in dormancy affects adult phenology independently of germination time; and whether environmental cues experienced by dormant seeds have an effect on adult life history.
- Dormancy conditioned the germination response to low temperatures, such that prolonged periods of chilling induced dormancy in nondormant seeds, but stimulated germination in dormant seeds. The alleviation of dormancy through after-ripening was associated with earlier flowering, independent of germination date. Experimental dormancy manipulations showed that prolonged chilling at the seed stage always induced earlier flowering, regardless of seed dormancy. Surprisingly, this effect of seed chilling on flowering time was observed even when low temperatures did not induce germination.
- In summary, seed dormancy influences flowering time and hence life history independent of its effects on germination timing. We conclude that the seed stage has a pronounced effect on life history, the influence of which goes well beyond the timing of germination.

Introduction

All plants and animals go through an embryonic phase, whose effects on later life stages can be highly consequential. The environmental conditions experienced during this period can dramatically alter the adult phenotype (Gilbert, 2001). In Gymnosperms and Angiosperms, embryo development happens within a seed and, for many plants, this phase represents a significant proportion of the life cycle, in some cases longer than any other life stage (Gutterman, 1994).

The seed stage can have pronounced effects on plant life histories. The timing of germination establishes the season in which plants begin their growth, which conditions subsequent survival and phenology, and, in turn, affects the reproductive output of the plant (Galloway, 2001; Donohue *et al.*, 2005a; Wilczek *et al.*, 2009).

Although it is well established that the seed stage influences life history through its effects on germination timing, the degree to which environmental inputs at the seed stage influence other life stages, independent of their effects on germination phenology, is poorly understood. However, the effect of selection on embryonic stages can have significant consequences on the expression of adult traits (Gilbert, 2001). In this study, we test how the physiology, in particular the level of dormancy, and environmental conditions of seeds influence the subsequent phenological transition of flowering, and thus the adult phenotype.

Germination can only occur after dormancy is lost and specific environmental conditions are present. Seed dormancy is therefore a primary determinant of the timing of germination. Dormancy is controlled by the maternal and embryonic genotypes, and their respective environments (Baskin & Baskin, 1998). It is recognized as a block to the completion of germination of a viable seed under conditions that are favorable for germination in nondormant seeds (Finch-Savage & Leubner-Metzger, 2006). The most prevalent form of seed dormancy is physiological dormancy (Baskin & Baskin, 2004). Physiological dormancy is a quantitative state, such that seeds can be more or less dormant in response to environmental cues (Baskin & Baskin, 1998). In addition, physiological dormancy decreases with time under dry storage conditions through a process called after-ripening (Holdsworth *et al.*, 2008).

Arabidopsis thaliana exhibits the most widespread form of physiological dormancy and constitutes a good model to examine the causes and consequences of dormancy (Baskin & Baskin,

2004). In *A. thaliana*, the loss of dormancy with after-ripening is concomitant with drastic changes in the individual's metabolism and transcriptome (Cadman *et al.*, 2006; Bassel *et al.*, 2008). In particular, after-ripening is linked to changes in the dynamic balance of, and sensitivity to, major hormones (i.e. gibberellin, abscisic acid and, possibly, ethylene; Iglesias-Fernandez *et al.*, 2011). When germination is forced following different after-ripening time lengths, the physiological variation among seeds results in heterogeneity in the phenotype of seedlings (Fox *et al.*, 1995; Cabin *et al.*, 1997).

In addition to after-ripening, temperature is an important factor regulating dormancy and germination. When a non-dormant seed is exposed to temperatures that are unfavorable for germination, it can cycle back into a dormant state, in a process called 'secondary' dormancy induction (compared with 'primary' dormancy, which is the dormancy state of the seed immediately after dispersal; Finch-Savage & Leubner-Metzger, 2006). In many temperate species, such as *A. thaliana*, germination is highest after exposure to periods of low temperature, and chilling is a common mechanism of dormancy breakage (Baskin & Baskin, 1998; Bentsink & Koornneef, 2008). However, prolonged periods of seed chilling can induce secondary dormancy (Baskin & Baskin, 1983; Nordborg & Bergelson, 1999). It is unclear what determines whether seed chilling induces or breaks dormancy (Penfield & Springthorpe, 2012). It has been suggested that the effect of chilling or other cues depends on the pre-existing dormancy level of the seed (see, for instance, Baskin & Baskin, 1984, 1986), but mechanistic evidence for this is lacking.

Chilling also affects flowering time in many temperate species, such as *A. thaliana* (Simpson & Dean, 2002; Baurle & Dean, 2006). The extent to which the seed and rosette responses to chilling overlap is unclear. In *A. thaliana*, the flowering time is accelerated after exposure to low temperatures at both the seed (stratification) and rosette (vernalization) stage, but, in closely related species, such as *Arabis alpina*, seed chilling has no effect on the flowering time (Wang *et al.*, 2011). Furthermore, there is natural variation in *A. thaliana* in how (or even if) seed chilling accelerates flowering, although longer periods of chilling generally result in earlier flowering (Nordborg & Bergelson, 1999). To our knowledge, it has never been investigated whether the variation in the response to seed chilling is associated with variation in dormancy. Specifically, it is not known whether the ability of seeds to integrate environmental cues for flowering depends on their dormancy state.

In short, the seed stage may influence subsequent phenology not only through its effects on germination timing, but also through physiological processes that may act independent of germination timing *per se*. The after-ripening or dormancy state may influence directly post-embryonic developmental transitions, such as flowering, and these may influence how environmental cues, such as chilling, experienced by seeds influence adult phenotypes.

Here, we investigate the interaction between seed dormancy and flowering time, the major developmental transitions and determinants of life history in annual plants. We used *A. thaliana* and its response to chilling to test whether seed dormancy influences phenology independently of its effects on the timing of germination. In addition, we investigated whether cues that are experienced by dormant seeds have consequences on the adult phenotype. We compared the flowering phenology of plants from seeds that experienced different durations of after-ripening and secondary dormancy treatments in order to determine how dormancy depth influences flowering time and its response to seed chilling. Specifically, we tested the following: how seed dormancy affects germination responses to chilling; whether dormancy alleviation by dry after-ripening affects flowering time independently of germination time; and how chilling at the seed stage affects flowering time under various seed dormancy conditions.

Materials and Methods

Genotypes used and maternal plant growth conditions

All experiments were conducted using five *Arabidopsis thaliana* (L.) Heynh ecotypes (Table 1) selected to encompass variation in geographic distribution and different combinations of primary dormancy levels, flowering time (authors' unpublished data) and level of expression of *Flowering Locus C* (*FLC*; according to data from Shindo *et al.*, 2005). This last criterion was included because FLC is a major regulator of flowering time responses to rosette chilling (Searle *et al.*, 2006) and can therefore be used as a proxy for the predicted response to vernalization. Moreover, this gene is also involved in temperature-dependent germination, which makes it additionally relevant for the present study (Chiang *et al.*, 2009).

