

Natural variation in germination responses of *Arabidopsis* to seasonal cues and their associated physiological mechanisms

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- **Background and Aims** Despite the intense interest in phenological adaptation to environmental change, the fundamental character of natural variation in germination is almost entirely unknown. Specifically, it is not known whether different genotypes within a species are germination specialists to particular conditions, nor is it known what physiological mechanisms of germination regulation vary in natural populations and how they are associated with responses to particular environmental factors.
- **Methods** We used a set of recombinant inbred genotypes of *Arabidopsis thaliana*, in which linkage disequilibrium has been disrupted over seven generations, to test for genetic variation and covariation in germination responses to distinct environmental factors. We then examined physiological mechanisms associated with those responses, including seed-coat permeability and sensitivity to the phytohormones gibberellic acid (GA) and abscisic acid (ABA).
- **Key Results** Genetic variation for germination was environment-dependent, but no evidence for specialization of germination to different conditions was found. Hormonal sensitivities also exhibited significant genetic variation, but seed-coat properties did not. GA sensitivity was associated with germination responses to multiple environmental factors, but seed-coat permeability and ABA sensitivity were associated with specific germination responses, suggesting that an evolutionary change in GA sensitivity could affect germination in multiple environments, but that of ABA sensitivity may affect germination under more restricted conditions.
- **Conclusions** The physiological mechanisms of germination responses to specific environmental factors therefore can influence the ability to adapt to diverse seasonal environments encountered during colonization of new habitats or with future predicted climate change.

Key words: Abscisic acid, dormancy, germination, gibberellic acid, hormonal sensitivity, natural variation, pleiotropy, *Arabidopsis thaliana*.

INTRODUCTION

Phenology is one of the most important determinants of plant performance in response to environmental change (Chuine and Beaubien, 2001; Walther *et al.*, 2002; Parmesan and Yohe, 2003; Menzel *et al.*, 2006; Parmesan, 2006; Bradshaw and Holzapfel, 2008). The seasonal timing of critical developmental transitions, such as germination or the initiation of reproduction, is under strong natural selection to coincide with favourable growing conditions, and seasonal phenology has a major influence on fitness, local demography and even local species persistence (Bradshaw and Holzapfel, 2008; Willis *et al.*, 2008). With climate change or dispersal into novel environments, the timing and duration of the growing season change and can create a mismatch between formerly adaptive cues of seasonal events and the actual timing of those events, resulting in novel selective pressures on phenological cueing.

As one of the earliest developmental transitions, germination timing is under extremely strong natural selection (reviewed in Donohue *et al.*, 2010) and is a major determinant of the expression of and selection on post-germination traits (Donohue, 2002; Weinig, 2000; Donohue *et al.*, 2005;

Korves *et al.*, 2007; Wilczek *et al.*, 2009; Huang *et al.*, 2010). The seasonal timing of germination is determined by the environmental conditions that are required to break dormancy and those that permit germination after dormancy is broken (reviewed in Bewley, 1997; Baskin and Baskin, 1998). The germination behaviour of seeds is, moreover, highly responsive to environmental conditions both during seed maturation and after dispersal (reviewed in Baskin and Baskin, 1998; Donohue, 2009). To predict the seasonal conditions of seed germination under any climatic scenario, it is therefore necessary to characterize germination responses to combinations of seed-maturation and post-dispersal conditions.

The environmental conditions during seed maturation, such as photoperiod and temperature, strongly influence dormancy and germination (e.g. Lacey, 1996; Munir *et al.*, 2001; Donohue *et al.*, 2005, 2007, 2008; Schmuths *et al.*, 2006; Dechaine *et al.*, 2009; reviewed in Gutterman, 1992; Baskin and Baskin, 1998; Donohue, 2009). Maternal control of dormancy and germination occurs in part through the seed coat, which is maternal tissue. The seed coat (testa) can be a physical constraint on germination (Dobrovolska and Cetl,

1966, Bewley, 1997; Koornneef *et al.*, 2002) and an environmental filter whose permeability mediates water and/or oxygen uptake by the seed and the light environment experienced by the embryo (Debeaujon *et al.*, 2000, 2007). The triploid endosperm, which has $2n$ maternal genotype and $1n$ paternal genotype, is also a strong regulator of germination in *A. thaliana* and other species (Bethke *et al.*, 2007, Linkies *et al.*, 2009), and in *Arabidopsis thaliana* it consists of a single layer of aleurone cells beneath the testa. In addition, maternal influences on germination and dormancy occur via the induction of primary seed dormancy during the late stages of seed maturation, the depth of which is at least in part controlled by the maternal plant (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). Abscisic acid (ABA) is the major plant hormone that induces and maintains dormancy, and ABA production in seeds increases late in development, during dormancy induction and just before seed desiccation (reviewed in Kucera *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Bentsink and Koornneef, 2008; Holdsworth *et al.*, 2008). As such, ABA production and sensitivity to ABA are major regulators of seed dormancy. Primary dormancy is gradually lost by a process of dry after-ripening (Baskin and Baskin, 1998; Bentsink and Koornneef, 2008).

Primary dormancy loss also occurs in response to environmental factors experienced after dispersal. In particular, chilling (seed imbibition at cold temperature) breaks dormancy in many species from temperate climates (Baskin and Baskin, 1998). Cold-stimulated dormancy loss and germination is regulated by a balance of the plant hormones, gibberellic acid (GA), which stimulates germination, and abscisic acid (ABA) which induces and maintains dormancy (Koornneef *et al.*, 2002; Kucera *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Bentsink and Koornneef, 2008; Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008). GA biosynthesis increases in response to chilling via known genetic pathways (Penfield *et al.*, 2005). Thus the synthesis and sensitivity to GA have a major role in regulating germination responses to temperature.

Seeds can also be induced into secondary dormancy by specific temperatures or temperature cycling (Baskin and Baskin, 1998). In *Arabidopsis thaliana*, for example, seeds re-enter dormancy in the autumn if they do not experience appropriate germination conditions (water, light and permissive temperatures) after primary dormancy is broken (Baskin and Baskin, 1972, 1983). Prolonged cold imbibition of non-dormant seeds (Rubio de Casas *et al.*, NESCent, NC, USA, unpubl. res.) and imbibition at warm temperatures (Donohue *et al.*, 2008; Heschel *et al.*, 2008) both induce secondary dormancy in *A. thaliana*.

The seasonal timing of germination is therefore the combined outcome of primary dormancy induction during seed maturation, primary dormancy breakage via after-ripening or chilling (or other environmental stimuli), secondary dormancy induction (and breakage), and the conditions that permit the germination of non-dormant seeds. These responses are regulated by numerous physiological pathways, among the most important of which are those regulating the endogenous levels of, and sensitivity to, GA and ABA, which in turn determine seed-coat/endosperm properties and embryonic growth potential (Koornneef *et al.*, 2002; Kucera *et al.*, 2005;

Bentsink and Koornneef, 2008; Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008).

In this study, we examined germination responses of *A. thaliana* to multiple seasonal environmental factors and tested for genetic variation in those responses and their underlying physiological mechanisms. Specifically, we examined germination responses to seed-maturation photoperiod, seed-maturation temperature, dormancy inducing and breaking treatments, and the temperature-dependent germination of non-dormant seeds. Previous studies have shown that the germination of *A. thaliana* seeds responds to the temperature during seed maturation and after dispersal (Donohue *et al.*, 2008; Donohue, 2009), and that genetic variation exists for these responses (Schmuths *et al.*, 2006). Here we extend these studies by investigating the effect on germination of additional environmental factors as well as physiological mechanisms associated with those germination responses. Specifically, we characterize the effects of, and genetic variation for, seed-coat (including endosperm) constraints and permeability as well as sensitivity to the phytohormones GA and ABA.

Arabidopsis thaliana is an appropriate species for addressing the genetic basis of natural variation in germination, both because of its genetic tractability and its ecological context. *Arabidopsis thaliana* is an annual mustard that exhibits pronounced variation in life history (Effmertova, 1967; Ratcliffe, 1976; Thompson, 1994; Griffith *et al.*, 2004) including natural variation in germination and dormancy (Van der Shaar *et al.*, 1997; Alonso-Blanco *et al.*, 2003; Clercx *et al.*, 2004; Schmuths *et al.*, 2006; Boyd *et al.*, 2007; Meng *et al.*, 2008; Chiang *et al.*, 2009; Bentsink *et al.*, 2010). *Arabidopsis thaliana* has a wide latitudinal distribution, and has adapted to a wide range of seasonal environments (Sharbel *et al.*, 2000; Vander Zwan *et al.*, 2000; Hoffmann, 2002). It has recently been introduced into North America, Australia and East Asia, and is in the process of expanding its geographic range. As a consequence of its wide dispersal, it frequently encounters novel climatic conditions to which it must adapt. Germination phenology is under intense, geographically variable natural selection in *A. thaliana*, as it is a major determinant of fitness during the earliest stages of colonization in experimental populations (Donohue *et al.*, 2005, Huang *et al.*, 2010). Therefore, characterizing germination responses to seasonal environmental factors and the genetic variation and covariation for these responses is relevant for predicting the adaptive dynamics of *A. thaliana* as it expands its range.

Plants must adapt to novel combinations of seasonal environmental factors as they expand their geographic range or as they experience altered seasonal regimes because of climate change. To do so may require the ability to evolve adaptive phenological responses to specific environmental cues independently. The degree to which responses to specific cues are genetically correlated versus independent can influence the flexibility of plants to adapt to novel seasonal environments that comprise novel combinations of different environmental cues. Studies of intact natural genotypes can characterize the genetic correlations among phenological responses to specific environmental factors and thereby reveal short-term constraints to evolving responses to novel combinations of seasonal factors. Such genetic associations

are the product of linkage or pleiotropy as well as linkage disequilibrium caused by demographic or selective processes independently of physical linkage. Experimental disruption of such linkage disequilibrium via experimental crosses can be a powerful approach for testing the genetic independence of traits that may be genetically associated in intact natural genotypes. Distinguishing between these causes of genetic correlations is important for predicting the longer-term potential to adapt to novel combinations of environmental conditions. This distinction is especially important within the context of range expansion, in which genotypes are newly encountering each other and interbreeding in novel environments.

Despite the intense interest in phenological adaptation to environmental change, the fundamental character of natural variation in germination is almost entirely unknown. Specifically, it is not known whether different genotypes within a species are germination specialists, with some germinating well under some conditions and poorly under other conditions, while other genotypes germinate well in other environments. Nor is it known what physiological mechanisms of germination regulation vary in natural populations and how they are associated with responses to particular environmental factors. In this study, we use a sample of recombinant inbred genotypes of *A. thaliana*, in which linkage disequilibrium has been experimentally disrupted through seven generations of recombination, to examine natural variation in and genotypic correlations of germination responses to multiple seasonal cues and their associated physiological mechanisms. We address the following questions. (a) Does genetic variation exist for germination responses to seed-maturation conditions, dormancy inducing and breaking temperatures, and germination temperature? If so, are these responses genetically correlated? (b) Does genetic variation exist in physiological pathways involved with germination; namely seed-coat/endosperm constraints and hormonal sensitivities? If so, are these physiological properties genetically correlated? (c) What physiological mechanisms are associated with germination responses to particular environmental factors, and which physiological properties are the best predictors of natural genetic variation in germination?

