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INVITED REVIEW AND RESEARCH OPINION

Seeds and seasons: interpreting germination timing in the field

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Abstract

This paper discusses how field and laboratory experiments, using a variety of genetic material, can be combined to investigate the genetic basis of germination under realistic ecological conditions, and it reviews some of our recent work on germination phenology of *Arabidopsis thaliana* in the field. Our results indicate that the genetic basis of germination depends on the environment. In particular, the conditions during seed maturation interact with post-dispersal environmental factors to determine germination phenology, and these interactions have a genetic basis. Therefore genetic studies of germination need to consider carefully the environment – both during seed maturation and after dispersal – in which the experiments are conducted in order to characterize genetic pathways involved with germination in the field. Laboratory studies that explicitly manipulate ecologically relevant environmental factors can be combined with manipulative field studies. These studies can identify the particular environmental cues to which seeds respond in the field and characterize the genetic basis of germination responses to those cues. In addition, a variety of genetic material – including mutant and transgenic lines, intact natural genotypes, recombinant genotypes, and near isogenic lines – can be used in field studies as tools to characterize genetic pathways involved in germination schedules under natural ecological conditions.

Keywords: *Arabidopsis thaliana*, dormancy, life history, maternal effects, phenotypic plasticity, photoperiod, seasonal cues, stratification

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Introduction

What ultimately matters to a seed in the field is when it germinates. That is because the conditions that enable germination are the same conditions that the germinant will be exposed to immediately upon germination, and those conditions can have lasting consequences throughout the life of the plant. Indeed, appropriate germination responses to environmental factors are the first requirement for successful growth and adaptation in any life-history trait; no subsequent life-history trait can even be expressed if the plant does not first survive past the germination stage. As such, germination timing can be a stringent selective sieve, determining which genotypes can establish in particular conditions. Therefore, determining the basis of variation in germination timing in the field contributes to our understanding of fundamental processes of population establishment, range expansion and geographic differentiation.

Despite their sessile habit, plants practice habitat choice through the exquisitely precise mechanism of germination cueing to environmental conditions (Donohue, 2003). Certain environmental conditions are necessary to break dormancy, and additional environmental conditions must be satisfied to permit germination after dormancy is broken (Léon-Kloosterziel *et al.*, 1996; Bewley, 1997; Foley, 2001). Therefore, germination cueing precisely determines a particular set of environmental conditions that a germinant will encounter. The season of germination, in particular, can determine the seasonal environment that a plant experiences throughout its life, since the course of seasons is, in a general manner, predictable. Seasonal habitat choice through germination phenology can determine whether a plant will express a winter or spring annual life history (Effmertova, 1967; Evans and Ratcliffe, 1972; Napp-Zinn, 1976; Ratcliffe, 1976; Nordborg and Bergelson, 1999), or even a biennial habit (Galloway, 2001, 2002).

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Investigating causes of seasonal variation in germination timing thereby provides information on variation in the overall life history of plants.

One challenge to the study of germination timing is to translate germination timing in the field into germination and dormancy responses to particular environmental stimuli. This paper suggests an approach to the study of germination that is ecologically grounded so that controlled studies in the lab can be used to interpret complex germination responses under natural seasonal conditions in the field, and vice versa. In particular, it is important to consider germination and dormancy not only in response to environmental conditions experienced directly by seeds after dispersal, but also responses to environmental conditions experienced during seed maturation on the maternal plant. Such maternal environmental effects on germination interact with seed responses to post-dispersal factors, to determine germination timing under natural ecological conditions. Through a combination of laboratory and field studies, we can analyse the genetic basis of specific germination responses to particular conditions experienced during seed maturation and after dispersal, and determine their effect on germination phenology under natural conditions.

An empirical example: combining field and laboratory studies to examine germination timing in the field

Here, some of our recent results are summarized that demonstrate how field and laboratory studies can be combined to investigate the environmental and genetic basis of germination timing in the field. We have conducted these studies in *Arabidopsis thaliana* because of the diverse genetic tools available in the species (Mitchell-Olds, 2001), and also because it represents a widely dispersed, introduced species that encounters, and must adapt to, a wide range of seasonal climatic conditions (Griffith *et al.*, 2004). In species encountering variable seasonal conditions throughout their range, natural selection on germination responses to seasonal environmental cues can be an especially significant determinant of which genotypes can establish in given seasonal environments.

We began with experimental manipulations of specific environmental factors under controlled conditions to determine their effect on germination percentages. We then conducted a field experiment to determine how pre-dispersal and post-dispersal seasonal environmental factors influence germination phenology in the field. Using a quantitative genetic design, we were able to test the evolutionary flexibility of germination responses to seasonal factors, and to examine the role of pleiotropy in integrating or constraining the evolution of germination responses to seasonally variable environments. That is, we were able to test whether the same or closely linked genes regulated germination phenology in multiple seasonal environments, or alternatively, whether different genes are associated with variation in germination in different seasonal environments. We found that the interaction between maternal pre-dispersal and seed post-dispersal seasonal environments has the potential to result in demographically important variation in overall life history and generation time.

How does germination respond to seasonal environmental factors?