Two sequential batches of maternal plants were grown in growth chambers under a 12-h photoperiod of full-spectrum light at 22°C, except for 7 d of vernalization at 6°C after the sixth true leaf had appeared. To control for environmental effects, plants were rotated at random every week until bolting (i.e. when the flower buds were clearly visible). The first batch

Table 1 *Arabidopsis thaliana* ecotypes used (flowering time, dormancy and *Flowering Locus C* (*FLC*) expression level values are relative to the ecotypes included in this study)

Stock no.	Line code	Origin	Latitude	Longitude	Flowering time	Dormancy	*FLC* expression
CS6688	Edi-0	Edinburgh, UK	50.95°N	3.22°E	Late	Nondormant	High
CS994	Br-0	Brno, Czech Republic	49.2°N	16.05°E	Intermediate	Intermediate	Very high
CS26649	Pro-0	Proaza, Spain	43.15°N	6°W	Early	Dormant	Low
CS1567	Tu-0	Turin, Italy	45.03°N	7.67°E	Early	Nondormant	Very low
CS6674	Ct-1	Catania, Italy	37.05°N	15.0°E	Early	Dormant	Very low

matured seeds during the spring of 2008, and the second during the autumn of 2008. Maternal plants were grown from seeds that had been harvested 6 wk after dehiscence of the first silique and kept at room temperature for 2 wk. These seeds were stratified in agar plates at 4°C for 1 wk in total darkness and exposed to a 12-h photoperiod with full-spectrum light (florescent plus incandescent) at 22°C, and seedlings were transplanted into pots filled with Metromix 360 (Scotts Sierra, Marysville, OH, USA).

Manipulation of after-ripening

Primary dormancy was studied by comparing fresh (planted 2 wk after harvest) and after-ripened (kept dry at room temperature for 5 months) seeds. This after-ripening period is similar to the span between dispersal and germination under natural conditions (Donohue *et al.*, 2005b; Huang *et al.*, 2010).

It is not possible to control simultaneously for the seed batch and timing of the germination assay when comparing fresh vs after-ripened seeds. Therefore, three different seed cohorts were tested. The first batch was harvested in the spring of 2008, and the germination assay was performed on after-ripened seeds during the autumn of 2008 (AR1). The second batch was harvested and its fresh seeds were assayed in the autumn of 2008 (Fresh) at the same time as the after-ripened seeds of the first batch. These two cohorts compare fresh and after-ripened seeds in germination assays conducted at the same time. Because these seeds came from different batches, we also assayed after-ripened seeds from the second batch during the spring of 2008 (AR2). All plants were grown in the same growth chambers under the same conditions. We tested for the effect of after-ripening in three ways: first, by comparing AR1 vs Fresh (controlling for the timing of the germination assay); second, by comparing AR2 vs Fresh (controlling for the seed batch); and third, as the mean between the difference between Fresh seeds and each after-ripened cohort: AR effect = ((Trait Value Fresh − Trait Value AR1) + (Trait Value Fresh − Trait Value AR2))/2.

Manipulation of secondary dormancy and seed chilling

Dormancy was experimentally manipulated through secondary dormancy induction and forced dormancy breakage. Secondary dormancy was induced by exposing agar plates containing seeds to 7 d at 31°C in the dark before subsequent experimental treatments (Donohue *et al.*, 2008). To break dormancy and induce germination, seeds were individually scarified with a needle and placed in clean plates containing 10 mM KNO_3 (Cadman *et al.*, 2006). Dormancy was experimentally broken either before or after seed chilling, according to the treatments below (Fig. 1).

To test whether dormancy influenced responses to seed chilling, we exposed seeds in different states of natural or experimentally manipulated dormancy to different durations of chilling. Imbibing seeds were kept at 4°C in total darkness for 0 d (No chilling), 3 d ('Short' chilling) or 21 d ('Long' chilling). The experiment was factorial with respect to after-ripening, dormancy manipulation (see next paragraph) and chilling treatment.

Dormancy treatments are schematically represented in Fig. 1. Table 2 gives the interpretation of comparisons between specific treatments. Control seeds (C) experienced neither experimental dormancy induction nor breakage. DBa experienced dormancy breakage after chilling. It is worth noting that, in both of these treatments (C and DBa), seeds were exposed to chilling at their endogenous, natural dormancy level. A minimally dormant control was provided by DBb seeds, which experienced dormancy breakage before chilling. Conversely, the maximum dormancy level was represented by DI seeds, which experienced dormancy induction before chilling. This treatment resulted in very low germination, and could not be used to assess flowering time (see next section). DI + DBa seeds experienced both dormancy induction before chilling and dormancy breakage after chilling. Only after-ripened seeds (AR2) were used for the DBa treatment, but all other treatments were applied to all three seed batches (AR1, AR2 and Fresh).

We used the following comparisons to test for the effects of the experimental manipulations (see Table 2 for interpretations of contrasts): C vs DI; C vs DBb; C vs DBa; and DI + DBa vs DI.

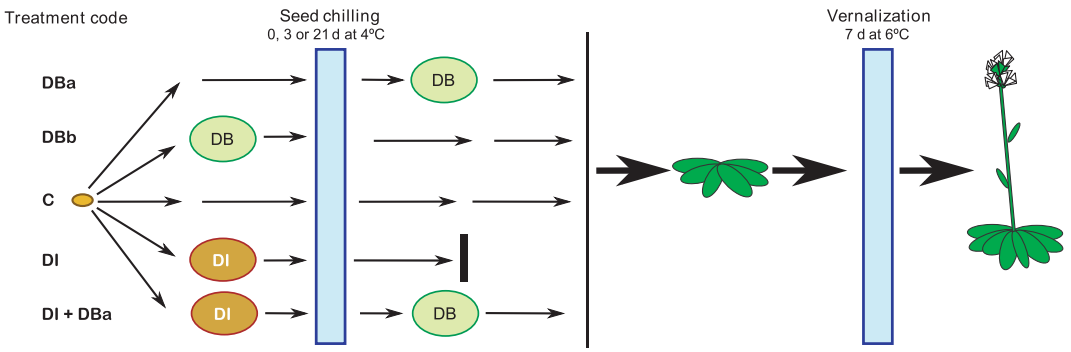

Fig. 1 Schematic representation of the experimental layout. This design was applied to all *Arabidopsis thaliana* seed batches (Fresh, AR1 and AR2), except for DBa (only AR2), and all chilling durations. AR, after-ripened; C, control seeds (no secondary dormancy manipulation); DBa, dormancy breakage after chilling; DBb, dormancy breakage before chilling; DI, secondary dormancy induction treatment, but no dormancy breakage; DI + DBa, secondary dormancy induction before, and dormancy breakage after, chilling. DB, dormancy breakage; seeds were scarified individually and exposed to 10 mM KNO_3. DI, dormancy induction; seeds were imbibed for 7 d at 31°C in total darkness. Seed chilling, seed imbibition at 4°C in the dark. Vernalization, rosette chilling for 7 d at 6°C.