MATERIALS AND METHODS

Plant material, growing conditions and germination assays

We compared germination responses of ten recombinant inbred lines (RILs) of *Arabidopsis thaliana*. These RILs were derived from two naturally occurring accessions of *A. thaliana*: Calver, England (Cal) and Tacoma, Washington (Tac) (Huang et al., 2010). These ten lines were chosen for detailed physiological studies from a larger set of RILs, based on their extreme germination phenotype in previous studies, both with respect to germination responses to cold imbibition and with respect to germination phenology in the field (Munir et al., 2001; Donohue, 2005; Huang et al., 2010). Three markers (two on Chromosome 3 and one on Chromosome 5, within the regions of the loci *DOG6* and *DOG1*, respectively) were shown to associate with germination under field and laboratory conditions (Huang et al., 2010). The

genotypes of this subset of RILs at those markers are shown in Supplementary Data Table S1 (available online).

Seeds were matured under three treatments in Conviron (Winnipeg, Canada) E7/2 environmental growth chambers. The ‘long-warm’ seed-maturation treatment imposed a 14-h light/10-h dark cycle of full-spectrum light (fluorescent plus incandescent) at 22 °C, and resembles seasonal conditions typically experienced during seed maturation in late spring in temperate climates. The ‘short-warm’ treatment imposed a 10-h light/14-h dark cycle at 22 °C. The ‘short-cold’ treatment imposed a 10-h light/14-h dark cycle at 10 °C, and resembles seasonal conditions typically experienced when plants mature seeds in autumn or very early spring. Plants were grown in four chamber compartments (blocks) per seed-maturation environment, and two replicates of each genotype were planted in each compartment. Plantings were conducted so that seeds in all treatments would mature simultaneously. Seeds were harvested over a period of 3–4 d, and seeds were pooled within blocks for germination assays.

Germination assays were conducted on ‘fresh’ seeds (harvested approx. 1 week previously) and on seeds that had been dry after-ripened at 22 °C for 4 and/or 12 months. For each germination assay, 12 seeds of a given genotype were used in a single Petri plate (50 × 9 mm) containing 0.5 % agar. Eight replicate Petri plates were used for each genotype in each seed-maturation and imbibition treatment, for a total of 96 seeds per genotype in each treatment. The number of seeds that germinated in periodic censuses was counted using a stereoscope, until germination plateaued at 14 d in the light. Germination was scored when radicle protrusion was visible. We estimated the germination proportion as the cumulative number of germinants after 14 d in the light, divided by the total number of viable seeds. Seed viability was assessed by testing firmness to touch (Baskin and Baskin, 1998).

Experiment 1: primary dormancy, secondary dormancy and dormancy breakage – response to seed imbibition conditions

To test for genetic variation and covariation in response to seed-maturation and imbibition conditions, germination assays were conducted on fresh, 4-month after-ripened and 12-month after-ripened seeds. Seeds were imbibed in the dark at various temperatures and then transferred to 22 °C in a 12-h photoperiod of white fluorescent light (with photon flux density of 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The five dark imbibition treatments included temperatures or temperature sequences that seeds experience during different seasons in the field. (a) ‘22–22’ (‘dark temperature–light temperature’) which consisted of 7 d in the dark at 22 °C (before being transferred to 22 °C in the light) and resembles the temperature immediately after dispersal in spring. It is also the most frequently used temperature in most other germination studies in *A. thaliana*. (b) ‘4–22’ consisted of 7 d at 4 °C, and resembles a brief cold period in the autumn or early spring. This temperature is often used as a standard dormancy-breaking treatment. (c) ‘31–22’ consisted of 7 d at 31 °C and resembles the hot temperatures of summer. (d) ‘31–4–22’ consisted of 7 d at 31 °C followed by 7 d at 4 °C and resembles a cycle of hot summer temperatures followed by cool autumn temperatures.

(e) '31–22–22,' consisted of 7 d at 31 °C followed by 7 d at 22 °C. This treatment was compared with the '31–4–22' treatment to determine the effect of a cold imbibition period following warm. Preliminary analyses showed that the '31–22–22' treatment did not differ from the '31–22' treatment (results not shown), so the '31–22–22' treatment was excluded from subsequent experiments to reduce the total number of treatments. Regarding the physiological state of the seeds, the '22–22' treatment assesses primary dormancy, the '4–22' treatment assesses the ability of cold to overcome primary dormancy, the '31–22' treatment assesses the ability of warm to induce secondary dormancy, and the '31–4–22' treatment assesses the ability of cold to break primary and/or warm-induced secondary dormancy.

To test for significant differences in response to imbibition treatments among the genotypes, we first conducted analyses with all the RILs in a full ANOVA model, with genotype (ten RILs) as a random factor, and seed-maturation environment (long-warm, short-warm and short-cold), imbibition treatment (4–22, 22–22, 31–22 and 31–4–22) and after-ripening (fresh, 4 month, and 1 year) as fixed factors. All seeds matured under short-cold days exhibited pronounced dormancy [$F_{(\text{short-warm vs short-cold})} = 32.3$, $P < 0.001$]. This dormancy was displayed by all genotypes, in all dark imbibition conditions and in all three after-ripening stages. Because of the lack of variation in seeds matured under short-cold conditions, they were excluded from the following analysis. Because the data were not always normally distributed, a series of nonparametric Kruskal–Wallis tests was conducted to verify the significance of the ANOVA results. Spearman correlations on genotypic means were calculated. Bonferroni corrections were performed to adjust the significance levels for multiple comparisons.

Experiment 2: Temperature-dependent dormancy breakage and temperature-dependent germination

Experiment 1 examined germination under the standard temperature of 22 °C. To test for genetic variation in temperature-dependent dormancy breakage and temperature-dependent germination of non-dormant seeds, fresh and 4-month after-ripened seeds were exposed to different temperatures in the dark and the light. First, we tested to see if genotypes differed in whether 4 °C or 10 °C was more effective in breaking primary dormancy. Five 'dark-light' treatments were imposed: '4–10', '4–22', '10–10', '10–22' and '22–22'. We compared the effect of dark imbibition temperature for each germination temperature ('4–10' vs. '10–10' and '4–22' vs. '10–22' vs. '22–22') as well as the effect of germination (light) temperature within each imbibition treatment ('4–10' vs. '4–22' and '10–10' vs. '10–22').

We further examined temperature-dependent germination by testing whether genotypes differed in their ability to germinate at 10 °C, 22 °C and 28 °C after dormancy was lost through 4 months of after-ripening and cold imbibition with the following 'dark-light' treatments (with some already mentioned above): '4–10'; '4–22' and '4–28'. The following constant-temperature treatments were also imposed to examine, in comparison with cold-imbibed seeds, the effect of cold imbibition

on temperature-dependent germination; '10–10', '22–22' and '28–28'.

To test for significant differences in response to dark imbibition temperature, we conducted analyses with an ANOVA model that included genotype (ten RILs) as a random factor, and seed maturation environment (long-warm, short-warm and short-cold), imbibition temperature (4 °C, 10 °C and 22 °C) and after-ripening (fresh and 4 months) as fixed factors. As in the previous experiment all seeds matured under short-cold days exhibited pronounced dormancy, and were excluded from the following analysis [$F_{(\text{short-warm vs short-cold})} = 17.05$, $P < 0.005$]. We also specifically tested whether dormancy breakage was more effective at 4 °C or 10 °C using an *a priori* planned contrast that included all the RILs, long-warm and short-warm maturation environments, and both after-ripening stages. Kruskal–Wallis tests were conducted to verify the significance of ANOVA results. To test for significant differences in response to germination temperature (using seeds in which primary dormancy was first weakened by 4 months of after-ripening and then by dark imbibition at 4 °C), we used a model with genotype (ten RILs) as a random factor, and seed maturation environment (long-warm and short-warm) and germination temperature (10 °C, 22 °C and 28 °C) as fixed factors. As before, seeds matured under short-cold days were excluded from the analysis. Spearman correlations were performed on genotypic means, and Bonferroni corrections were used to adjust the significance levels for multiple comparisons.

Experiment 3: seed-coat properties

To examine the degree to which the seed coat/aleurone layer contributes to germination differences among lines, we scarified (pricked) fresh seeds to reduce mechanical constraints to germination and imbibition. We also examined seed-coat permeability. To reduce mechanical constraints, seeds were gently pricked with a fine hypodermic syringe. Germination was compared between pricked and non-pricked seeds. We compared effects of pricking between seeds that were matured under 'short-warm' vs. 'short-cold' seed-maturation conditions and between seeds given two dark imbibition treatments: '22–22', which consisted of 7 d at 22 °C; and '4–22', which consisted of 7 d at 4 °C. Seeds were then exposed to light at 22 °C.

To examine seed-coat permeability, we observed tetrazolium dye uptake for fresh seeds that were matured under 'short-warm' and 'short-cold' maternal environments. Seeds were incubated in a 0.1 % tetrazolium dye solution at 22 °C in the dark, and the dye uptake was measured by scoring the percentage of seeds that were stained. To verify that tetrazolium dye uptake reflects seed-coat permeability, we compared the two genotypes with the lowest (RIL 58) and highest (RIL 112) uptake. After scarification of their seed coat, both genotypes had indistinguishable dye uptake (Supplementary Data Fig. S1, available online), supporting the interpretation that variation in dye uptake among genotypes is a good indicator of variation in seed-coat permeability.

Differences in germination responses to scarification among the genotypes were examined using analysis of variance, with genotype (ten RILs) as a random factor, and seed-maturation

environment (short-warm and short-cold) and scarification treatment (control and scarified) as fixed factors.

Experiment 4: hormone sensitivity I

Because most genetic variation for germination was for responsiveness to cold imbibition and after-ripening (see Results), we conducted assays to determine the sensitivity of germination to GA and ABA in fresh, and 4-month after-ripened seeds that had been given a cold imbibition treatment. All seeds imbibed in the dark at 4 °C for 7 d before being transferred to the light at 22 °C. For the GA response assays, 10⁻⁵ M uniconazol (Valent USA Chemicals, FL, USA) was used to inhibit endogenous GA synthesis, and for the ABA response assays 10⁻⁵ M fluridone (Westchester Chemicals, PA, USA) was used to inhibit endogenous ABA synthesis. Uniconazol, fluridone, GA₄₊₇ (Duchefa, The Netherlands) and ABA (mixed isomers, Sigma-Aldrich, MO, USA) were first dissolved in 100 % ethanol to make stock solutions, and then diluted to the appropriate concentration with distilled water. The inhibitors and hormone solutions at the appropriate concentrations were added to the liquid agar when it reached a temperature of 48–49 °C. The final concentration of ethanol in the agar was 0.2 %. Plates containing 0.2 % ethanol were used to control for the effect of alcohol on germination. As the seed coats of these seeds were permeable to uniconazol and fluridone (see Results) they would also be permeable to exogenously applied hormones.