The study species

Arabidopsis thaliana is a highly selfing (Abbott and Gomes, 1989) annual mustard species, and successive generations of self-pollination create genomes that are primarily homozygous (Todokoro *et al.*, 1995; Berge *et al.*, 1998; Bergelson *et al.*, 1998). Phylogeographic studies have indicated that populations that are geographically close are not necessarily closely related genetically (Todokoro *et al.*, 1995; Sharbel *et al.*, 2000; Hoffmann, 2002), such that uncommon outcrossing events between members of adjacent populations can create recombinant genotypes that provide diverse genetic material necessary for evolutionary responses to natural selection (Lexer *et al.*, 2003; Rieseberg *et al.*, 2003a, b; Weinig *et al.*, 2003; Griffith *et al.*, 2004). Therefore, experimental hybrid segregants offer a tool that is valuable for characterizing both the genetic basis and adaptive significance of characters, since such lines comprise new character combinations that represent genetic and phenotypic diversity that may have already been eliminated from populations by natural selection (Jordan, 1991; Schemske and Bradshaw, 1999). Recombinant inbred lines, in particular, disrupt linkage disequilibrium more completely than F_2 hybrids and, thereby, enable us to distinguish genetic constraints on phenotypes that are due to pleiotropy, as opposed to linkage disequilibrium (although very tight linkage would not be detected). In addition, recombinant inbred lines allow replicates of essentially identical genotypes to be grown under diverse environmental conditions, enabling a direct evaluation of environment-dependent genetic expression. In the studies discussed here, we used a set of recombinant inbred lines derived from two natural ecotypes: one from Calver, England (acquired through the Arabidopsis Biological Resource Center at Ohio State University: Stock #CS1062), and the other from Tacoma, Washington, USA (collected by T. Mitchell-Olds).

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Previous field observations of *A. thaliana* have documented extensive variation in overall life-history expression (Effmertova, 1967; Evans and Ratcliffe, 1972; Napp-Zinn, 1976; Ratcliffe, 1976; Nordborg and Bergelson, 1999). The most frequently documented is the winter annual life history, in which seeds germinate primarily in the autumn, rosettes overwinter, and reproduction and dispersal occur in the spring (Baskin and Baskin, 1972, 1983). Several populations also exhibit a spring annual life history, in which seeds germinate in the spring, and plants reproduce and disperse seeds later that same spring (Effmertova, 1967; Ratcliffe, 1976). Some mixed spring and winter annual populations have also been reported (Effmertova, 1967). Germination studies of winter annuals of *A. thaliana* determined that it displays an annual cycle of dormancy and non-dormancy. Freshly dispersed seeds were dormant, but lost dormancy gradually throughout the summer, first germinating at lower temperatures, later acquiring the ability to germinate at higher temperatures, and losing dormancy by October (Baskin and Baskin 1972, 1983). Ungerminated seeds re-entered dormancy in late autumn and winter, first losing the ability to germinate at high temperatures, then losing the ability to germinate at low temperatures, so that they became dormant by spring. This pattern explains why *A. thaliana* germinates in autumn in the location in which it was studied; it is dormant and incapable of germinating under warm conditions during the summer, and seeds are dormant in the spring.

In some populations, plants have the ability to germinate and reproduce in the autumn as well as during the spring (Thompson, 1994; Griffith *et al.*, 2004), and this ability very likely results in two generations per year rather than the typical one generation, although we are still in the process of documenting the demography of this particular life history. This variation in reproductive phenology results in variation in the seasonal conditions during which seeds are matured and dispersed. For example, plants that flower during the more typical spring season mature their seeds under long, warm days, and seeds experience warm conditions soon after dispersal. In contrast, plants that flower during the autumn mature their seeds under short, cool days, and seeds experience cold winter conditions soon after dispersal. Therefore, the seasonal conditions – specifically the photoperiod and temperature – during seed maturation and immediately after dispersal depend on the reproductive phenology of the maternal plant. We have been studying how the seasonal environmental factors of photoperiod and temperature influence dormancy and germination in *A. thaliana*. Our approach was to manipulate these factors first under controlled conditions and then in the field.

Manipulating seasonal cues under controlled conditions

In one experiment (Munir *et al.* 2001), we used 40 recombinant inbred lines and examined their germination responses to seasonal cues experienced both during seed maturation and after dispersal under controlled conditions. We manipulated the photoperiod during seed maturation on the maternal plants to resemble the photoperiod during spring (14 h of full-spectrum light followed by 10 h of darkness) and during autumn (10 h of full-spectrum light followed by 14 h of darkness). We collected seeds from these plants and manipulated the temperature experienced by seeds after a period of dry after-ripening; seeds were either placed directly at permissible conditions for germination (22°C), or they were first stratified for 5 days at 4°C and then placed at 22°C. We found that seeds that were matured under short days germinated to higher percentages when they received cold stratification, whereas seeds matured under long days did not respond strongly to cold stratification (Fig. 1). These results suggest that seeds matured during the short days of autumn could be stimulated to germinate quickly after receiving a cold period, either over winter or even during a warm spell in late autumn. This response could contribute to a successful spring annual strategy by promoting germination in the spring. It could also contribute to a novel bivoltine life history, whereby seeds matured in autumn can germinate either in late autumn or spring,

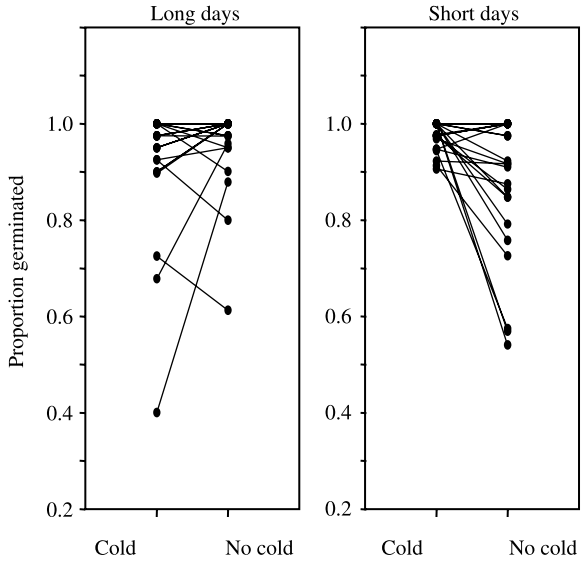


Figure 1. Proportion of seeds matured under long days (left panel) and short days (right panel) that germinated after dry after-ripening, followed by cold stratification or no cold stratification. Lines connect the means of a given recombinant inbred line in each treatment. (From Munir *et al.*, 2001.)