Table 2 Relevant contrasts of experimental treatments

Contrasts	Treatments compared	Interpretation
Effect of experimental manipulations		
C vs DI	Control (unmanipulated) dormancy level vs dormancy induction before chilling	Effect of dormancy induction before chilling
C vs DBb	Control dormancy vs dormancy breakage before chilling	Effect of dormancy breakage before chilling
C vs DBa	Control dormancy vs dormancy breakage after chilling	Effect of dormancy breakage after chilling
DI vs DI + DBa	Dormancy induction vs dormancy induction followed by dormancy breakage after chilling	Effect of dormancy breakage after chilling
Effect of dormancy status during chilling		
DBa vs DBb	Dormancy breakage after vs before chilling	Natural dormancy level vs minimum (broken) dormancy
DBa vs DI + DBa	Dormancy breakage after chilling vs dormancy induction before chilling with dormancy breakage after chilling	Natural dormancy vs maximum (induced) dormancy
DBb vs DI + DBa	Dormancy breakage before chilling vs dormancy induction before chilling with dormancy breakage after chilling	Minimum dormancy vs maximum dormancy

In addition, the following contrasts estimated how the dormancy status of seeds influenced responses to seed chilling: DBa vs DBb; DBa vs DI + DBa; DBb vs DI + DBa (Table 2).

Assessment of germination and flowering time

Germination assays were conducted using 12 seeds of a given genotype in a single Petri plate (35 mm × 9 mm) containing 0.5% agar. We used 32 replicate Petri plates for each genotype in each chilling and dormancy treatment, with a total of 384 seeds per genotype in each treatment (12 seeds × 32 plates × 3 chilling treatments). A preliminary experiment with three of the genotypes (Br-0, Ct-1, Pro-0) did not reveal any significant differences in percentage germination after 7 d, whereas longer periods inside the Petri plates resulted in fungal growth (data not shown). Germination experiments were therefore limited to 1 wk, with censuses conducted at 0, 3 and 6 d after the end of chilling and transfer into light at 22°C. The proportion of germination was estimated as the total number of germinants after the last census, divided by the total number of viable seeds (i.e. seeds that had a living embryo). Viable seeds that did not germinate were considered to be dormant. The germination date was estimated as the day at which 75% of the final germination proportion was reached. Germination was scored when radicle protrusion was visible. Seed viability was assessed by testing firmness to touch (Baskin & Baskin, 1998).

After the germination assays, germinants were removed from the agar and planted in pots, with a total of eight pots per genotype × treatment combination. Treatments that had four or fewer germinants (≤ 5% germination) and seeds that germinated in the dark were not transplanted into pots. Plant growth conditions were the same as described for maternal plants. The bolting time was measured as the number of days spent at 22°C between germination and bolting. It is therefore a measure of bolting speed independent of the timing of germination. Bolting and flowering time, considered to be the day on which the first petals were visible, were highly correlated (R^2 = 0.98), and hence the bolting time was used as the measure of reproductive phenology.

Statistical analyses

First, to characterize the effect of after-ripening and seed chilling on germination and flowering without the effects of experimental dormancy modifications, only unmanipulated seeds (C dormancy treatment) were included in the analyses. We used a priori directed contrasts comparing fresh seeds with each of the after-ripened cohorts to distinguish differences attributable to after-ripening from those caused by maternal plant batch. Tukey honestly significant difference (HSD) *post-hoc* tests were used to compare the mean differences between the three after-ripening treatments (Fresh, AR1, AR2).

We then estimated the effects of after-ripening and chilling on germination and flowering of seeds in different experimental dormancy treatments. We employed the contrasts listed in Table 2 to compare specific treatments.

Germination analyses were performed using generalized linear models (GLMs) with a log link function and percentage germination as a quasi-binomial response. Bolting time was analysed using linear models. In all models, after-ripening, chilling and dormancy treatment were used as categorical independent variables, with all interaction terms included. Comparisons across specific dormancy treatments were conducted using submodels analogous to the full model, except for the levels of the secondary dormancy factor. All models were calculated using the 'glm' and 'lm' functions of the 'stats' R package (R Development Core Team, 2009). The correlation between germination and flowering responses to after-ripening was assessed by comparing the AR effect metric described above with Spearman tests of independence with 9999 Monte Carlo resamplings using the package 'coin' in R (Hothorn *et al.*, 2008).

Results

Effect of after-ripening and dormancy manipulations on germination

After-ripening After-ripening and chilling were found to affect germination speed, with a variation of up to 6 d in how fast a

given genotype reached 75% of the total percentage germination (e.g. Edi-0 Fresh vs AR2; Supporting Information Table S1), but no clear trend or directionality was observed in these effects. Conversely, the germination proportion of Fresh unmanipulated seeds was unambiguously lower than that of either after-ripened set (Table 3a; Fig. 2a,c,e: Treatment C). The effect of chilling was also highly significant and dependent on the after-ripening status of the seeds (Chill and Chill × AR significant effects in Table 3a). Fresh seeds germinated to the highest percentages after experiencing a short chilling treatment, and germinated the least if they did not experience any chilling. The effect of after-ripening was most pronounced in nonchilled seeds and least pronounced in seeds that were chilled for 21 d. As a result, long chilling increased germination relative to no chilling in Fresh seeds, but reduced germination relative to no chilling in after-ripened seeds. Long chilling seeds of both AR cohorts had the lowest germination proportion of the three temperature treatments (Fig. 2a,c,e). Thus, although a period of short chilling stimulated germination in both fresh and after-ripened seeds, after-ripening determined whether prolonged chilling broke or induced seed dormancy.