Seeds matured under the ‘short-cold’ environment showed extreme dormancy, reduced sensitivity to hormones and their inhibitors, and reduced seed-coat permeability (see expt 3 and Results). As a follow-up to examine whether the low hormone sensitivity of ‘short-cold’ matured seeds was caused by the lack of permeability to the exogenously applied hormones and inhibitors, we examined how scarification of the seed coat affected germination responses to GA and fluridone, in fresh ‘short-cold’ matured seeds.

To test for significant differences in GA and ABA responses among the genotypes, we analysed germination percentages with an ANOVA model with genotype (ten RILs) as a random factor, and seed maturation environment (long-warm and short-warm), hormone concentration (either GA or ABA) and after-ripening as fixed factors. To ask whether the GA and ABA responses were correlated, we examined Spearman correlations between genotypic mean germination at GA and ABA concentrations that exhibited the greatest variation. Spearman correlations were also calculated between genotypic mean germination of seeds matured under short vs. long days and of fresh vs. after-ripened seeds within hormonal treatments. Bonferroni corrections were used to adjust the significance levels for multiple comparisons.

Experiment 5: hormone sensitivity II

In expt 4, while examining the response of GA or ABA, the endogenous level of the other hormone was not controlled, but instead had its genotype-specific level. In this experiment we sought to control the endogenous levels of both GA and ABA simultaneously and measure hormonal sensitivities in a common hormonal environment. This experiment was

conducted on seeds matured under ‘short-warm day’ conditions that were 4 months after-ripened. Short-warm day seeds were used because they exhibited a very similar response to seeds matured under long-warm days, and the short-cold matured seeds did not have much variation because they were very dormant.

Endogenous synthesis of both ABA and GA was first inhibited. The GA response was then examined by supplying a range of GA concentrations to seeds at two controlled ABA concentrations: no exogenous ABA and 10⁻⁵ M exogenous ABA. The ABA response was examined by supplying a range of ABA concentrations to seeds at one controlled concentration of GA: 10⁻⁵ M. (We could not examine the ABA response in the absence of GA as these seeds did not show any germination.)

To test for significant differences in germination responses to the different hormone treatments among the genotypes, we used an ANOVA model, with genotype (ten RILs) as a random factor and hormone treatment as fixed factors. Spearman correlations of genotypic means tested for correlations of GA sensitivity in different ABA backgrounds and of ABA sensitivity in different GA backgrounds (expt 4 vs. expt 5).

As the most accurate index of true GA sensitivity (not confounded by variation in ABA levels or sensitivity), we used the genotypic germination in the absence of any *de novo* endogenous or exogenous ABA, averaged across the two intermediate GA concentrations that exhibited the most variation in germination. To estimate genotypic ABA sensitivity, we used average genotypic germination at a concentration of 10⁻⁴ M ABA while GA concentrations were controlled at 10⁻⁵ M, since we observed the most genotypic variation in germination at this concentration and because all genotypes exhibited close to 100 % germination with no ABA.

Comparing hormone sensitivities between expts 4 and 5 allowed us to opportunistically infer the approximate relative amounts of endogenous GA and ABA. To estimate endogenous ABA for each line, we evaluated its germination with 10⁻⁵ M exogenous GA, common to both assays, and compared its germination in 10⁻⁴ M exogenous ABA (expt 5) with germination in native endogenous levels of ABA (expt 4). We estimated that the difference in germination would be due to the difference in genotype-specific endogenous ABA content. Higher germination is indicative of lower amounts of endogenous ABA. Thus for each RIL at 10⁻⁵ M exogenous GA: [relative endogenous ABA] = germination_(10⁻⁴ M ABA) – germination_(native ABA). A positive value indicates that germination at the native content was lower than at 10⁻⁴ M, so the ABA content was higher. A similar method was used to estimate the relative endogenous levels of GA. At 10⁻⁵ M exogenous ABA: [relative endogenous GA] = germination_(native GA) – germination_(10⁻⁵ M GA). A positive value indicates that germination was higher in seeds with native GA content than at 10⁻⁵ M, so the GA content was higher. To test whether hormone sensitivity and/or relative content is the best predictor of variation in germination, we performed a multiple regression analysis of germination with estimated GA and ABA content and sensitivity.

We also tested for associations between marker genotypes at three QTLs and germination phenotypes, including: GA

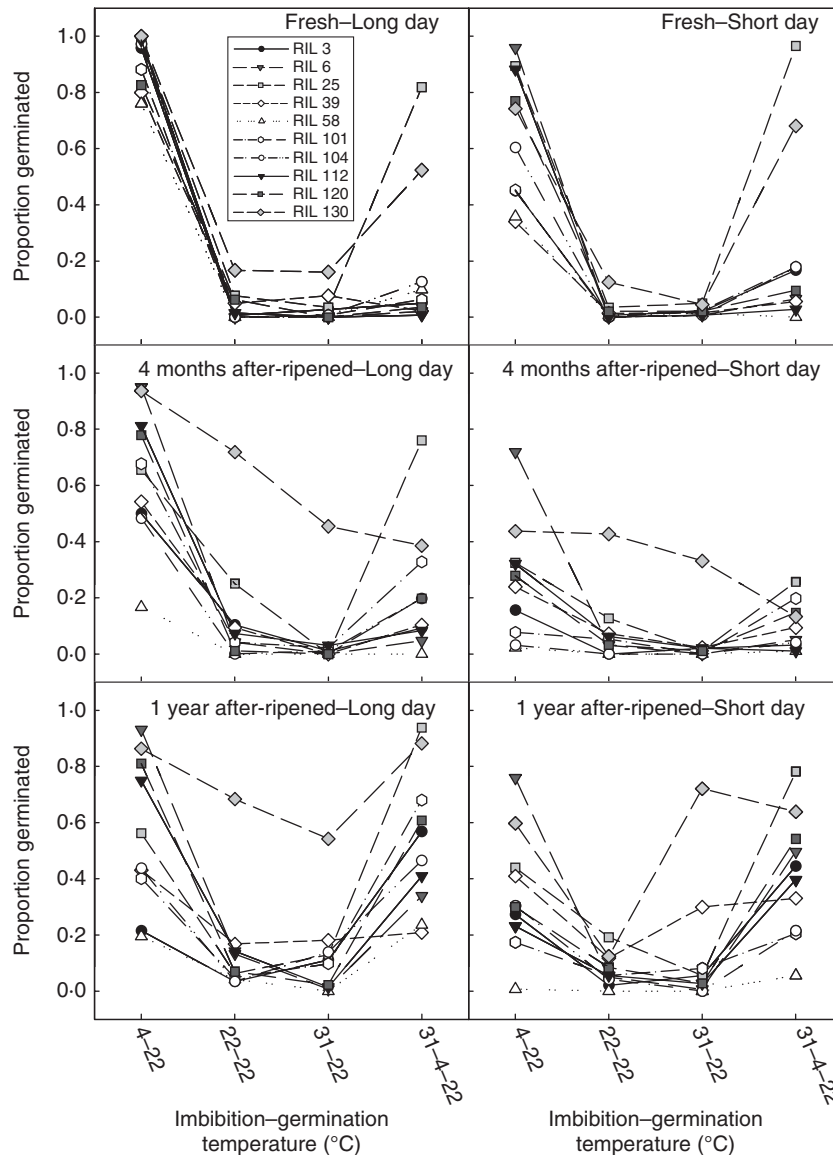


FIG. 1. Proportion of seeds that germinated for the different RIL genotypes under the different dark imbibition conditions (dark–light temperatures: 4–22; 22–22; 4–22; 31–4–22). Seeds were matured under long-warm and short-warm days, and after-ripened for different durations – fresh, 4 months and 1 year. Seeds were exposed to the different imbibition temperatures in the dark, and then transferred to 22 °C in the light. Final germination proportions were determined after 14 d in the light.

sensitivity, ABA sensitivity, estimated GA and ABA content, scarification responses (in short-warm seeds at 22 °C and short-cold seeds at 4–22 °C: those treatments that showed genetic variation); germination proportions of short-warm seeds (because they exhibited genetic variation and resembled long-warm seeds) from expt 4; germination proportions of short-warm seeds from expt 5 (because they exhibited genetic variation and resembled long-warm seeds).

RESULTS

Environmental responses of germination and their genetic independence

Primary and secondary dormancy induction and breakage. For seeds matured under warm conditions (long and short days),

all environmental factors interacted significantly to influence germination [$F_{(\text{photoperiod} \times \text{imbibition} \times \text{after-ripening})} = 2.48, P < 0.05$]. A significant four-way interaction with genotype [$F_{(\text{genotype} \times \text{photoperiod} \times \text{imbibition} \times \text{after-ripening})} = 3.73, P < 0.05$] indicated that significant genetic variation exists for responses to different combinations of environmental conditions (Fig. 1).

Genetic variation for germination proportions was detected in all treatments, with one exception (fresh, short-warm days, 31–22). In fresh seeds, genotypic differences were most pronounced when seeds were cold-treated, but in after-ripened seeds some genotypic differences were also apparent in non-cold-treated seeds. Thus, the genotypes here did not differ much in degree of primary dormancy at the time of seed shed. Instead genotypes differed in the rate of dormancy loss with after-ripening and in the responsiveness to cold or warm imbibition, as discussed next.

TABLE 1. Genotypic correlations of germination proportions across environmental treatments

(A) Correlations between short vs. long days							
Imbibition conditions		Fresh		4 months			1 year
4–22		0.69		0.81			0.72
22–22		0.95		0.98			0.37
31–22		0.61		0.99			0.95
31–4–22		0.98		0.88			0.69
(B) Correlations across after-ripening duration							
Photoperiod	Imbibition conditions	Fresh vs. 4 months		Fresh vs. 1 year		4 months vs. 1 year	
Long day	4–22	0.61		0.46		0.91	
Long day	22–22	0.91		0.84		0.92	
Long day	31–22	0.87		0.96		0.91	
Long day	31–4–22	0.90		0.78		0.87	
Short day	4–22	0.75		0.60		0.88	
Short day	22–22	0.97		0.53		0.57	
Short day	31–22	0.59		0.55		0.94	
Short day	31–4–22	0.72		0.73		0.55	
(C) Correlations between imbibition treatments							
After-ripening	Photoperiod	4–22 vs. 22–22	4–22 vs. 31–22	4–22 vs. 31–4–22	22–22 vs. 31–22	22–22 vs. 31–4–22	31–22 vs. 31–4–22
Fresh	Long day	0.21	0.19	0.41	0.86	0.66	0.48
Fresh	Short day	0.20	0.22	0.38	0.74	0.65	0.92
4 months	Long day	0.44	0.44	0.24	0.94	0.49	0.23
4 months	Short day	0.37	0.30	0.08	0.96	0.36	0.14
1 year	Long day	0.51	0.21	0.25	0.89	0.37	0.53
1 year	Short day	0.47	0.42	0.66	0.44	0.77	0.34

Spearman correlations are given for germination proportions in the different treatments. Values in bold are significant at $P < 0.05$, after Bonferroni correction.