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rather than waiting until the following autumn to germinate with the spring-flowering cohort.

The precise effect of short days during seed maturation on germination timing in the field will depend on post-dispersal after-ripening conditions and on annual temperature cycles in the field. While the experiment above showed that short days increased germination proportions after cold at 22°C, suggesting the increased likelihood of spring germination of such seeds, this needs to be tested in the field under natural after-ripening conditions and temperature cycles.

Germination phenology in the field

To test how maternal photoperiod and post-dispersal seasonal conditions influence germination phenology in the field, we conducted a field study using 120 recombinant lines (Donohue et al., 2005a, b). We manipulated, under controlled conditions, the photoperiod under which seeds were matured (10 h versus 14 h, as above) and the season during which seeds were dispersed (June versus November), and we dispersed seeds in two locations: Rhode Island (RI) and Kentucky (KY), USA. In KY, natural populations flower only during the spring, so we dispersed seeds in KY only during the late spring (June). In RI, populations flower both during the spring and the autumn, so we dispersed seeds in RI both during the late spring (June) and autumn (November). This design enabled us to determine whether maternal seasonal effects influence germination in the field, and if so, whether maternal effects operate more strongly because of the photoperiod during seed maturation or the seasonal environment experienced immediately after dispersal.

Experimental details can be found in Donohue *et al.* (2005b). Briefly, seeds of all lines were harvested as close in time to each other as possible, after staggering planting to synchronize the time of seed maturation across lines and treatments. Plants were watered as needed until seed collection, rather than imposing sudden drought conditions which would accelerate the ripening of seeds at different stages of development. It was necessary to include a two- to three-week period of dry after-ripening in the lab during the time that it took us to aliquot and randomize the seeds for field dispersal. Such an after-ripening period would accelerate the loss of dormancy, but our results suggest that this period was not sufficient to explain the germination phenology in the field; the seeds that after-ripened slightly longer (those dispersed in RI) exhibited less early germination than those that after-ripened for a shorter period (those dispersed in KY). In addition, because these lines are recombinant, we did not expect them all to exhibit natural germination behaviour, and indeed, they did not. Seeds were dispersed on to the soil surface and protected from mechanical disturbance with lids fixed with wire screens.

We found that maternal seasonal effects strongly influenced germination phenology, but they did so primarily by determining the post-dispersal seasonal conditions rather than because of the photoperiod during seed maturation (Donohue *et al.*, 2005b). Maternal photoperiod effects were subtle and detectable only when seeds were dispersed in KY (in June). Moreover, in this sample maternal photoperiod influenced the germination only of strongly dormant seeds. Similar to the results of the laboratory study, dormant seeds that were matured under short days germinated to higher percentages than those matured under long days after temperatures began to become cool in the autumn. While the maternal photoperiod effects that were detected in the laboratory did appear in the field, their effects were tempered by the specific seasonal conditions experienced upon dispersal.

The post-dispersal seasonal environment, in contrast, strongly influenced germination phenology. Seeds dispersed in June exhibited a pronounced pulse of germination immediately after dispersal, and they continued to germinate at a low level throughout the summer (Fig. 2, lower left panel). Another pulse of germination occurred in mid October, and seeds continued to germinate at a low level throughout the winter and into spring. The earliest pulse of germination in the summer was apparently due to seeds with weak dormancy induction or maintenance. In contrast, seeds that germinated in the spring had strong dormancy that required cold conditions to break it; this being apparent because these seeds experienced similar permissible temperatures in the autumn as in the spring, yet they did not germinate in the autumn. Autumn-germinating seeds may have required a short cold treatment to overcome dormancy, or they may have simply had a lower permissible temperature for germination than summer germinants.

The huge pulse of germination of seeds dispersed in November (Fig. 2, lower right panel) shortly after dispersal indicates that a combination of weak dormancy (resembling the pulse of germination in the early summer by seeds dispersed in June), dormancy-breaking conditions, and permissible temperatures all coalesced. There were still some strongly dormant seeds that required cold conditions to break dormancy and/or that required warmer temperatures than were present in the autumn in order to germinate after dormancy was broken. In short, germination phenology in the field indicated that the recombinant lines varied in dormancy induction or maintenance, dormancy breakage, and likely varied in the conditions that permit germination after dormancy was broken.