The response to after-ripening and chilling was genotype dependent (Gen factor in Table 3a). Differences in the effect of after-ripening were significant among genotypes (Gen × AR $F_{AR\ effect}$ [4,484] = 51.5, $P < 0.0001$; Gen × AR effect in Table 3a; Fig. 3a) and chilling treatments (AR × Chill $F_{AR\ effect}$ [2,484] = 296.0, $P < 0.0001$; Table 3a; Fig. 3a). The combined effect of chilling and after-ripening on germination was also genotype dependent (Gen × Chill × AR $F_{AR\ effect}$ [8,484] = 42.3, $P < 0.0001$; Table 3a; Fig. 3a) and appeared to be contingent on the level of seed dormancy. Short stratification was effective in enhancing germination relative to no stratification in all genotypes, except in AR seeds of Br-0 and Tu-0, which were already highly nondormant (Table S1). Long chilling was less effective at promoting germination than short chilling in most cases (except AR1 Edi-0), and resulted in lower germination than no chilling (i.e. induced dormancy) in several genotypes when seeds were after-ripened (e.g. AR1 Ct-1 & Pro-0; AR2 Edi-0; Table S1). In Tu-0, in particular, long periods of chilling resulted in reduced germination relative to no chilling in every cohort (Table S1). After long periods of chilling, the effects of after-ripening were diminished in some, but not all, genotypes, and were even reversed in one genotype (Pro-0), such that after-ripened seeds were more dormant than fresh seeds when they experienced prolonged stratification (Fig. 3a). The two genotypes with the lowest *FLC* expression (Tu-0 and Ct-1) were also those that exhibited the least change in percentage germination through after-ripening (Fig. 3a).

Table 3 Effect of genotypic differences (Gen), chilling (Chill) and after-ripening (AR) on germination proportion (a) and bolting time (b)

	Fresh vs AR1		Fresh vs AR2		
Factor	df	*F*	df	*F*	*F*
(a)					
AR	2	1208.557**	1	63.615**	2353.499**
Chill	2	401.687**			
Chill × AR	4	159.324**	2	32.969**	285.678**
Gen	4	775.885**			
Gen × AR	8	75.824**	4	91.361**	60.287**
Gen × Chill	8	142.82**			
Gen × Chill × AR	16	31.955**	8	19.221**	44.688**
Residuals	1548				

	Partial contrasts		Fresh vs AR1		Fresh vs AR2
Factor	df	*F*	df	*F*	*F*
(b)					
AR	2	51.09**	1	29.02**	73.16**
Chill	2	35.70**			
Chill × AR	4	6.07**	2	5.04*	7.10*
Gen	3	428.91**			
Gen × AR	6	5.59**	3	1.34	9.83**
Gen × Chill	6	16.09**			
Gen × Chill × AR	12	12.88**	6	14.00**	11.76**
Residuals	244				

Results of a priori contrasts to determine the effect of after-ripening are included. Data for these analyses are presented in Fig. 2. Only data from the control (C) treatment were used, that is, no manipulation of seed dormancy other than dry after-ripening and chilling.
*, $P < 0.01$; **, $P < 0.001$.

Dormancy manipulations The treatments used to manipulate dormancy did not affect seed viability (Table S1), but were highly effective in modifying germination speed and proportion (dormancy treatment (DT) effect in Table 4a; Fig. 2a,c,e; Table S1). The effect of dormancy manipulations on germination speed exhibited no clear pattern. In general, dormancy breakage appeared to accelerate germination, and dormancy induction to delay it (Table S1). Conversely, secondary dormancy treatments had a strong effect on the germination proportion. Dormancy induction decreased the percentage germination significantly (Table 4a, treatment C vs DI), except when seeds were already highly dormant (i.e. in fresh, nonchilled seeds). The dormancy-breaking treatment was effective at breaking natural and induced dormancy (Table 4a; Fig. 2a,c,e), and this was true whether it occurred before or after chilling (DBb or DBa). The total effect of dormancy breakage was dependent on how dormant the seeds were before the treatment; it was more effective if performed after chilling. This might be caused by an alleviation of dormancy by chilling, although the difference between DBa and DBb was not found to be affected by chilling duration (nonsignificant Chill and DT × Chill effects in Table 4a, DBa vs DBb, Fig. 2a,c,e).

The effect of chilling on germination was dependent on its duration and the dormancy level of the seeds (Chill and DT × Chill factors in Table 4a, Fig. 2a,c,e). Short chilling always increased germination relative to no chilling, except when seeds already had very shallow (e.g. DBb seeds) or very deep (Fresh DI) dormancy before the chilling treatment. Long chilling stimulated germination in seeds that were induced into dormancy (DI and DI + DBa treatments) and its effect was comparable with that of short chilling (Fresh and AR1 DI + DBa; AR2 DI) or even higher (AR1 DI; AR2 DI + DBa). This result parallels the effect of prolonged chilling on fresh and after-ripened seeds: prolonged