(A) Genotypic correlations between seeds matured under different photoperiods (long-warm vs. short-warm days) in the different imbibition conditions. Bonferroni with $\alpha = 0.05/4$. (B) Genotypic correlations between after-ripening stages for seeds matured in long and short days (at 22 °C), and under the different imbibition conditions. Bonferroni with $\alpha = 0.05/3$. (C) Genotypic correlations between imbibition treatments for seeds matured in long and short days (at 22 °C), and after-ripened for different durations. Bonferroni with $\alpha = 0.05/6$.

Seeds matured under long-warm days exhibited greater germination than those matured under short-warm days ($F_{\text{photoperiod}} = 47.87$, $P < 0.05$), and this was consistent across the different genotypes [$F_{(\text{genotype} \times \text{photoperiod})} = 1.8$, $P > 0.05$; Fig. 1). The effects of seed-maturation photoperiod were especially pronounced for those seeds that received a cold treatment [$F_{(\text{photoperiod} \times \text{imbibition})} = 34.41$, $P < 0.05$]. Thus, seeds matured in long days (e.g. summer) may germinate to high percentages after exposure to cold (e.g. in autumn).

After-ripening was associated with increased germination ($F_{\text{after-ripening}} = 8.71$, $P < 0.01$), except in seeds that were given a cold imbibition (4–22) treatment [$F_{(\text{after-ripening} \times \text{imbibition})} = 29.07$, $P < 0.0001$; Fig. 1). After-ripened cold-treated seeds actually germinated to lower proportions than fresh cold-treated seeds. This effect was not seen in seeds given cold after secondary (as opposed to primary) dormancy induction. After-ripening was also slightly more effective at alleviating dormancy in seeds matured under long days than short days [$F_{(\text{photoperiod} \times \text{after-ripening})} = 3.64$, $P < 0.05$], but this effect also depended on the imbibition treatment [$F_{(\text{photoperiod} \times \text{after-ripening} \times \text{imbibition})} = 2.48$, $P < 0.05$]. Thus, seeds matured in summer may after-ripen more quickly.

Imbibition treatment strongly influenced germination ($F_{\text{imbibition}} = 37.4$, $P < 0.05$). Cold imbibition stimulated germination (in 4–22 and 31–4–22 treatments; Fig. 1),

especially in fresh seeds (see above). The response to cold imbibition was significantly greater for seeds matured under long-warm days than short-warm days. Warm imbibition (31–22) usually induced dormancy, but the effect was observed only in after-ripened seeds, since fresh, non-cold-treated seeds (22–22) were already extremely dormant. Warm imbibition actually released dormancy in two genotypes when matured under short days. Thus significant genetic variation was detected for response to imbibition conditions.

We observed significant genotypic correlations across seed-maturation photoperiods (Table 1A) and after-ripening durations (Table 1B). In contrast, genotypic correlations across imbibition conditions were weak (Table 1C). The exceptions were the significant correlations between the 22–22 and 31–22 imbibition conditions, but seeds in both of these imbibition conditions were very dormant. In particular, germination with vs. without cold treatments had weak genotypic correlations. Surprisingly, genotypic correlations were not high between the 4–22 and the 31–4–22 treatments (Fig. 1 and Table 1C), suggesting that breakage of primary dormancy with exposure to cold may be genetically distinct from breakage of secondary warm-induced dormancy with exposure to cold. A few other treatments had statistically significant genotypic correlations, but these correlations were highly

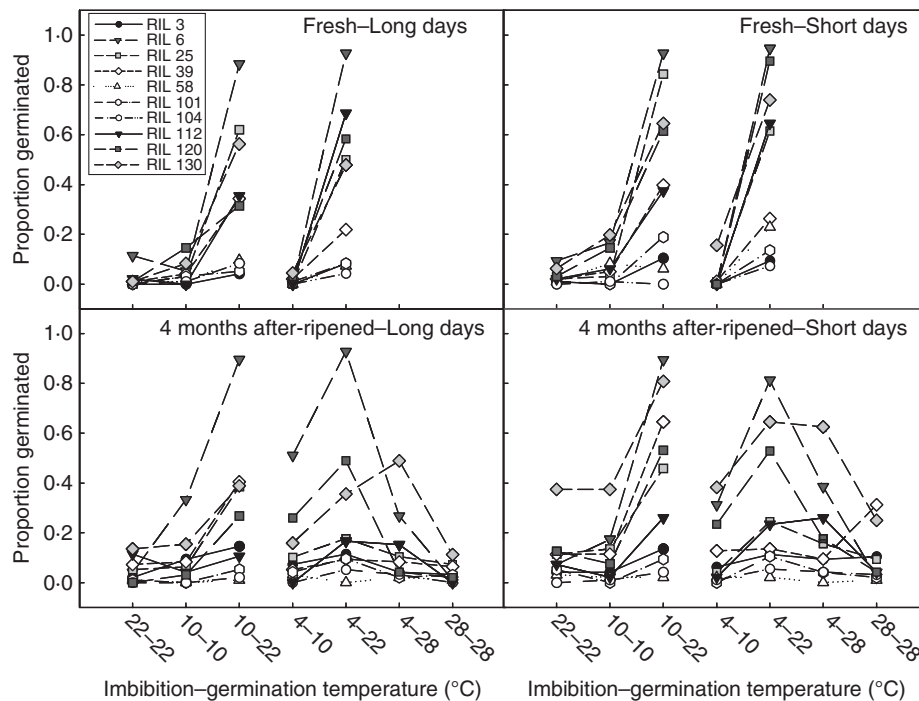


FIG. 2. Proportion of seeds that germinated for the different RIL genotypes under the different imbibition conditions. Seeds were matured under long-warm and short-warm days, and after-ripened for different duration – fresh and 4 months. Seeds were exposed to the dark imbibition temperatures of 4 °C, 10 °C, 22 °C or 28 °C, and then transferred to different germination temperatures of 10 °C, 22 °C or 28 °C in the light ('dark–light' temperatures). Final germination proportions were determined after 14 d in the light.

influenced by a single genotype or they included a treatment with very low, and probably not biologically significant, variation in one treatment.

Temperature-dependent dormancy breakage. As before, all seeds matured under short-cold days exhibited pronounced dormancy [$F_{(\text{short-warm vs. short-cold})} = 17.11, P < 0.05$], and were therefore excluded from the following analysis (Fig. 2). For some genotypes a 4 °C dark imbibition was more effective at breaking dormancy, while for others 10 °C was more effective [$F_{\text{imbibition}} = 0.05, P > 0.05$; $F_{(\text{genotype} \times \text{imbibition})} = 6.37, P < 0.05$]. The response to imbibition at 4 °C vs. 10 °C was similar in seeds matured under short and long days [$F_{(\text{photoperiod} \times \text{imbibition})} = 2.15, P > 0.05$], and genotypes did not differ in this regard [$F_{(\text{genotype} \times \text{photoperiod} \times \text{imbibition})} = 0.97, P > 0.05$]. Responses to imbibition temperature depended on after-ripening stage [$F_{(\text{after-ripening} \times \text{imbibition})} = 8.11, P < 0.05$], the differences being evident only after 4 months of dry after-ripening, but genotypes did not differ in the degree to which after-ripening increased their responsiveness to imbibition temperature [$F_{(\text{genotype} \times \text{after-ripening} \times \text{imbibition})} = 2.56, P > 0.05$]. Therefore, the temperature at which dormancy was broken most effectively depended on genotype and after-ripening period (see Supplementary Data Table S3 and Appendix S1 for additional results). Germination proportions of seeds given a 4 °C and a 10 °C imbibition treatment were highly correlated (Supplementary Data Table S2), suggesting a similar genetic basis to germination responses to 4 °C and 10 °C. Correlations ranged from 0.83 to 0.88 in all cases ($P < 0.05$)

except for fresh, short-warm day seeds at germination temperatures of 10 °C in the light ($r = 0.12, P > 0.05$).

Temperature-dependent germination. We examined temperature-dependent germination (at 10 °C, 22 °C and 28 °C) in 4-month after-ripened seeds that were either given a cold treatment or not (expt 2, Fig. 2). When not given a 4 °C cold treatment (10–10, 22–22, 28–28), seeds were highly dormant [$F_{(\text{cold imbibition})} = 5.67, P < 0.05$], but in a genotype-specific manner [$F_{(\text{cold imbibition} \times \text{genotype})} = 3.95, P < 0.05$]. Because of uniformly high dormancy, seeds without cold imbibition exhibited limited genetic variation.

In cold-imbibed seeds, most genotypes showed maximal germination at 22 °C [$F_{(\text{germination temp})} = 4.70, P < 0.05$], but there was significant genetic variation for the response to germination temperature [$F_{(\text{genotype} \times \text{germination temp})} = 11.43, P < 0.001$; see Supplementary Data Table S3 for specific contrasts]. This pattern was consistent across photoperiods [$F_{(\text{germination temp} \times \text{photoperiod})} = 2.45, P > 0.05$; $F_{(\text{genotype} \times \text{germination temp} \times \text{photoperiod})} = 0.8, P > 0.05$].

As seen before, the germination responses of seeds matured under long-warm and short-warm days were highly correlated (Supplementary Data Table S4a), as were seeds in different stages of after-ripening (Supplementary Data Table S4b). Also as before, correlations for germination with vs. without a cold treatment were weak for seeds germinating at both 22 °C ($r_{\text{short-warm}} = 0.58, P > 0.05$; $r_{\text{long-warm}} = 0.10, P > 0.05$) and 28 °C ($r_{\text{short-warm}} = 0.36, P > 0.05$; $r_{\text{long-warm}} = 0.59, P > 0.05$; expt 2, Fig. 2). At 10 °C, however, there were significant correlations with and without a cold, dark imbibition ($r_{\text{short-warm}} = 0.85, P < 0.05$; $r_{\text{long-warm}} = 0.85, P < 0.05$).