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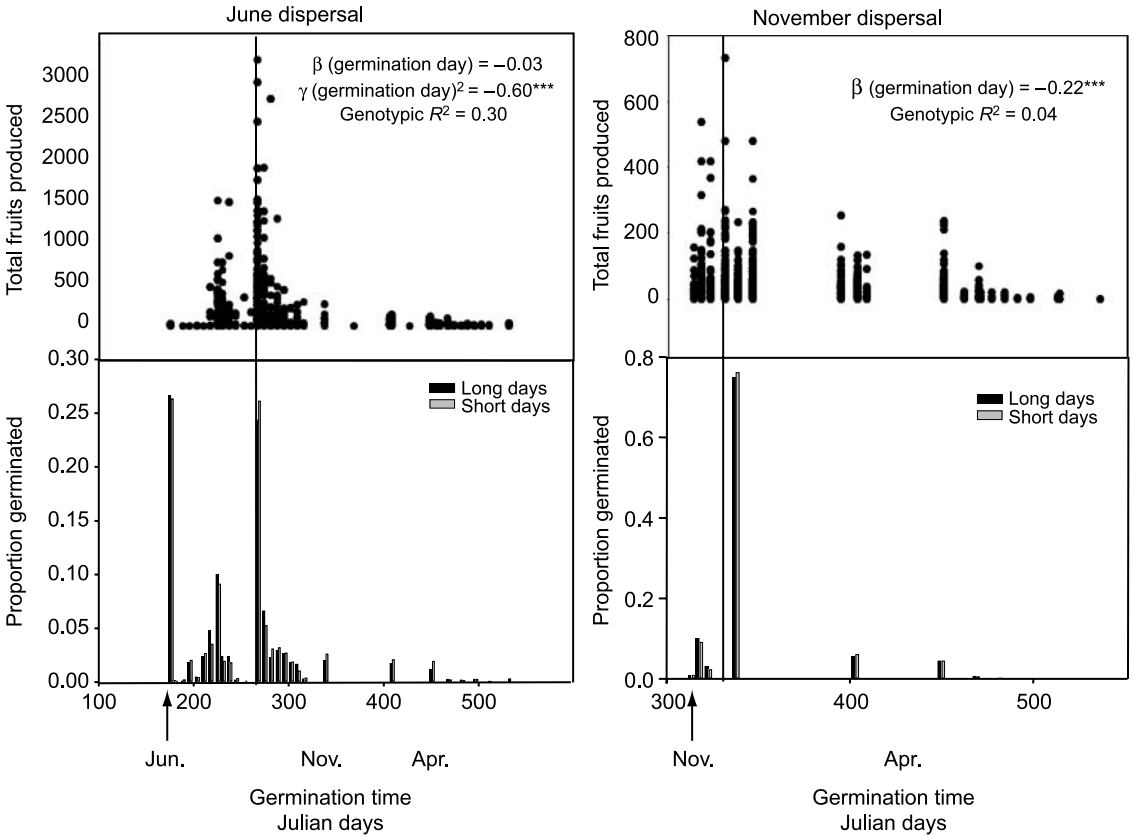


Figure 2. Germination schedules of seeds dispersed in Rhode Island (RI) during June (left) and November (right) are shown in the lower panels as the percentage of all seeds that germinated (*y* axis) over time (*x* axis). Days are in Julian days, with January 1, 2001 being the first day. Black bars represent seeds matured under long days, and grey bars represent seeds matured under short days. A scatter-plot of germination timing (*x* axis) versus the total number of fruits produced by each germinant (*y* axis) is shown in the upper panel. Each point represents the value for an individual plant. The vertical line indicates the date on which fruit production was highest. The arrow indicates the time of seed dispersal into the field. β is the standardized directional selection gradient (regression coefficient) indicating the strength of selection on germination timing, based on individual phenotypes. γ is the standardized quadratic selection coefficient indicating the strength of stabilizing selection on germination timing. ***$P < 0.001$. 'Genotypic R^{2}' indicates the proportion of the variation in fitness (fruit production) among genotypes that is explained by variation among genotypes in germination timing. (Adapted from Donohue *et al.*, 2005a.)

Considering natural selection on germination timing, we found strong stabilizing selection favouring intermediate germination timing in mid October for seeds dispersed in June (Fig. 2, fruit production, upper panels; Donohue *et al.*, 2005a). In contrast, we found weak directional selection favouring earlier germination in seeds dispersed in November. The important contrast is that weakly dormant or non-dormant seeds that were dispersed in June had zero fitness, whereas weakly dormant or non-dormant seeds that were dispersed in November had the maximal fitness. Therefore, the direction of selection on dormancy induction and maintenance is expected to vary strongly depending on the season of seed dispersal.

The challenge from these studies is obvious. Germination phenology of a population of seeds in the field is determined by a combination of mechanisms of dormancy induction, dormancy maintenance, dormancy breakage and permissible conditions for germination after dormancy is broken. How each of these physiological mechanisms contributes to germination phenology in the field requires more study, and can be approached with a variety of environmental manipulations to test dormancy status and the permissive conditions for germination after different environmental challenges. What this approach gives us is a set of genotypes that vary in these attributes in an ecologically interpretable manner. We can challenge these variable genotypes with specific controlled environmental treatments to determine more precisely which mechanisms contribute to their observed germination timing under natural ecological conditions. A good place to start, for example, would be to compare the cumulative cold

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requirement for dormancy breakage among summer-, autumn- and spring-germinating genotypes, as well as to compare the optimal germination temperatures of non-dormant seeds of these germination classes.

The genetic basis of germination responses in the field

Two fundamental, yet related, questions concerning the genetic basis of germination responses are: how stable are germination responses in the presence of gene flow and recombination, and how evolutionarily flexible are germination responses to diverse seasonal conditions? The genetic design of the field experiment enabled us to address these questions.

First, a look at the germination phenotypes of the recombinants compared to the parental lines (Fig. 3; Donohue *et al.*, 2005b) reveals that recombination creates germination phenotypes more extreme (that is, germinating sooner or later) than either parent. However, the degree of transgressive segregation depends on the particular geographic location and season of dispersal. For most of the treatments, transgressive recombinants tended to germinate earlier than either parent (Fig. 3) – which would be maladaptive if they were dispersed in June, but adaptive if they were dispersed in November. The large number of non-dormant or weakly dormant recombinants suggests that dormancy induction and maintenance requires the coordinated action of multiple genes. Therefore, outcrossing events are expected to disrupt dormancy induction and maintenance, but the adaptive consequence of this disruption would depend on the season during which seeds are dispersed.