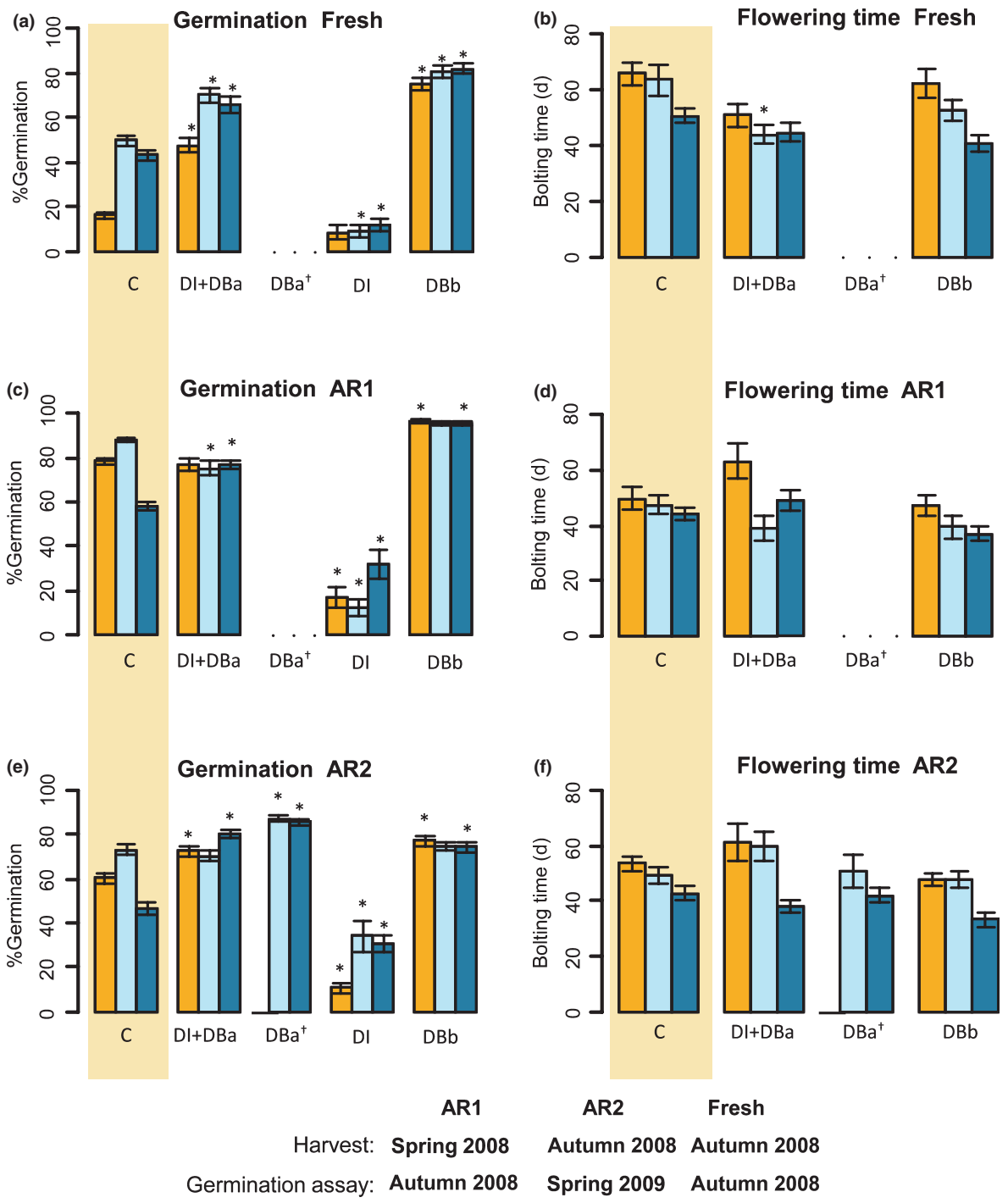

Fig. 2 Effect of chilling and secondary dormancy on germination and bolting in *Arabidopsis thaliana*. Mean percentage germination (%Germination) or number of days at 22°C before bolting (± SE) for fresh and after-ripened (AR) seeds. (a) %Germination of fresh seeds. For each bar, n = 24.6 ± 1.3. (b) Bolting time of plants from fresh seeds, n = 7.6 ± 0.4. (c) %Germination of AR1, n = 22.9 ± 1.4. (d) Bolting of AR1, n = 7.7 ± 0.4. (e) %Germination of AR2, n = 22.4 ± 1.4. (f) Bolting of AR2, n = 6.9 ± 0.4. †The DBa treatment for AR2 seeds only. 'No chilling' of DBa would have been identical to DBb. Asterisks indicate significant differences from the respective control (C, shaded). DBb, dormancy breakage before chilling; DI, secondary dormancy induction treatment, but no dormancy breakage; DI + DBa, secondary dormancy induction before, and dormancy breakage after, chilling. Orange bars, 0 d at 4°C (no chilling); light blue bars, 3 d (short chilling); dark blue bars, 21 d (long chilling).

chilling increased germination only in more dormant seeds, regardless of whether the seeds had primary or secondary dormancy.

Genotypic differences in germination responses to dormancy manipulations were highly significant (Gen × DT effect in Table 4a; Fig. 3a; Table S1). Ct-1 was the most dormant genotype in the absence of dormancy manipulation (C treatment). However, in all other treatments, Edi-0 had the lowest percentage germination (pooled over all stratification treatments: DI + DBa, 44.0 ± 34.8; DBa, 80.3 ± 15.7; DI, 10.2 ± 23.2; DBb, 61.72 ±

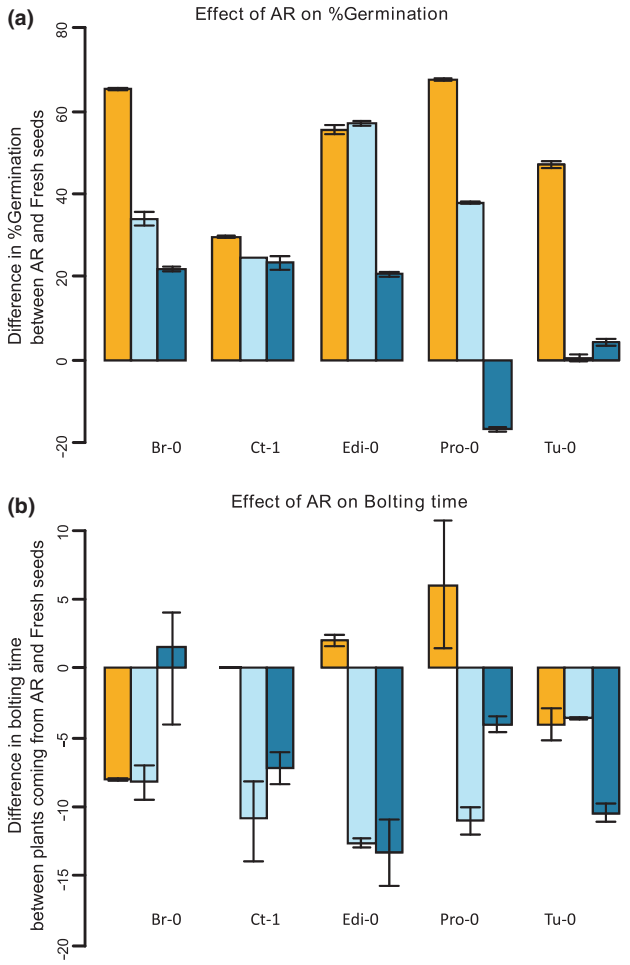

Fig. 3 Relative change induced by after-ripening on germination and bolting time in five *Arabidopsis thaliana* genotypes. Only unmanipulated seeds from the control (C) treatment are presented. Difference in percentage germination (%Germination) (a) and difference in bolting time (expressed in days at 22°C) (b) between after-ripened (AR) and fresh seeds. Bars represent the mean (± SE) of the difference between fresh seeds and the mean of the two AR cohorts for the trait in every treatment combination. (a) Each bar n = 26.63 ± 1.58; (b) n = 7.8 ± 0.19. Orange bars, 0 d at 4°C (no chilling); light blue bars, 3 d (short chilling); dark blue bars, 21 d (long chilling).

30.8), which resulted in a very small number of adult plants and forced its exclusion from the analysis of flowering time. By contrast, Tu-0 had the highest germination in all treatments other than C (DI + DBa, 90.5 ± 6.2; DBa, 98.8 ± 4.0; DI, 20.8 ± 21.1; DBb, 99.4 ± 3.1). Tu-0 seeds germinated so readily after dormancy breakage that most of the AR1 DBb seeds of this genotype exposed to long chilling germinated during the cold treatment and had to be removed from subsequent analyses.