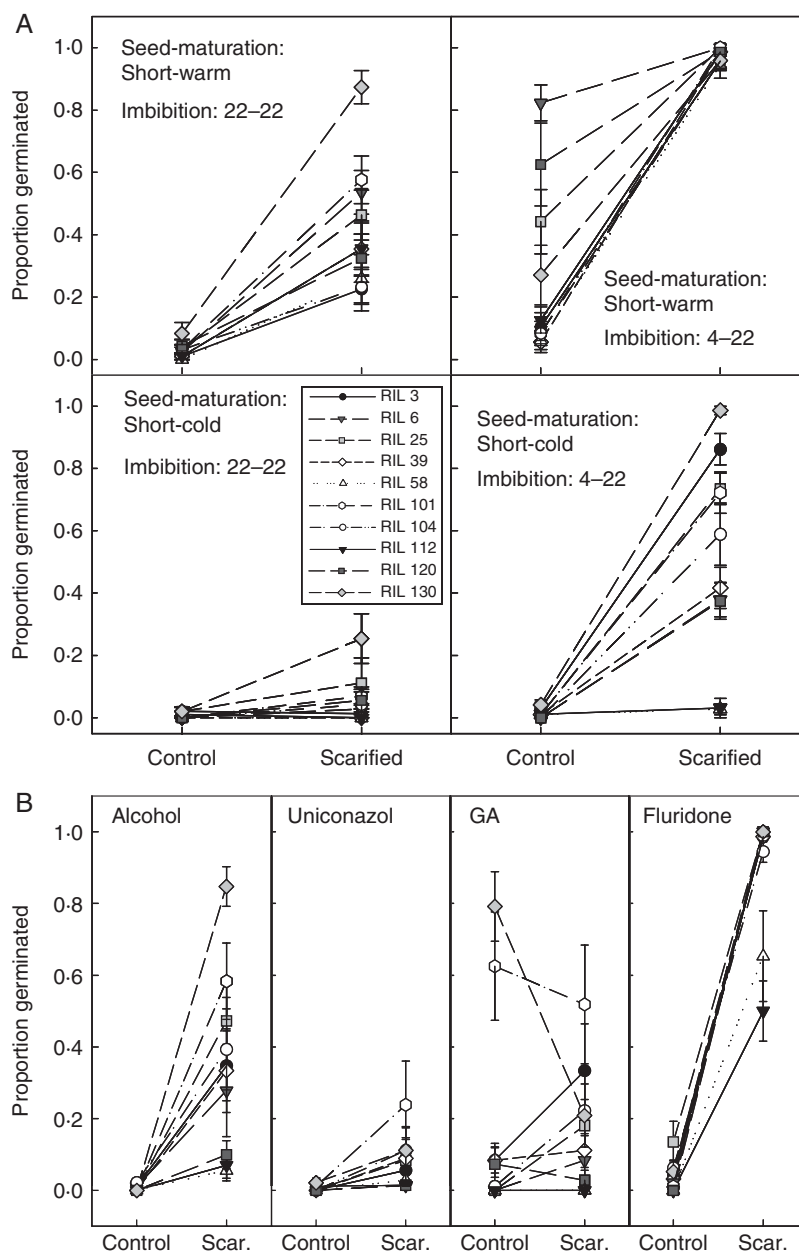


FIG. 3. Effect of scarification. (A) Seeds were matured under short-warm and short-cold days and given a 4 °C or 22 °C dark imbibition treatment before being transferred to light at 22 °C ('dark-light' temperatures). (B) Effect of scarification on hormone responses of cool-matured seeds for solvent control (0.2 % ethanol), uniconazol (10^{-5} M), GA (10^{-5} M) and fluridone (10^{-5} M).

For cold-imbibed seeds, genotypic correlations of germination at 10 °C, 22 °C and 28 °C were significant when seeds were matured under short-warm days [$r_{(10 \text{ vs. } 22)} = 0.91$, $P < 0.05$; $r_{(10 \text{ vs. } 28)} = 0.85$, $P < 0.05$; $r_{(22 \text{ vs. } 28)} = 0.84$, $P < 0.05$]. When seeds were matured under long-warm days, the genotypic correlations between germination at 10 °C and 22 °C was significant [$r_{(10 \text{ vs. } 22)} = 0.98$, $P < 0.05$], but the correlations with germination at 28 °C were not significant [$r_{(10 \text{ vs. } 28)} = 0.43$, $P > 0.05$; $r_{(22 \text{ vs. } 28)} = 0.51$, $P > 0.05$], probably because germination and variation for germination at 28 °C was less in short-day seeds than in long-day seeds. In general, genotypes that germinated to high degrees at low temperature also germinated well at high temperatures, and

in no case were genotypic correlations negative. Thus we found no evidence that these genotypes have specialized in their germination temperature.

Seed-coat properties

We tested for effects of seed-coat/aleurone constraints on germination and for genetic variation in seed-coat properties. Scarification of the seed coat significantly increased germination [$F_{\text{scarification}} = 97.5$, $P < 0.05$; Fig. 3]. The effect of scarification was particularly pronounced for seeds exposed to cold imbibition [$F_{(\text{scarification} \times \text{imbibition})} = 31.6$, $P < 0.05$], indicating that the primary constraint to germination of cold-imbibed seeds

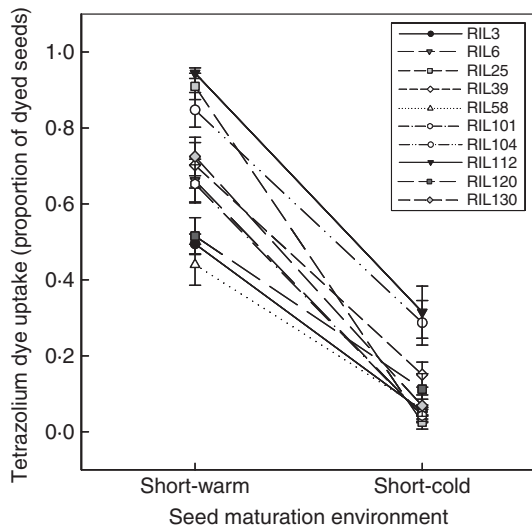


FIG. 4. Tetrazolium dye uptake assayed in seeds matured under short-warm and short-cold days.

appears to be the seed coat. The effect of scarification was stronger in seeds that were matured under warmer temperatures [$F_{(\text{scarification} \times \text{maturation temp})} = 17.9, P < 0.05$], suggesting that the reduced germination of cool-matured seeds is not due entirely to constraints of the seed coat. These effects of scarification were consistent across genotypes [$F_{(\text{scarification} \times \text{genotype})} = 2.6, P > 0.05$; $F_{(\text{scarification} \times \text{genotype} \times \text{maturation temp})} = 0.7, P > 0.05$; $F_{(\text{scarification} \times \text{genotype} \times \text{imbibition})} = 0.8, P > 0.05$].

Seeds matured under cool temperatures exhibited dramatically lower tetrazolium dye uptake [$F_{(\text{maturation temp})} = 160.2, P < 0.05$; Fig 4]. The effect of genotype on dye uptake was marginal [$F_{(\text{genotype})} = 3.0, P = 0.058$], but this was dependent on the seed maturation temperature [$F_{(\text{genotype} \times \text{maturation temp})} = 4.1, P < 0.05$].

Thus the seed coat constrains germination and appears to be the only constraint to germination of warm-matured, cold-imbibed seeds. Dormancy imposed by cool seed-maturation temperatures involves decreased permeability of the seed coat and can be alleviated in part by disruption of the seed coat, particularly if the seeds are also physiologically stimulated to germinate by a cold treatment. However, other physiological pathways prevent full germination of cold-matured seeds even without seed-coat constraints.

Sensitivity to GA and ABA

Effectiveness of the experimental treatments. We examined germination sensitivity to the hormones GA and ABA in seeds exposed to a 4 °C dark imbibition (expt 4, Fig. 5). Seeds matured in cool temperatures were very dormant in control and solvent-control treatments. In seeds matured under warm days, germination generally was higher in the solvent-control treatment than in the control [$F_{(\text{control vs. solvent-control})} = 8.55, P < 0.05$]. This effect of ethanol differed among genotypes [$F_{(\text{control vs. solvent-control} \times \text{genotype})} = 3.64, P < 0.05$] but was not affected by maternal photoperiod [$F_{(\text{control vs. solvent-control} \times \text{photoperiod})} = 0.01, P > 0.05$] or after-ripening [$F_{(\text{control vs. solvent-control} \times \text{after-ripening})} = 0.58, P > 0.05$].

Exposure to 10^{-5} M uniconazol (GA synthesis inhibitor) completely inhibited germination in all genotypes indicating that *de novo* synthesis of GA is required for germination (Fig. 5). Addition of exogenous GA restored germination completely in at least some genotypes (see below). Exposure to 10^{-5} M fluridone (ABA synthesis inhibitor), increased germination to near 100 % in most of the lines matured under warm temperatures. Therefore, dormancy in these lines is maintained by *de novo* ABA synthesis. At 10^{-4} M ABA, germination was completely inhibited in all lines in all seed-maturation environments, indicating that exogenous ABA effectively induced dormancy.

Genetic variation and genotypic correlations of hormonal sensitivities. Genotypes differed significantly in their sensitivity to exogenous GA [$F_{(\text{genotype} \times [\text{GA}])} = 3.71, P < 0.05$], but the degree of sensitivity to GA also depended on the experimental treatments (see below). Likewise, the application of exogenous ABA induced dormancy, but genotypes differed significantly in their sensitivity to ABA [$F_{(\text{genotype} \times [\text{ABA}])} = 2.6, P < 0.05$; and see below]. Thus genetic variation exists for responses to both GA and ABA. Sensitivity to GA was not significantly correlated with sensitivity to ABA in any treatment, except for after-ripened seeds matured under cool temperatures (Table 2A). The following analyses investigate how environmental conditions alter hormonal sensitivities and their genetic variation.

Response to after-ripening. The effect of after-ripening on sensitivity to GA [$F_{(\text{after-ripening} \times [\text{GA}] \times \text{mat})} = 3.20, P = 0.001$] and sensitivity to ABA [$F_{(\text{after-ripening} \times [\text{ABA}] \times \text{mat})} = 8.90, P < 0.001$] depended on seed-maturation conditions (Fig. 5 and Table 2B). In warm-matured seeds, after-ripening altered sensitivity to GA in a genotype-dependent manner [$F_{(\text{after-ripening} \times [\text{GA}])} = 3.00, P < 0.05$; $F_{(\text{after-ripening} \times [\text{GA}] \times \text{genotype})} = 3.52, P < 0.05$]. Genotypic correlations of germination at a non-saturating GA concentration (10^{-5} M; the concentration that exhibited the most genetic variation) in fresh vs. after-ripened seeds were strong, suggesting a common genetic basis of variation in GA sensitivity regardless of after-ripening. Sensitivity to ABA was increased by after-ripening in warm-matured seeds [$F_{(\text{after-ripening} \times [\text{ABA}])} = 3.3, P < 0.05$], and this did not differ among genotypes [$F_{(\text{after-ripening} \times [\text{ABA}] \times \text{genotype})} = 0.9, P > 0.05$]. As with GA sensitivity, correlations of germination at a non-saturating ABA concentration (10^{-5} M; the concentration that exhibited the most genetic variation) in fresh vs. after-ripened seeds were strong, suggesting a common genetic basis of variation in ABA sensitivity regardless of after-ripening. Thus, for warm-matured seeds, after-ripening is accompanied by an increase in ABA sensitivity, but genotype-specific changes in GA sensitivity.