Secondly, genetic correlations across sites (Fig. 4A; Donohue *et al.*, 2005b) indicate that germination in different locations has some common genetic basis (that is, the correlations are significantly positive), but that they also have some degree of genetic independence (that is, the correlations are also significantly different from unity). Because these lines are highly recombinant, the shared genetic basis is due to the same, or closely linked, genes that regulate germination in both environments, rather than due to non-physical linkage disequilibrium. What this shared genetic basis means is that natural selection on germination of plants in KY will cause a correlated change in germination if those genotypes are dispersed into a more northerly location such as RI. However, despite the correlated response to selection, germination timing retains some ability to evolve independently in the different geographic locations; eventually, some genotype could exist that is capable of exhibiting adaptive germination in multiple environments. In contrast, genetic correlations across

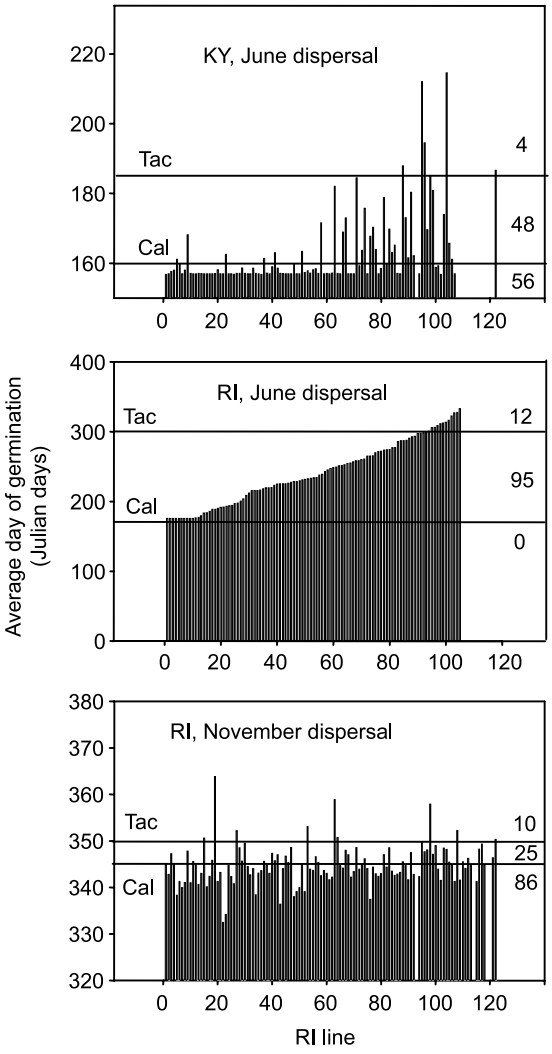


Figure 3. Average day of germination of seeds dispersed in Kentucky (KY) during June (upper), Rhode Island (RI) during June (middle), and RI during November (lower). Each bar represents the mean phenotype of a given recombinant inbred line. The order of the genotypes is the same in all graphs. Horizontal lines indicate the mean germination day of the parental lines (Cal and Tac), and the numbers to the right indicate the number of genotypes with germination days later (above), between, or earlier (below) than those of the parental lines. (Adapted from Donohue *et al.*, 2005b.)

dispersal season were not significant (Fig. 4B), suggesting that the germination of autumn-dispersed seeds can evolve without causing correlated changes in the germination of those genotypes if they were to be dispersed in June.

We found evidence for another form of pleiotropy, or shared genetic basis of germination behaviour. Namely, delayed germination in the autumn was significantly genetically correlated with a propensity

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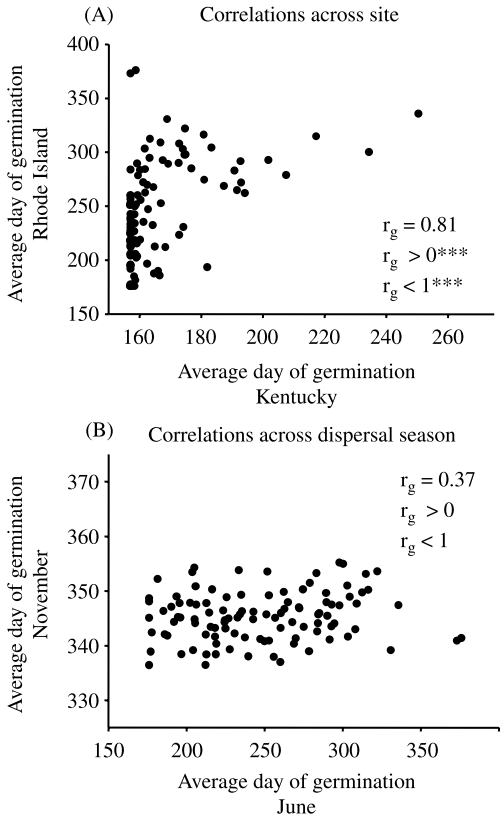


Figure 4. Genetic correlations across sites (A) for day of germination of seeds dispersed in June. Genetic correlations across dispersal seasons (B) for day of germination of seeds when dispersed in RI. r_g indicates the genetic correlation; $r_g > 0$ and $r_g < 1$ give the significance of the test that $r_g > 0$ and that $r_g < 1$, respectively. ***$P < 0.001$. Results are shown only for seeds that were matured under short days, but the results were very similar for seeds matured under long days. Each point represents the mean phenotype for one genotype. (Adapted from Donohue *et al.*, 2005b.)