Effect of after-ripening and dormancy manipulations on bolting time

After-ripening The effect of after-ripening on bolting time was assessed only if at least three plants of each genotype and treatment combination reached bolting. This requirement led to the exclusion of Ct-1 from these analyses. After-ripening, chilling, genotype and their interactions had highly significant effects on bolting time (AR, Chill and Gen effects in Table 3b, Fig. 2b,d,f, Treatment C; note that the five genotypes were included in this figure). When considering the pooled results for all genotypes, long chilling of seeds resulted in earlier bolting in all batches and cohorts. As expected, the effect of long chilling was stronger on the genotypes with higher FLC expression levels: mean difference in bolting time across cohorts (in days at 22°C) between long and no chilling: Edi-0 = − 27.9; Br-0 = − 15.6; Pro-0 = − 4.8; Tu-0 = 2.2; Ct-1 = − 4.2 (Table S1).

After-ripening accelerated significantly flowering in plants derived from nonchilled and short-chilled seeds (Figs 2b,3b). The effect of seed chilling on flowering time was most pronounced in plants from fresh seeds (Fig. 2b,d,f: Treatment C). Significant differences were found using a priori contrasts between fresh seeds and each of the after-ripened cohorts (AR1 and AR2, Table 3b), whereas *post-hoc* Tukey HSD tests detected no significant differences between the two after-ripened cohorts. Alleviation of primary dormancy by after-ripening resulted in an acceleration of flowering that was independent of germination timing. This effect was lost in long-chilled seeds, because chilling had already accelerated flowering time and after-ripening could not accelerate it further.

The effect of after-ripening and seed stratification on bolting time differed among genotypes (significant Gen × AR and Gen × Chill interactions in Table 3b; Fig. 3b; Table S1). For example, long cold stratification generally accelerated flowering, but, in TU-0 and after-ripened Br-0 plants, it had the opposite effect and delayed bolting. Overall, stratified seeds of Edi-0 had the strongest response to after-ripening, which caused bolting time to occur 2 wk earlier. When seeds experienced no stratification, Br-0 and Pro-0 had the strongest response, but in opposite directions (an acceleration of 7 d and a delay of 5 d in bolting time, respectively; Fig. 3b). In addition, the response of the different genotypes to after-ripening differed between the two AR cohorts (significant Gen × AR interaction for Fresh vs AR2, but not for Fresh vs AR1; Table 3b).

Flowering responses to after-ripening and chilling differed from those of germination (Figs 3, S1). After-ripening increased germination most significantly in nonchilled seeds, whereas its influence on bolting time was comparable for all chilling treatments. The genetic variation observed for the response of bolting to after-ripening also differed from that of germination, and no clear pleiotropic pattern could be observed (Spearman test of independence, Z = − 0.6, P = 0.6; Figs 3, S1). The germination response of a genotype to after-ripening was not a good predictor of its flowering response.

Dormancy manipulations The analyses of the effect of dormancy manipulation on bolting time, shown in Table 4b, were necessarily limited to the Fresh and AR2 batches, and excluded the genotype Edi-0, because of an insufficient number of plants. Seed chilling duration resulted in a significant acceleration of bolting time, with longer chilling inducing earlier bolting (Table 4b, Fig. 2b,d,f; Table S1).

Table 4 Comparison of changes in germination and flowering time caused by after-ripening (AR), cold chilling (Chill) and dormancy manipulation (DT): (a) ANOVA table of the generalized linear model (GLM) of germination results and (b) results of the analysis of flowering time

Factor (df full | partial comparisons*)	Full model	C vs DI	C vs DBb	C vs DBa	DI vs DI + DBa	DBa vs DBb	DBa vs DI + DBa	DBb vs DI + DBa
(a) Germination proportion								
NULL	3105.2	1315.17	1648.15	1072.27	1296.83	209.84	873.52	1343.3
AR (2 | 2)	**2687.49**	**981.83**	**1207.7**		**1092.91**			**998.37**
Chill (2 | 2)	**1972.69**	**724.12**	**903.22**	**489.68**	**688.79**	96.55	**618.24**	**929.67**
AR × Chill (4 | 4)	**989.92**	**348.13**	**444.93**		**405.89**			**492.48**
Gen (4 | 4)	**2908.15**	**1169.14**	**1415.26**	**852.11**	**1132.29**	**107.56**	**699.27**	**1085.45**
Gen × AR (8 | 8)	**1723.86**	**672.75**	**773.17**		**511.74**			**643.27**
Gen × Chill (8 | 8)	**1047.62**	**400.49**	**497.47**	**311.76**	**421.05**	**86.72**	**342.71**	**513.21**
Gen × AR × Chill (16 | 16)	**738.91**	**265.09**	**331.76**		**315.76**			**393.02**
DT (4 | 1)	**2007.02**	**783.01**	**950.63**	**757.08**	**702.3**	**97.44**	**661.74**	**936.33**
AR × DT (6 | 2)	**1169.9**	**504.46**	**609.02**		**465.46**			**541.58**
DT × Chill (7 | 2)	**940.67**	**319.6**	**425.73**	**310.15**	**400.11**	86.6	342.17	**485.4**
AR × DT × Chill (12 | 4)	**677.81**	**240.67**	**323.49**		**298.19**			**379.12**
Gen × DT (16 | 4)	**1247.04**	**512.04**	**651.78**	**472.94**	**486.08**	94.62	**572.11**	**579.34**
Gen × × AR × DT (24 | 8)	**832.31**	**304.37**	**378.86**		**381.56**			**463.25**
Gen × DT × Chill (28 | 8)	**700.87**	**254.99**	**324.75**	**242.15**	**306.13**	84.67	**322.81**	**387.71**
Gen × AR × DT × Chill (45 | 13)	**635.18**	**235.21**	**317.83**		**289.05**			**371.67**

Residual deviances for each model are reported. Full model: all treatments and seed cohorts were included in the analysis. Partial comparisons are tests for the effect of dormancy depth during chilling on germination (Full model, C vs DI; Db vs DI + DBa) or for the effectiveness of the experimental manipulation of dormancy induction and breakage (DI vs DI + DBa; C vs DBb; DBb vs DBa). Values in bold were significant at $P = 0.05$ after Bonferroni correction according to chi-squared tests. Experimental treatments are described in Table 2.