In cool-matured seeds, after-ripening did not significantly alter germination sensitivity to GA [$F_{(\text{after-ripening} \times [\text{GA}])} = 0.87, P > 0.05$], and there was a significant genotypic correlation for germination in non-saturating GA concentrations (10^{-4} M; the concentration that exhibited the most genetic variation) across fresh vs. after-ripened seeds (Table 2B). Four-month after-ripened seeds were more sensitive to ABA [$F_{(\text{after-ripening} \times [\text{ABA}])} = 6.65, P < 0.05$] than fresh seeds, but this effect differed among genotypes [$F_{(\text{after-ripening} \times \text{ABA} \times \text{genotype})} = 3.61, P < 0.05$]. Genotypic

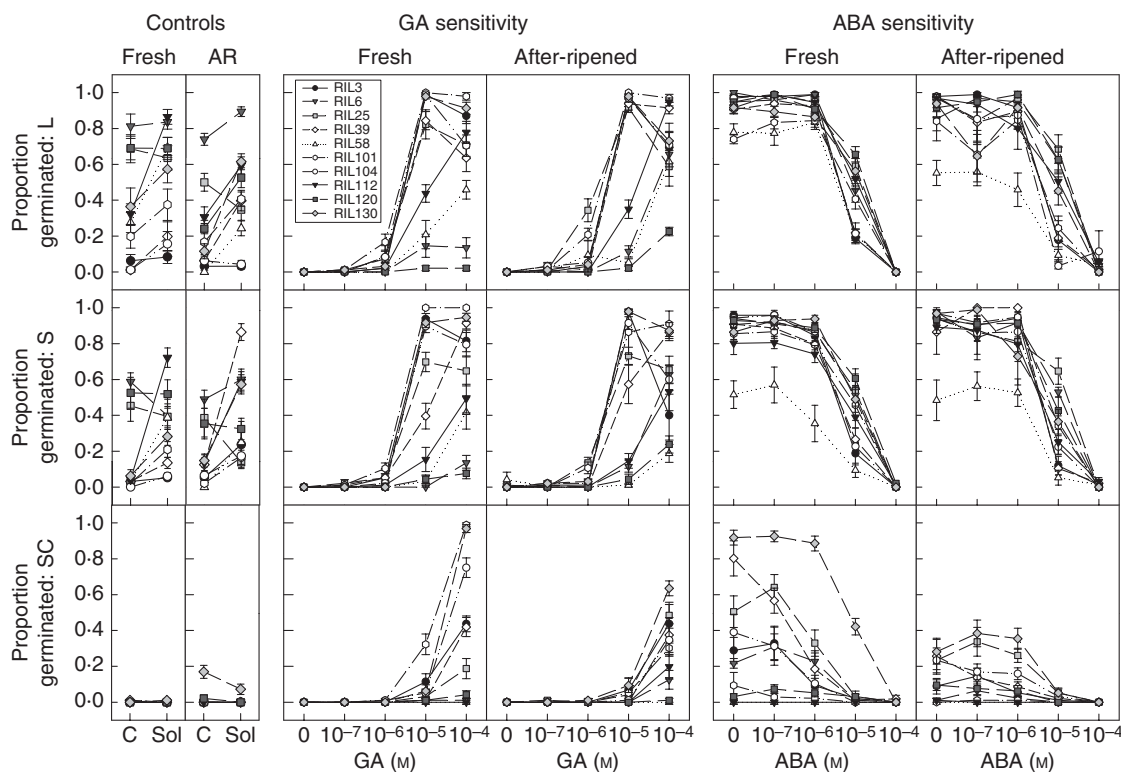


FIG. 5. Germination response to gibberellic acid (GA) and abscisic acid (ABA). Control (C) germination was assayed in agar; Solvent control (Sol) germination was assayed in agar that contained 0.2% ethanol. GA responses were assayed after inhibiting endogenous GA synthesis with 10^{-5} M uniconazole. ABA responses were assayed after inhibiting endogenous ABA synthesis with 10^{-5} M fluridone. Seeds were matured under long-warm (L) and short-warm (S) days or short-cold (SC) days, and after-ripened for 0 or 4 months. Seeds imbibed at 4°C for 7 d and then were transferred to 22°C in the light. Final germination proportions were determined after 14 d in the light.

correlations for germination in a non-saturating ABA concentration (10^{-7} M; the concentration that exhibited the most genetic variation) were strong across fresh vs. after-ripened seeds (Table 2B). Thus in cool-matured seeds, after-ripening does not significantly alter GA sensitivity, but it increases ABA sensitivity in a genotype-specific manner.

Response to seed-maturation photoperiod. As before, germination was lower for seeds matured under short-warm days than long-warm days (Fig. 5). Seeds matured under long days were more sensitive to GA than those matured under short days [$F_{(\text{photoperiod} \times [\text{GA}])} = 3.0$, $P < 0.05$], with seeds matured under long days germinating to higher proportions at lower GA concentrations than those matured under short days. This effect differed among genotypes [$F_{(\text{photoperiod} \times [\text{GA}] \times \text{genotype})} = 3.52$, $P < 0.0001$], but the genotypic correlations of germination at non-saturating GA concentrations were high across photoperiod treatments (Table 2C), indicating that the rank order of the genotypes did not change across photoperiods.

The effect of exogenous ABA application on germination did not differ across photoperiods [$F_{(\text{photoperiod} \times [\text{ABA}])} = 1.9$, $P > 0.05$], nor did the responses of genotypes to ABA concentration [$F_{(\text{genotype} \times \text{photoperiod} \times [\text{ABA}])} = 0.8$, $P > 0.05$]. The genotypic correlations of germination at non-saturating ABA concentrations were also high across photoperiod treatments (Table 2C). Thus the increased germination of control

long-day seeds is accompanied by increased sensitivity to GA but not changes in sensitivity to ABA.

Response to seed-maturation temperature. Seeds matured under short-cold days exhibited pronounced dormancy, as was observed previously (Fig. 5). GA was significantly less effective in restoring germination in seeds matured in short-cold days than in short-warm days [$F_{(\text{maturation temperature} \times [\text{GA}])} = 13.85$, $P < 0.0001$], and genetic variation for sensitivity to GA also depended on seed maturation temperature [$F_{(\text{maturation temperature} \times [\text{GA}] \times \text{genotype})} = 6.14$, $P < 0.0001$]. The effect of seed-maturation temperature on GA sensitivity was especially pronounced in after-ripened seeds (see above). There were significant genotypic correlations across seed-maturation temperature of germination proportions at non-saturating GA concentrations (Table 2C) for both fresh and after-ripened seeds. Thus, the inability of cold-matured seeds to germinate appears to be due in part to their decreased sensitivity to GA.

Fluridone increased germination in some of the lines matured in cool temperature, but to a much lesser degree than seeds matured in short-warm conditions [Fig. 5; $F_{(\text{maturation temperature} \times \text{Fluridone})} = 93.55$, $P < 0.05$]. Some lines showed no response to fluridone. Addition of exogenous ABA decreased germination, and seed-maturation temperature altered the response to ABA application in a genotype-dependent manner [$F_{(\text{maturation temperature} \times [\text{ABA}])} = 51.9$,

TABLE 2. Genotypic correlations of hormonal sensitivities across environmental treatments

(A) GA vs. ABA sensitivity					
After-ripening	Seed-maturation environment				$r_{(GA\ vs.\ ABA)}$
Fresh	Long-warm				-0.33
Fresh	Short-warm				-0.08
Fresh	Short-cold				0.40
4 months	Long-warm				-0.34
4 months	Short-warm				-0.02
4 months	Short-cold				0.82
(B) Fresh vs. 4-month after-ripened					
Seed-maturation environment	Control	Solvent control	GA	ABA	
Long-warm	0.83	0.75	0.99	0.91	
Short-warm	0.96	0.17	0.98	0.88	
Short-cold	-0.13	0.99	0.68	0.93	
(C) Seed-maturation conditions					
After-ripening	Seed-maturation environment	Control	Solvent control	GA	ABA
Fresh	Long-warm vs. short-warm	0.93	0.92	0.94	0.93
Fresh	Short-warm vs. short-cold	-0.22	-0.06	0.87	-0.02
Fresh	Long-warm vs. short-cold	-0.40	0.08	0.82	0.14
4 months	Long-warm vs. short-warm	0.88	0.57	0.96	0.85
4 months	Short-warm vs. short-cold	-0.02	0.26	0.88	0.39
4 months	Long-warm vs. short-cold	-0.16	0.27	0.89	0.24

Spearman correlations are given for germination proportions in the different treatments. 'GA' uses germination proportions at the GA concentration that exhibited the most genetic variation: 10^{-5} M for seeds matured under warm temperature, and 10^{-4} M for seeds matured under cool temperature. 'ABA' uses germination proportions at the ABA concentration that exhibited the most genetic variation: 10^{-5} M for seeds matured under warm temperature, and 10^{-7} M for seeds matured under cool temperature. Values in bold are significant at $P < 0.05$.

(A) Genotypic correlations of germination sensitivity to GA vs. ABA. (B) Genotypic correlations in fresh vs. after-ripened seeds for germination sensitivity to GA and ABA. (C) Genotypic correlations across seed-maturation environments, for fresh and 4-month after-ripened seeds.

$P < 0.05$; $F_{(\text{maturation temperature} \times [\text{ABA}] \times \text{genotype})} = 4.5$, $P < 0.05$]. The effect of seed-maturation temperature on ABA sensitivity was especially pronounced in after-ripened seeds (see above). We did not detect significant genotypic correlations of the ABA response across seed maturation temperatures (Table 2C).

Scarification of the seed coat of cool-matured seeds significantly increased the response to exogenously applied fluridone (Fig. 3B), indicating that reduced seed-coat permeability of cool-matured seeds could have inhibited its uptake. Nevertheless, scarification alone did not completely restore germination of cool-matured seeds, and fluridone was also required for full germination. Therefore, decreased permeability of the seed coat of cool-matured seeds (see above) contributes to the lack of germination of those seeds, but the synthesis of ABA in those seeds also prevents their germination even when seed coat constraints are removed.

Hormone sensitivity controlling for endogenous hormone synthesis (expt 5). Because endogenous levels of ABA might alter sensitivity to GA and vice versa, we measured sensitivity of after-ripened short-warm-matured seeds to GA and ABA while controlling the endogenous levels of both GA and ABA simultaneously. Fluridone increased germination to high levels in all of the lines except RIL58 (Fig. 6A). The addition of 10^{-5} M uniconazol completely inhibited germination, even when ABA synthesis was inhibited as well. In the

presence of both fluridone and uniconazol, addition of 10^{-5} M GA restored germination to near 100 % in all genotypes. The addition of 10^{-5} M ABA had no effect, since seeds were already completely dormant.