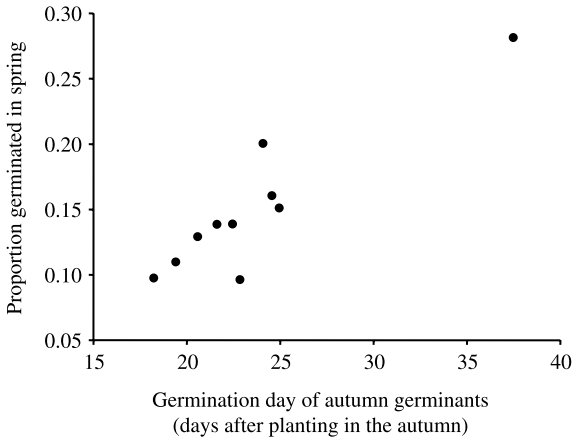


Figure 5. Relationship between germination timing in the autumn and the percentage of spring germinants. Each point represents the mean of a population. Spearman correlation $= 0.77$, $P = 0.01$. Spearman correlation without outlier $= 0.68$, $P < 0.05$. (From Griffith *et al.*, 2004.)

to germinate in the spring for seeds dispersed in RI (June-dispersed seeds: $r = 0.53$, $P < 0.001$; November-dispersed seeds: $r = 0.82$, $P < 0.001$; based on genotypic means). We found the same result in a set of intact natural genotypes grown in KY (Fig. 5; Griffith *et al.*, 2004). This result suggests a common physiological basis to delayed germination in the autumn and germination in the spring. Specifically, it suggests a cumulative requirement for cold might be necessary to break dormancy in some genotypes; if too much cold is required, then seeds may experience prohibitively cold conditions before they can germinate in the autumn and must then wait until permissive temperatures arrive in the spring. If such is the case, then seeds that are selected to have later germination in the autumn (for example because of hot, dry autumns typical of southerly locations in North America) may exhibit a higher proportion of spring germination under conditions in which non-permissive winter conditions come sooner (for example after dispersal to more northerly latitudes). Remarkably, the tendency to germinate in the spring in more northerly latitudes in North America may conceivably be due to the northerly migration of seeds that have evolved to require cold in the south, rather than due to local adaptation to northerly conditions. Given the extreme mobility of *A. thaliana*, due to its efficient seed dispersal and apparent common transport by humans, this possibility cannot be neglected. These are testable hypotheses that require, first, controlled laboratory studies to determine cold requirements for dormancy breakage and permissive temperatures of non-dormant seeds, and, secondly, field studies to determine the adaptive significance of those requirements.

In short, the degree of pleiotropy in germination under diverse environmental conditions can determine the evolutionary flexibility of germination. Specifically, the degree of pleiotropy determines whether germination can evolve independently in different environments, or whether an evolutionary response to selection on germination in one environment will cause the correlated evolution of germination in a different environment. Pleiotropy can also determine the facility of evolving a coordinated response to integrated environmental cues. Testing for pleiotropy within natural environments is necessary to make conclusions about how germination can evolve under ecologically realistic conditions, since our results indicate that the genetic basis of germination depends on the environment. Such tests can provide valuable information on which responses are most genetically independent (and thus may involve independent genetic pathways of regulation), and

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which environmental challenges are likely to constrain responses to other environmental factors. Quantitative genetic studies of the sort just described can be refined by conducting quantitative trait locus (QTL) analysis to identify specific chromosomal regions and eventually genes associated with natural variation in germination under different environments (see below).

Summary of field and laboratory studies

The above example illustrates how laboratory studies of germination can be effectively combined with field studies to provide novel information on both the ecological context of germination behaviour and the genetic basis of relevant germination responses. First, we found that appropriate responses of germination timing to geographically variable seasonal conditions may be the first requirement for establishment of populations in new locations. Natural selection on germination timing can be an extremely strong sieve that determines which genotypes can establish in a given location with a given seasonal schedule.

Secondly, it is clear that we need to know more about the physiological and genetic basis of maternal effects on germination. The strong influence of flowering and dispersal phenology on germination phenology suggests that, in fact, under natural ecological conditions, genes with the largest effect on germination timing may actually be flowering-time genes. Much of the variation in germination phenology that is so easily observable in the field may be as much due to genetically based differences in flowering phenology as genetically based differences in germination behaviour *per se*. However, we also detected abundant genetic variation for germination after controlling for differences in seed maturation conditions and dispersal phenology. Genetic variation for dormancy induction and/or maintenance, dormancy breakage and germination requirements of non-dormant seeds appears to be abundantly present in these lines. This genetic variation provides useful material for conducting more detailed studies of the physiological mechanisms underlying variation in germination phenology. Specifically, we can contrast conditions for dormancy induction, requirements for dormancy breakage and permissible temperatures of germination after different environmental challenges between lines known to have ecologically relevant variation in germination timing in the field.

Thirdly, the observed maternal effect – that of flowering and dispersal timing influencing germination phenology – can contribute to important demographic and evolutionary dynamics. It is this effect of maternal reproductive phenology on germination that likely leads to a very interesting demographic innovation for a weedy plant: namely the potential ability to complete two generations in a single year instead of one. This may contribute to the ability of *A. thaliana* to be so successful in establishing throughout its range. By studying explicitly the effects of reproductive timing and conditions during seed maturation, we can better understand the physiological, genetic and evolutionary mechanisms of their variation, and we can also gain ecological insight.