Factor (df full | partial comparisons*)	Full	C vs DBb	C vs DBa	DBa vs DBb	DBa vs DI + DBa	DBb vs DI + DBa
(b) Flowering time						
AR (1 | 1)	**97.94**	**113.36**				**26.41**
Chill (2 | 2)	**181.81**	**136.53**	**56.42**	**53.04**	**56.90**	**111.70**
Chill × AR (2 | 2)	**5.29**	1.13				**6.49**
Gen (3 | 3)	**1349.12**	**622.18**	**454.32**	**308.06**	**407.42**	**890.98**
Gen × AR (3 | 3)	**4.67**	**8.05**				**9.96**
Chill × Gen (6 | 6)	**56.85**	**33.31**	**15.67**	**8.11**	**38.96**	**44.40**
Chill × Gen × AR (6 | 6)	**3.86**	**4.14**				**4.66**
DT (3 | 1)	**20.19**	**25.81**	7.69	0.26	**14.22**	**15.03**
DT × AR (2 | 1)	4.06	**2.97**				1.72
Chill × DT (5 | 2)	2.10	4.54	0.02	0.47	6.32	2.10
Chill × DT × AR (4 | 2)	**10.96**	**5.55**				**17.38**
DT × Gen (9 | 3)	**21.23**	**7.40**	**6.75**	**7.51**	**15.17**	**22.17**
DT × Gen × AR (6 | 3)	**13.97**	**15.98**				**15.60**
Chill × DT × Gen (15 | 6)	**6.54**	**7.49**	2.61	2.25	**9.58**	2.13
Chill × DT × Gen × AR (11 | 6)	**12.25**	2.42				**17.31**
Residuals df	524	307	221	221	229	314

F values for the full model including all secondary dormancy treatments and the partial contrasts testing the effect of the level of dormancy during chilling (DBb vs DI + DBa; C vs DBb and DBa vs DI + DBa) and the possible differences in flowering time induced by the dormancy manipulation treatments (C vs DBa, DBa vs S). Flowering time analyses were conducted using only AR2 and Fresh cohorts and excluding Edi-0 because of insufficient plant numbers. Partial comparisons that include the DBa treatment were only conducted with AR2 data, the only cohort in which that treatment was used. Values in bold were significant at $P = 0.05$ after Bonferroni correction. Experimental treatments are described in Table 2.
*All partial comparisons of the effect of chilling (Chill) using the DBa treatment, df = 1.

Manipulated dormancy had a significant effect on bolting time (main effect of DT in Table 4b), but this effect was dependent on both after-ripening and chilling duration (Table S1). Secondary dormancy induction before chilling delayed flowering for *c.* 4 d (flowering time pooled means ± SD: DI + DBa = 39.2 ± 23.0; DBa = 35.0 ± 16.1), whereas dormancy breakage resulted in a symmetrical acceleration of bolting (C = 39.7 ± 15.3; DBb = 36.0 ± 18.1). The effect of dormancy breakage appeared to be slightly different if performed before vs after chilling: C vs DBb was significant, whereas C vs DBa was not, although DBa vs DBb and the difference between C and DBb within cohort classes were also not significant (Table 4b; Fig. 2). We detected no significant effect of the level of dormancy on the flowering response to seed chilling; none of the Chilling × DT terms was significant (Table 4b). Thus, seeds appear to perceive and integrate chilling signals that stimulate flowering, whether or not they

are dormant, and even if they do not germinate in response to the same chilling treatment.

After-ripening did not influence the response of bolting to secondary dormancy induction (nonsignificant DT × AR in Table 4b). Therefore, primary and secondary dormancy appear to have different effects on how chilling alters reproductive timing.

Genotypes differed significantly in the degree to which dormancy treatment, stratification duration and after-ripening influenced bolting time. For instance, dormancy induction (DI + DBa treatment) appeared to delay flowering in the genotype Br-0 under all chilling and after-ripening conditions, but such an effect was not apparent in the other genotypes (Table S1). No significant genotypic differences were found in how dormancy manipulation treatments interacted with stratification in the DBa vs DI + DBa, DBb vs DBa or DBb vs DI + DBa contrasts (nonsignificant Chill × DT × Gen factor in Table 4b).

Discussion

The seed stage represents a significant part of the life span of annual plants. This study proves that it can be highly consequential for overall life history, and that its effects transcend germination timing. Dormancy loss caused earlier flowering, independent of its effects on germination. Moreover, we found that both dormant and nondormant seeds perceive and integrate environmental cues, such as chilling, which modify life stages beyond germination. These results show that phenotypic and environmental variation at the seed (embryonic) stage can have significant repercussions on the expression of adult traits, and that ontogenetic transitions and environmental signals are integrated across the life history.

After-ripening and dormancy effects on the life history of plants

The alleviation of primary dormancy by after-ripening resulted in significant changes in the two life history traits studied. After-ripened seeds showed higher germination percentages and also produced plants that flowered more rapidly. Genotypic differences were also a significant source of variation for germination and flowering responses to after-ripening.

An increase in the germination proportion with after-ripening is common in seeds with physiological dormancy (Finch-Savage & Leubner-Metzger, 2006 and references therein), and has been interpreted as a mechanism to ensure that seeds do not germinate under ephemeral favorable conditions shortly after dispersal. For example, the requirement for after-ripening prevents seeds that are dispersed during the summer from germinating in response to short rain spells (Gutterman, 2002; Merritt *et al.*, 2007).

However, after-ripening varied considerably with seed chilling, such that short periods of cold increased germination, but prolonged chilling could both enhance and hinder germination. The heterogeneous response to prolonged chilling has been extensively documented previously (Baskin & Baskin, 1983, 1984; Nordborg & Bergelson, 1999). In a recent paper, Penfield & Springthorpe (2012) found that the effect of long periods of chilling on *A. thaliana* seeds was contingent on the dormancy state of the seeds, with less dormant seeds germinating in response to the treatment. Our results are similar, in that longer chilling resulted in slightly higher germination proportions in after-ripened seeds than in fresh seeds, but the difference was drastically smaller than that observed in the no chilling treatment. Moreover, in fresh seeds, long chilling stimulated germination relative to no chilling, but had the opposite effect in after-ripened cohorts. After-ripening reversed the response to the same environmental cue (i.e. long periods of cold). To summarize, our results showed that, in fresh seeds, prolonged chilling increases germination, but, in after-ripened seeds, it induces dormancy.

It has been shown recently that winter temperatures can deepen, and spring temperatures alleviate, the dormancy of *A. thaliana* seeds in the seed bank (Footitt *et al.*, 2011). This can be an adaptive mechanism that enables seeds that have been after-ripening in the soil for some time and, consequently, have a relatively greater tendency to germinate. If germination does not occur before the onset of winter, the cold season will induce secondary dormancy. Small differences in the speed of germination, like those observed in our experiment, can have significant consequences for survival and fitness, particularly during the autumn and spring (Donohue, 2002; Donohue *et al.*, 2005a). Selection is thus expected to favor mechanisms that enable seeds to cycle timely in and out of dormancy in response to environmental fluctuations (Vleeshouwers *et al.*, 1995). The results presented here demonstrate that it can be the after-ripening history of a seed that determines whether it germinates or cycles back into dormancy in response to a given cue.