Genotypes differed in sensitivity to GA in the absence of ABA (Fig. 6B). Exogenous GA restored germination to nearly 100 %, suggesting that the insensitivity to GA observed in expt 4 in some lines could be due to *de novo* ABA synthesis. The sensitivity of germination to GA changed in the presence of exogenous ABA (Fig. 6B vs. C); with the addition of 10^{-5} M ABA, more GA was required to achieve comparable levels of germination [$F_{(\text{ABA presence} \times [\text{GA}])} = 5.3$, $P < 0.05$]. Genotypes responded differently to GA depending on whether or not ABA was present [$F_{(\text{ABA presence} \times [\text{GA}] \times \text{genotype})} = 13.2$, $P < 0.05$]. Greater genetic variation in GA sensitivity was observed when both GA and ABA were present than when ABA was absent. This result suggests genetic variation for sensitivity to ABA as well as genetic variation for sensitivity to GA. However, sensitivity to GA, as measured by the degree of germination at a non-saturating GA concentration, was positively correlated across all ABA treatments, including the absence of ABA, 10^{-5} M ABA and native ABA concentrations (Supplementary Data Appendix S2).

Likewise, sensitivity to ABA depended on exogenous GA application. Seeds were less sensitive to ABA at a constant [GA] of 10^{-5} M (Fig. 6D), compared with the previous

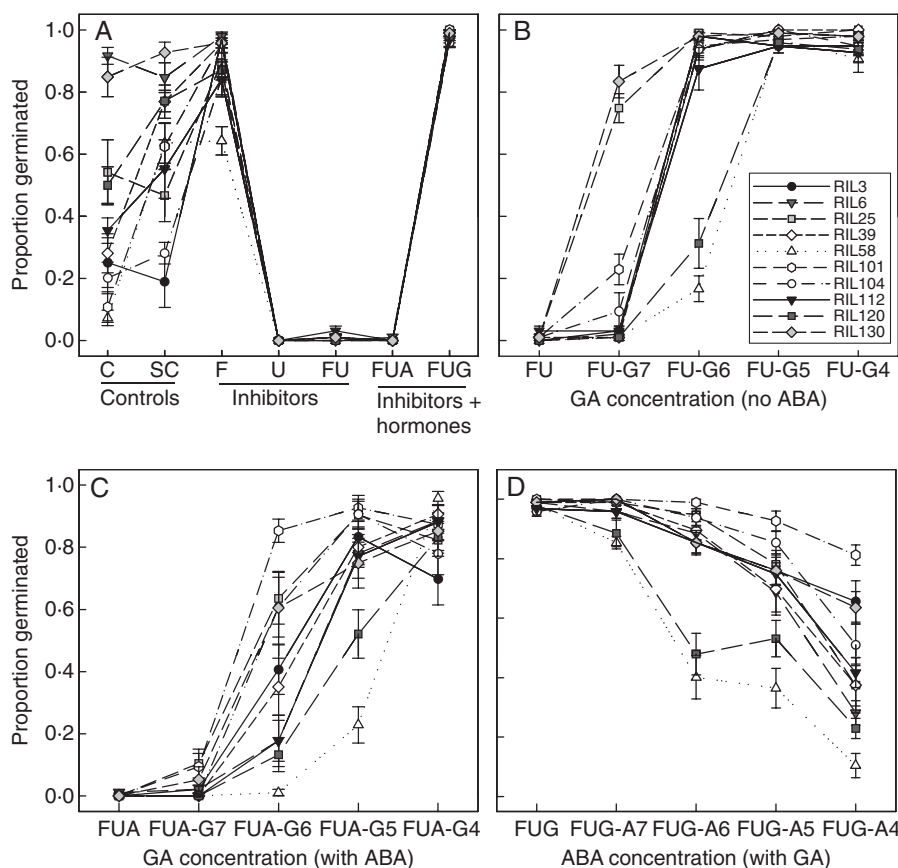


FIG. 6. Germination response to gibberellic acid (GA) and abscisic acid (ABA) while inhibiting endogenous synthesis of both hormones simultaneously. (A) C, control germination; Sol, solvent control, germination assayed in agar that contained 0.2% ethanol; F, fluridone (10^{-5} M); U, uniconazol (10^{-5} M); FU, fluridone and uniconazol; FUA, fluridone, uniconazol and ABA (10^{-5} M); FUG, fluridone, uniconazol and GA (10^{-5} M). (B) GA response in the presence of fluridone and uniconazol (both at 10^{-5} M), and increasing concentrations of GA (from 10^{-7} M to 10^{-4} M). (C) GA response in the presence of fluridone, uniconazol and ABA (all at 10^{-5} M), and increasing concentrations of GA (from 10^{-7} M to 10^{-4} M). (D) ABA response in the presence of fluridone, uniconazol and GA (all at 10^{-5} M), and increasing concentrations of ABA (from 10^{-7} M to 10^{-4} M). Seeds were matured under short-warm days, after-ripened for 4 months, and given a cold (4°C) imbibition treatment in the dark for 7 d before being transferred to light at 22°C . Final germination proportions were determined after 14 d in the light.

experiment in which GA levels were allowed to remain at the genotype-specific levels. The highest concentration of ABA, 10^{-4} M, failed to completely inhibit germination as it had previously done. Significant genetic variation for ABA sensitivity was detected when endogenous GA was held constant [$F_{(\text{genotype} \times [\text{ABA}])} = 6.25$, $P < 0.001$]. While this variation in ABA sensitivity may be due partly to variation in sensitivity to the exogenously applied GA, it cannot be due to differences in endogenous GA content, the synthesis of which is inhibited in the presence of uniconazol. Unlike sensitivity to GA, germination proportions with native endogenous GA levels (10^{-5} M ABA) were not significantly correlated with germination at fixed GA levels and ABA concentrations (from Fig. 6D: 10^{-5} M ABA, $r = 0.24$, $P > 0.05$; 10^{-4} M ABA, $r = 0.07$, $P > 0.05$). Therefore, much of the variation in ABA sensitivity observed in the native ABA assay could be due to variation in GA content.

In short, measures of GA and ABA sensitivities depended on the concentration of the other hormone, indicating that variation in hormonal sensitivity in assays that do not control for endogenous concentrations of hormones may be caused both by variation in sensitivity and by variation in endogenous

hormonal concentrations. Even when controlling for endogenous hormonal levels, however, we found genetic variation for hormonal sensitivities.

GA and ABA sensitivity and estimated endogenous levels: predicting natural variation in germination. Estimated GA content was positively correlated with ABA content and ABA sensitivity (Tables 3 and 4). GA sensitivity was not significantly correlated with estimated endogenous GA content. In contrast, higher GA sensitivity was associated with lower ABA sensitivity and lower ABA content, suggesting that high ABA-imposed dormancy may mask natural variation in GA content. However, this correlation is in part spurious as our method of estimation of relative endogenous ABA uses GA sensitivity. ABA sensitivity (measured under controlled GA concentrations) was not significantly correlated with ABA content. ABA sensitivity was positively correlated with GA content, suggesting variation in ABA sensitivity may be more apparent in the presence of GA, but this correlation is in part spurious (as mentioned above).

Some germination characteristics were significantly associated with allelic variation at quantitative trait loci (QTLs) for field germination behaviour based on a previous study

TABLE 3. Estimated GA and ABA sensitivity, and relative endogenous levels for the different RIL genotypes

Genotype	GA sensitivity	ABA sensitivity	Relative GA content	Relative ABA content
RIL3	0.51	0.34	-0.29	-0.32
RIL6	0.48	0.72	0.35	0.17
RIL25	0.87	0.63	0.01	-0.36
RIL39	0.47	0.63	-0.25	-0.20
RIL58	0.09	0.90	0.04	0.09
RIL101	0.59	0.19	-0.51	-0.05
RIL104	0.52	0.49	-0.38	-0.40
RIL112	0.45	0.58	0.07	0.27
RIL120	0.16	0.77	0.29	0.19
RIL130	0.91	0.36	-0.24	-0.34

See text for details on estimations.

TABLE 4. Pearson genotypic correlations between relative hormone content and sensitivity

	GA sensitivity	GA content	ABA sensitivity	ABA content
GA sensitivity	1.00	-0.40	-0.61*	-0.65**
GA content		1.00	0.79**	0.67**
ABA sensitivity			1.00	0.49
ABA content				1.00

** $P < 0.05$; * $P < 0.1$.

(Huang *et al.*, 2010; Supplementary Data Table S5). The Calver alleles at the two (adjacent) markers on Chromosome 3 were associated with delayed germination in the field. In this study they had significantly higher sensitivity to ABA, higher estimated GA and ABA content, and a lower response to scarification. The Calver allele of the QTL on Chromosome 5 was associated with delayed germination in the field, and in this study was associated with higher GA content. Genotypes with higher GA content also had higher ABA sensitivity and higher ABA content, however (Table 4). Thus for these three QTLs, alleles that increase sensitivity to ABA and ABA content may delay the germination of seeds in the field even if seed-coat constraints are relieved or GA content is higher.

In one treatment (seeds matured under warm conditions and given a cold, dark imbibition treatment), we were able to test the ability of each of the hormonal sensitivities and estimated concentrations to predict germination. These physiological parameters combined accounted for up to $\geq 80\%$ of the variation in germination across genotypes. The estimated relative endogenous level of GA was the best and only significant predictor of germination in a multiple regression (Table 5). There was no significant relationship between germination and ABA levels, ABA sensitivity or GA sensitivity in this treatment.

DISCUSSION

Abundant genetic variation for germination responses to different environmental conditions was detected, even within this small sample of ten RILs derived from two natural

TABLE 5. Multiple regression of germination with GA and ABA sensitivity and content for two different batches of seeds: Batch I and Batch II, and the average germination of the two batches

	β	s.e. of β	t	P
Batch I: $R = 0.9119$; $R^2 = 0.8316$				
Intercept	0.2782	0.2874	0.9682	0.3773
GA sensitivity	0.1825	0.2264	0.8059	0.4568
Endogenous GA	0.7820	0.2665	2.9338	0.0324*
ABA sensitivity	-0.2535	0.3367	-0.7529	0.4853
Endogenous ABA	-0.1995	0.2362	-0.8446	0.4368
Batch II: $R = 0.8938$; $R^2 = 0.7990$				
Intercept	0.5175	0.5377	0.9623	0.3800
GA sensitivity	0.5906	0.4237	1.3936	0.2221
Endogenous GA	1.2188	0.4987	2.4439	0.0583**
ABA sensitivity	-0.5738	0.6301	-0.9106	0.4042
Endogenous ABA	-0.2374	0.4419	-0.5372	0.6141
Average of Batch I and II: $R = 0.9358$; $R^2 = 0.8758$				
Intercept	0.3978	0.3169	1.2554	0.2647
GA sensitivity	0.3865	0.2497	1.5478	0.1823
Endogenous GA	1.0004	0.2939	3.4038	0.0191*
ABA sensitivity	-0.4137	0.3713	-1.1140	0.3159
Endogenous ABA	-0.2184	0.2604	-0.8388	0.4398

* $P < 0.05$; ** $P < 0.01$.

ecotypes. Germination responses to different environmental conditions sometimes had different physiological mechanisms, and physiological mechanisms associated with germination responses also exhibited genetic variation. Thus, germination responses to different environmental cues have evolutionary potential in the form of genetic variation, and some, but not all, responses appear to have at least partially independent genetic bases.