Methodological considerations

Choosing ecologically relevant environmental treatments

Combining laboratory and field environments

The choice of ecologically realistic environmental manipulations is necessary to determine the genetic and physiological basis of germination behaviour in the field. A remarkable example of the importance of choosing appropriate environments is a set of studies of phytochrome regulation of flowering time. Until recently, the function of particular phytochromes had been characterized under standard laboratory conditions of 22°C. These studies were repeated at 16°C, a temperature very likely to be experienced by *A. thaliana* in the field (Halliday and Whitelam, 2003; Halliday *et al.*, 2003). It was determined that the phytochrome previously reported to control flowering time (phyB) no longer exhibited this function at the lower temperature, and that other phytochromes, not previously thought to be as important in flowering-time regulation, played important roles at lower temperatures. This was discovered simply by changing the temperature by 6°C. Therefore, molecular genetic studies need to be carefully calibrated to realistic ecological conditions if their goal is to explain phenotypic expression of organisms in natural environments.

In the case of germination, a particularly obvious conclusion from the empirical studies above is that it is necessary to consider environmental conditions during seed maturation as well as environmental conditions after seed dispersal (or collection). Maternal environmental effects on germination are well documented (reviewed in Gutterman, 1992b; Baskin and Baskin, 1998; Donohue and Schmitt, 1998). Seasonal factors such as photoperiod and temperature (Gutterman, 1992a, 1996), and factors such as light quality, which can vary seasonally as the vegetation canopy develops (McCullough and Shropshire, 1970; Gutterman 1974; Hayes and Klein, 1974; Gutterman *et al.*, 1975), are known to alter the conditions required to break dormancy and permit germination in diverse species. Explicit studies of the physiological and genetic basis of these maternal effects are necessary to

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predict germination under ecologically realistic conditions, even if the genetic basis of germination under different post-dispersal conditions is well characterized under a set of standard laboratory conditions.

Combining laboratory manipulations with field manipulations can identify precisely the effect of specific environmental cues on germination behaviour under natural conditions (see also Schmitt *et al.*, 1999). For example, one can manipulate a specific environmental factor during seed maturation and determine how that factor influences germination in the field. Conversely, one can manipulate the ecological conditions during seed maturation in the field and then assay germination responses to particular environmental factors in the lab. In addition, imposing environmental manipulations within field studies can be an especially powerful approach. For example, experimental manipulations of an ecologically relevant environmental factor such as density, vegetation shade, nutrient or water environment, or season of dispersal can reveal germination responses to particular ecological factors under ecologically realistic conditions.

A combination of field and laboratory studies can be especially effective when the same genetic material is exposed to multiple field and laboratory environments. Quantitative genetic approaches can test specifically whether germination responses to controlled laboratory manipulations share a genetic basis with germination responses to field conditions. In this manner, the particular environmental stimulus that most strongly regulates germination under natural conditions can be identified.

Replication

For experimental studies using environmental manipulations of maternal and progeny conditions, it is important to realize the need to replicate maternal environments as well as offspring environments. Adequate replication requires that, for each maternal or progeny environmental treatment, maternal plants or seeds be placed in multiple independent chamber compartments, unless treatments can be randomized within a chamber compartment. If space is limiting, then multiple temporal blocks may be required instead. This makes for larger, as well as slower, experiments. However, the importance of seed maturation conditions on germination justifies the extra space or time required to investigate these factors directly.

Selection of genetic material

Mutants or transgenic lines

The most appropriate genetic material to use in studies of dormancy and germination depends on the question being asked. To characterize physiological pathways involved in germination behaviour, mutant or transgenic genotypes are invaluable for testing specific hypotheses concerning the role of particular genes in dormancy and germination. Combining mutants can test hypotheses about the structure of pathways of dormancy and germination regulation. While most such studies are conducted under controlled laboratory conditions, exposing such genotypes to different natural perturbations could provide valuable information on how specific pathways are involved in determining germination timing in the field (Schmitt and Dudley, 1996; Choi *et al.*, 2003; Cipollini *et al.*, 2003; Galen *et al.*, 2004).

Intact natural genotypes

A different question is: which genes account for the *variation* in germination behaviour that is observed in natural populations? That is, many genes may be part of a pathway that regulates germination, but not all of those genes are variable and segregating within natural populations. A large effect of an artificially induced mutation in a particular gene does not imply that large differences in germination among ecotypes are due to alterations of that gene. Indeed, one of the fundamental questions concerning general evolutionary dynamics is how variation in particular genes within a specific pathway structure leads to phenotypic variation in multiple traits. Therefore, it is important to identify natural genetic variants and to characterize the functional significance of that variation.

The use of intact natural genotypes is valuable for characterizing natural variation in germination behaviour. These are simply genotypes collected directly from the field and which are used directly or maintained in a manner (e.g. single-seed descent as opposed to bulk maintenance) that does not erode genetic variation. Conducted within a geographical or ecological context, studies using natural genotypes can provide important information on how particular ecological factors influence the evolution of dormancy and germination, and what agents of natural selection might be most important in determining the relative success of different natural germination genotypes. Reciprocal transplants in the field, and controlled environmental manipulations either in the field or lab, can determine how particular natural genetic variants perform under specific ecological environments.

Quantitative genetic analyses of such natural genotypes can characterize the degree of pleiotropy or linkage disequilibrium in germination responses to diverse environmental cues, as illustrated above. In particular, a pleiotropic association between germination under field conditions and germination in response to a specific experimental environment

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would indicate that the particular experimental environmental factor is important in determining germination differences among natural genotypes in the field. This can be a very effective method for narrowing down the possibilities for further genetic investigation by identifying the most relevant environmental manipulations. Quantitative genetic analyses can also provide insight on the physiological mechanisms of germination by identifying potential crosstalk between pathways. For example, if the same genotypes display high levels of germination in short days and in cool conditions, then that result suggests that the same or linked genes are involved in responses to both day length and temperature, and that these genes vary in natural populations.