In addition to increasing the proportion of seeds that germinated, after-ripening accelerated significantly the flowering time – a previously undocumented phenomenon to our knowledge. Plants derived from fresh seeds bolted later than those from after-ripened seeds, except when seeds experienced prolonged periods of low temperature. The lack of an effect of after-ripening in these seeds is probably because prolonged cold itself had a vernalizing effect and accelerated flowering, and after-ripening could not accelerate it further.

Some seeds will readily germinate in the presence of high water availability and light, even in the absence of cold spells. The delayed flowering of seedlings that germinate shortly after dispersal, and the accelerated flowering of plants that emerge from after-ripened seeds, might lead to a more synchronized flowering season among individuals. Theoretical models predict a correlation between the timing of germination and flowering to maximize reproductive output (Ritland, 1983), and recent results by Toorop *et al.* (2011) seem to indicate that a correlation exists between dormancy and flowering times in *Capsella bursa-pastoris*. At this point, it is hard to establish the fitness consequences of the different germination and flowering time combinations. Field experiments are necessary to determine the adaptive value of the various phenological arrangements and whether synchronized flowering has any effect on individual fitness or population dynamics.

Some authors have described changes in life history associated with seed aging that are independent of germination (Napp-Zinn, 1960, 1964). These changes are usually attributed to a loss of vigor with aging (Lysgaard, 1991; Rice & Dyer, 2001). Our results cannot plausibly be explained by such a process; plants produced by fresh and after-ripened seeds did not differ in their size or total leaf number (data not shown), and the time of dry after-ripening used is well within that which is usually considered to be optimal for *A. thaliana*. It is interesting to note that, as a plant grows in the vegetative state, the responsiveness to cold or photoperiod stimuli increases, and flowering becomes more probable (Simpson, 2004). Thus, the maturation of the seed and the plant both influence flowering. The final flowering time may be a genotype-specific response to environmental conditions (e.g. chilling) and total plant age, *including* the time spent as a seed.

After-ripening entails significant shifts in the content of and sensitivity to major hormones, and a concomitant loss of dormancy (Iglesias-Fernandez *et al.*, 2011). Moreover, the physiology of secondary dormancy breakage is very similar to that of after-ripening (Cadman *et al.*, 2006). Both primary and secondary dormancy can be overcome and germination induced using hormonal treatments, but this leads to aberrant seedling phenotypes (Fox *et al.*, 1995; Cabin *et al.*, 1997). Thus, it is conceivable that seedlings that emerge from fresh and after-ripened seeds have different hormone equilibria and metabolism overall. In such a case, it is possible that the physiology associated with dormancy is what influences flowering time, rather than seed age *per se*. In either case, primary dormancy is widespread among plants (Keeley, 1991; Merritt *et al.*, 2007; Van Assche & Vandelook, 2010), and therefore its consequences beyond germination and across life history should not be overlooked.

Dormant seeds perceive and integrate environmental cues

The effect of prolonged chilling of seeds on germination was dependent on after-ripening. By contrast, its effect on bolting time was consistent across after-ripening treatments. In agreement with the results obtained by other authors, plants that experienced prolonged chilling as seeds flowered earlier than those that underwent short or no chilling (Nordborg & Bergelson, 1999; Stinchcombe *et al.*, 2004).

Strikingly, no significant difference was found among seeds at different dormancy levels in how chilling affected flowering time. In other words, seeds perceive and integrate environmental cues, independent of how dormant they are, and modify subsequent life history accordingly. The ecological and evolutionary consequences of this process are, as yet, unexplored, although it can be anticipated that cue sensing during dormancy probably increases the chance of completing the life cycle successfully once germination occurs (Vleeshouwers *et al.*, 1995). Although it is well known that seed chilling accelerates flowering (Chouard, 1960; Nordborg & Bergelson, 1999), it has never been shown previously that even fully dormant seeds can retain the cueing provided by environmental stimuli and respond by shifting adult phenology.

The photoperiod is another important factor regulating flowering time in *A. thaliana*. In this plant, exposure to increasing day length accelerates flowering. However, the response to photoperiod is not independent of the effect of chilling temperature, that is, natural ecotypes demonstrate greater sensitivity to photoperiod after chilling and, in some cases, respond to photoperiod only after chilling (Lempe *et al.*, 2005; Zhao *et al.*, 2007). Our results show that the cold exposure during the seed stage would substitute the chilling requirements of the rosette, even if seeds were dormant during the chilling period. This decouples the flowering pathways along the ontogeny: chilling can happen during the seed stage (even if seeds are dormant), enabling the adult to respond to photoperiod cues.

Furthermore, our results demonstrate that the environment experienced by the plant embryos can condition the adult life stages, even independent of their immediate effects on the embryo (i.e. on germination). These results have important implications for understanding the adaptive significance of dormancy and germination responses, and the correlated evolution of dormancy and post-germination traits.

Conclusions

Seed dormancy has traditionally been studied as a mechanism that determines germination time. Although this is important for the survival of individuals, the results presented here show that dormancy *per se* can influence significantly post-germination life history. Changes in the level of dormancy are associated with shifts in flowering time. In addition, seeds appear to be able to integrate abiotic cues regardless of their dormancy state, changing adult phenology in response to the environment experienced during the embryo stage.

The type of dormancy exhibited by *A. thaliana* is prevalent among seed plants; hence, the major effects described here are probably generalizable, at least to other annual temperate species. Shifts in germination time caused by after-ripening or other factors are expected to have repercussions on flowering time because both transitions share important metabolic pathways. Cue perception and integration by dormant seeds are predicted to be general phenomena because they provide a mechanism for the integration of life history and environment across the ontogeny.

The findings of this study open up a range of hitherto unexplored possibilities in the control and shaping of plant life histories. More research is needed to characterize the mechanisms linking changes in dormancy with adult traits and the effects of the environment experienced by individuals whilst in the seed bank on their post-germination life history.

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Supporting Information

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

Fig. S1 Correlation between the germination and bolting time responses to after-ripening of five *Arabidopsis thaliana* genotypes.

Table S1 Germination time (days after the end of chilling), percentage germination (%Germination; mean ± SD) and bolting time (mean ± SD) of the five genotypes for every chilling and secondary dormancy treatment and seed cohort

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