Genetic variation for germination responses to environmental factors

Genetic variation for germination (of warm-matured seeds) was detected in nearly every experimental treatment, but it was most pronounced in cold-treated and after-ripened seeds. There was no evidence of environmental specialization of germination behaviour, such that some genotypes germinated to higher percentages in some conditions while others germinated to higher percentages in others. Strong positive genetic correlations in germination across environmental treatments indicate that some genotypes consistently germinate better than others, and the lack of any negative correlation across environments indicates a complete lack of detectable trade-offs in environment-dependent germination. Genetic variation was not detected for primary dormancy at the time of seed shed; instead genetic variation for loss of dormancy with after-ripening or cold treatment predominated. These results suggest that a significant amount of genetic variation in germination phenology in *A. thaliana* could be caused by variation in dormancy loss, as opposed to variation in environment-dependent germination *per se*. This interpretation is consistent with a QTL study of germination phenology in the field using these same RILs (Huang *et al.*, 2010), which identified two major QTL for germination phenology—both collocating with dormancy loci (*At-DOG1* and *At-DOG6*). These two

QTLs are consistently associated with dormancy (at least in RILs containing the Ler parental line; Bentsink *et al.*, 2010).

Genotypic correlations of germination proportions were significant and strong across seed-maturation photoperiod, after-ripening status, cold-imbibition temperature (4 °C vs. 10 °C) and germination temperature, suggesting that germination under these sets of conditions shares a common genetic basis. In some cases, genotypic correlations across environments were low, most likely because genetic variation was low in one of the environments. However, some non-significant genotypic correlations of germination proportions are likely to indicate partially independent genetic bases for germination under different conditions. Specifically, non-significant correlations were found for germination after different imbibition treatments, especially between seeds given a cold imbibition and those that were not. A specific genetic pathway, involving GA synthesis regulated through the cold-sensitive gene *SPATULA*, regulates germination responses to cold imbibition (Penfield *et al.*, 2005). These results indicate that one or more genes in the pathway of cold-stimulated germination exhibits natural variation. This interpretation is consistent with a QTL study of these (Huang *et al.*, 2010) and other RILs (Meng *et al.*, 2008) that found large-effect QTLs for germination responses to cold imbibition. Interestingly, the cold-induced breakage of primary and secondary (warm-induced) dormancy appears to have distinct genetic bases, as genotypic correlations across these imbibition temperatures were not significant, despite significant genetic variation within each of those conditions.

In summary, we found no evidence for specialization of germination to different conditions. We did find genetic variation in dormancy and in germination responses to cold imbibition, and these two sources of variation appear to have at least partially independent genetic bases.

The physiological basis of germination responses to particular environmental factors

Germination responses to particular environmental factors sometimes had distinct physiological mechanisms. For example, the observed increase in germination of seeds matured under long days as opposed to short days appears to be associated with an increase in the sensitivity to GA in those seeds, but not to changes in sensitivity in ABA. In addition, seed-coat permeability did not appear to play a significant role in the difference in germination behaviour of seeds matured under different photoperiods.

The pronounced decrease in germination of seeds matured under a cool temperature as opposed to a warm temperature also appears to involve decreased sensitivity to GA, but the seed coat/aleurone was also a significant constraint to germination of these seeds. In cold-matured seeds, the seed coats were significantly less permeable than those matured under warm temperature; GA is known to stimulate a weakening of the seed coat, so decreased sensitivity to GA, likely by the aleurone layer (Bethke *et al.*, 2007), could account for more persistent seed-coat effects in these seeds (Bewley, 1997; Debeaujon *et al.*, 2000, 2007; Koornneef *et al.*, 2002). The decreased sensitivity to GA of cold-matured seeds could in part be because of higher endogenous ABA levels (Koornneef *et al.*, 2002;

Kucera *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Bentsink and Koornneef, 2008; Finkelstein *et al.*, 2008; Holdsworth *et al.*, 2008). In addition, cold-matured seeds exhibited increased sensitivity to ABA. In these seeds, ABA appears to maintain dormancy even when seed-coat constraints are relieved. Thus germination responses to seed-maturation temperature involve additional mechanisms of dormancy induction and maintenance than germination responses to seed-maturation photoperiod.

Germination in after-ripened seeds that are exposed to cold imbibition appears to be accompanied by increased sensitivity to ABA and genotype-specific changes in GA sensitivity. Note that GA and ABA sensitivity were measured in cold-treated seeds in this study, and that the cold treatment induced dormancy in after-ripened seeds (Rubio de Casas *et al.*, NESCent, NC, USA, unpubl. res.). Thus, the increased sensitivity to ABA in these after-ripened seeds could be caused by dormancy induction by the cold treatment. It has also been shown that ABA levels decline with after-ripening (Cadman *et al.*, 2006; Bentsink and Koornneef, 2008; Holdsworth *et al.*, 2008), which can account for the typical loss of dormancy in after-ripened seeds.

This study provides evidence that germination responses to different environmental factors can involve different physiological mechanisms. For example, while evolutionary changes in seed coat and ABA sensitivity are expected to influence germination responses to the seed-maturation temperature, they may not alter germination responses to the seed-maturation photoperiod. In contrast, evolutionary changes in GA sensitivity would be expected to alter germination responses to both cues. Therefore, the physiological basis of germination responses to particular cues may influence the degree to which the evolution of germination under particular environmental conditions effects changes in germination under other conditions.

Genetic variation for physiological mechanisms and its relation to genetic variation in germination

Different physiological mechanisms exhibited different degrees of genetic variation. We did not detect significant genetic variation for seed-coat permeability or for the effect of seed-coat/endosperm disruption on germination. In contrast, significant genetic variation was found for sensitivity to both GA and ABA, whether or not endogenous hormonal levels were controlled. Genotypes also differed in the degree to which complete inhibition of ABA synthesis promoted germination. Therefore, genetic variation exists for sensitivity to GA, sensitivity to ABA, and for the level of dormancy without *de novo* ABA synthesis.

Significant genetic variation was detected for changes in GA sensitivity with after-ripening, seed-maturation photoperiod, and seed-maturation temperature. In contrast, while ABA sensitivity did change with after-ripening and seed-maturation temperature, no significant genetic variation was detected for changes in ABA sensitivity with either of these factors. GA sensitivity depended on ABA concentration and vice versa. Therefore, genetic variation in GA sensitivity and its environmental responses could be caused, in part, by genetic variation in ABA synthesis and environment-dependent ABA synthesis,

respectively. Likewise, genetic variation in ABA sensitivity could be caused, in part, by variation in GA levels. In one treatment (warm-matured, cold-imbibed) in which both hormonal sensitivity and endogenous hormonal levels were estimated, the estimated GA concentration was the best predictor of variation in germination. While this is very approximate indicator of GA content in seeds, it, combined with the above evidence, is nonetheless suggestive that allelic variants of genes in GA synthesis pathways may be especially important contributors to natural variation in germination of cold-imbibed seeds. Studies that directly measure hormonal content and germination behaviour in natural ecotypes would be especially informative.

Alleles at markers that were associated with delayed germination in the field (Huang *et al.*, 2010) were also associated with increased ABA content and sensitivity to ABA, but higher GA content and somewhat lower scarification response. Therefore, while GA content may be the best predictor of germination under certain laboratory conditions, ABA sensitivity and content may be an important predictor of germination timing in the field.

These markers were located on Chromosome 3, within the general region of the QTL *Delay of Germination 6* (*DOG6*; Alonso-Blanco *et al.*, 2003; Bentsink *et al.*, 2010), whose identity has not yet been determined. The third marker, in which the Calver allele was associated with higher estimated GA content, is located within the region on Chromosome 5 of *Delay of Germination 1* (*DOG1*), a gene whose expression increases dormancy levels. ABA increases *DOG1* expression in *A. thaliana* and other mustards (Graeber *et al.*, 2010), via the sugar-signalling pathway (Teng *et al.*, 2008). Increased *DOG1* expression is associated with GA catabolism during seed maturation when primary dormancy is induced (Kendall *et al.*, 2011). Counter-intuitively, the allele that was associated with delayed germination in the field was also associated with higher GA content in imbibed seeds in this experiment. The association of the late-germinating *DOG1*-associated allele with high GA in this experiment could be accounted for, in part, by the positive association between GA content, ABA sensitivity and ABA content. Moreover, the reported increase in GA catabolism during high *DOG1* expression was found during seed maturation and dry seeds, but it has not been documented for imbibed seeds, as were used to estimate GA content in this experiment. Further work on *DOG1* expression dynamics throughout germination and their association with hormonal dynamics, with endogenous hormonal concentrations measured directly, would be most informative.

GA sensitivity depended on ABA concentrations and vice versa, and sensitivities to, and relative content of, GA and ABA were negatively correlated. Thus, hormonal sensitivities may evolve in concert, and the evolution of hormone synthesis is likely to cause changes in sensitivities as well.

Conclusions

Genetic variation for germination is environment-dependent, but no evidence for germination specialization to different conditions was found. Much variation in germination appears to be in the ability to germinate in multiple environmental conditions. Strong genotypic correlations across some environments would

constrain the evolution of distinct germination behaviour in those environments, but germination with versus without a cold treatment appears to have distinct genetic mechanisms. Some physiological mechanisms, such as GA sensitivity, are associated with germination responses to different environmental factors, but others, such as seed-coat permeability and ABA sensitivity, are specific to particular germination responses. The particular physiological mechanisms regulating germination under different conditions can therefore influence the genetic independence of germination under different conditions and consequently the potential to evolve adaptive germination in novel combinations of environmental conditions.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consists of the following. Table S1: marker information for RILs and genotypic means of variables that differed significantly between marker genotypes. Table S2: genotypic correlations between seeds that were exposed to dark imbibition at 4 °C vs. 10 °C. Table S3: test for genetic variation for responses to germination temperatures (genotype × temperature) for specific contrasts. Table S4: (a) genotypic correlations of germination proportions across seed-maturation photoperiod, for different imbibition and germination temperature and after-ripening durations; (b) genotypic correlations between seeds after-ripened for different durations. Table S5: means of germination characteristics that differed across markers, with *F*-tests of differences between marker genotypes. Appendix S1: temperature-dependent dormancy breakage. Appendix S2: correlations of GA sensitivities in different ABA backgrounds. Figure S1: effect of scarification on tetrazolium dye uptake for two RIL genotypes (RIL58 and RIL112), and one reference genotype.

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