Recombinant lines

Recombinant lineages can be extremely useful for studying natural selection (Jordan, 1991; Schemske and Bradshaw, 1999). They enable us to examine natural selection on naturally possible phenotypes, some of which may not be recoverable directly from the field because of previous natural selection. Even when it is impractical to create inbred lineages such as those used in the above studies, F_2 recombinants frequently exhibit widely variable phenotypes that can be used in field studies of natural selection. However, inbred recombinant lineages have the added virtue of being able to be replicated over different environments.

Recombinant lines can be used in studies like those described above, but such lines with linkage maps are also used in quantitative trait loci (QTL) analysis, which is another method that has been extremely useful in characterizing the genetic basis of natural variation in germination (e.g. Alonso-Blanco and Koornneef, 2000; Foley, 2001; Alonso-Blanco *et al.*, 2003; Gu *et al.*, 2004). QTL studies can be conducted on F_2 hybrids between divergent lineages or on recombinant inbred lines derived from such crosses. Recombinant inbred lines used in QTL analyses are especially useful for studies of plastic responses to environmental stimuli because identical genotypes can be grown in different environments. The goal of QTL analysis is to identify chromosomal regions, and ultimately genes, associated with natural variation in a phenotype of interest; that is, to identify the genes that actually vary among natural populations and that contribute to natural variation in the phenotype. QTL analysis can also test whether the same chromosomal region is associated with variation in germination timing under different environmental conditions, or whether the same chromosomal region is associated with variation in germination timing and some other trait of interest.

QTL analysis has been an enormously effective method for identifying novel genes and novel alleles associated with variation in the character of interest. It has become an invaluable approach for characterizing novel gene function (Alonso-Blanco and Koornneef, 2000).

Near isogenic lines

Once chromosomal regions associated with the trait of interest (e.g. germination responses to photoperiod) have been identified, near isogenic lines (NILs) can be created by successive backcrossing of the QTL into a given genetic background, creating genetically homogeneous lines in which only the QTL varies (Alonso-Blanco *et al.*, 2003). This sort of genetic material is ideal for determining the phenotypic effects of specific natural allelic substitutions under diverse environmental conditions. In short, it is probably the most evolutionarily relevant genetic material to use because it simulates a new spontaneous mutation, enabling precise investigations of the phenotypic effects of new variants and precise measurements of how it will increase or decrease in frequency under specific ecological environments of natural selection.

NILs will be most useful for ecological and evolutionary studies if they are created on relevant genetic backgrounds. Epistasis among QTL is not uncommon (although difficult to detect statistically). That is, a particular QTL allele can influence a trait in different ways, depending on the alleles that are present at different loci. This means that the genetic background of NILs can determine the effect of the QTL that has been introgressed. This is a fascinating issue in itself, and researchers can tackle this complexity by introgressing particular QTL alleles on to a range of ecotypic backgrounds. Currently, the Landsberg *erecta* background in *A. thaliana* has been used as the background for several sets of NILs, due to the convenience of its attributes for rapid growth under laboratory conditions. While ideal for efficient physiological and molecular genetic investigations, this background is less desirable for ecological studies, since several aspects of its fundamental life history and morphology – both known and probably unknown – have been bred for ecologically aberrant attributes. NILs on unselected ecotypic backgrounds would be more realistic for ecological studies, and especially for studies of natural selection.

The presence of epistasis also implies that very interesting results can come from studying different combinations of alleles at different loci. The creation of double (or multiple) NILs, i.e. material that differs in alleles at only two loci (or a few), can enable explicit tests of how particular QTL alleles interact to influence phenotypes, and also how specific combinations of QTL alleles influence fitness under different ecological conditions. With such studies, we can finally begin to address fundamental issues concerning how genetic

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changes at particular loci, and combinations of loci, influence evolutionary dynamics of specific characters.

Potential for collaboration

I hope to have illustrated how some of the common tools used for molecular genetic or physiological analysis can be integrated into ecological studies, and, likewise, how an ecological context can be crucial for investigating fundamental questions of gene function. Both genetic and ecological research is labour intensive, and it is quite unlikely that a single lab can efficiently create the best genetic material *and* conduct the most ecologically sophisticated experiments. Collaboration is necessary to integrate genetics, physiology and ecology.

There is an unprecedented opportunity for ecologists to make use of the genetic tools developed by their molecular colleagues to address fundamental ecological and evolutionary questions. Molecular geneticists, in turn, can benefit from collaboration with ecologists who have insight into what experimental environments are most relevant and what the potential targets and agents of natural selection are. They, and organismal biologists, can also provide information on appropriate genetic backgrounds for realistic assessment of gene function in ecologically functional organisms. The ubiquity of environment-dependent and genetic background-dependent gene expression is too well documented to ignore, but collaboration between molecular geneticists and ecologists can confront this complexity directly and effectively.

Such collaboration requires not only friendly dialogue but also concerted efforts to combine and share resources. New genetic material using appropriate genetic backgrounds can be created by geneticists for collaborative studies with ecologists and evolutionary biologists. Ecologists, in turn, can cultivate a literacy in molecular genetics and physiology in order to make use of, or guide the construction of, genetic material that can contribute to our knowledge of organismal *and* molecular function. Of course, not all molecular genetic studies require ecological realism to elucidate biochemical pathways and mechanisms of gene regulation. And not all ecological studies need to investigate phenotypes down to the level of specific genes. But we can now combine genetic and ecological studies in a way that has not been possible before. For the first time, we can ask how specific natural genetic variants alter phenotypes and fitness; how they are expected to increase in frequency in different ecological contexts; how combinations of variants influence these processes; and how, ultimately, molecular genetic changes account for phenotypic evolution.

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