

Contrasting germination responses to vegetative canopies experienced in pre- vs. post-dispersal environments

Lindsay D. Leverett^{1,2,*}, Gabriela A. Auge², Aman Bali² and Kathleen Donohue²

¹University Program in Ecology, Duke University, Durham, NC 27708, USA and ²Department of Biology, Duke University, Durham, NC 27708, USA

*For correspondence. E-mail ldl11@duke.edu

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• **Background** Seeds adjust their germination based on conditions experienced before and after dispersal. Post-dispersal cues are expected to be more accurate predictors of offspring environments, and thus offspring success, than pre-dispersal cues. Therefore, germination responses to conditions experienced during seed maturation may be expected to be superseded by responses to conditions experienced during seed imbibition. In taxa of disturbed habitats, neighbours frequently reduce the performance of germinants. This leads to the hypotheses that a vegetative canopy will reduce germination in such taxa, and that a vegetative canopy experienced during seed imbibition will over-ride germination responses to a canopy experienced during seed maturation, since it is a more proximal cue of immediate competition. These hypotheses were tested here in *Arabidopsis thaliana*.

• **Methods** Seeds were matured under a simulated canopy (green filter) or white light. Fresh (dormant) seeds were imbibed in the dark, white light or canopy at two temperatures (10 or 22 °C), and germination proportions were recorded. Germination was also recorded in after-ripened (less dormant) seeds that were induced into secondary dormancy and imbibed in the dark at each temperature, either with or without brief exposure to red and far-red light.

• **Key Results** Unexpectedly, a maturation canopy expanded the conditions that elicited germination, even as seeds lost and regained dormancy. In contrast, an imbibition canopy impeded or had no effect on germination. Maturation under a canopy did not modify germination responses to red and far-red light. Seed maturation under a canopy masked genetic variation in germination.

• **Conclusions** The results challenge the hypothesis that offspring will respond more strongly to their own environment than to that of their parents. The observed relaxation of germination requirements caused by a maturation canopy could be maladaptive for offspring by disrupting germination responses to light cues after dispersal. Alternatively, reduced germination requirements could be adaptive by allowing seeds to germinate faster and reduce competition in later stages even though competition is not yet present in the seedling environment. The masking of genetic variation by maturation under a canopy, moreover, could impede evolutionary responses to selection on germination.

Key words: Seed germination, dormancy, maternal environmental effects, shade, R:FR, *Arabidopsis thaliana*.

INTRODUCTION

Organisms can adjust their phenotypes in response to conditions experienced by their parents (parental environmental effects) and by themselves (Schlichting, 1986; Roach and Wulff, 1987; West-Eberhard, 1989; Sultan, 2000; Snell-Rood, 2013). While parental environmental effects can produce adaptive responses in offspring (Mousseau and Fox, 1998; Herman and Sultan, 2011), cues experienced by offspring themselves are likely to be more reliable predictors of their immediate environments, and in turn performance, than cues in the parental environment (DeWitt *et al.*, 1998). Therefore, it is expected that responses to conditions experienced by an individual in its immediate environment will over-ride responses to cues that were experienced during its maturation in the parental environment. However, parental environments may predict future conditions that are not yet present in an individual's immediate environment. If so, then plastic responses to parental environments could increase performance and would be expected to over-ride responses to the immediate environment.

Seed germination responds to cues experienced before and after dispersal in a manner that prevents seedlings from emerging under sub-optimal conditions (Guterman, 2000; Donohue, 2009; Donohue *et al.*, 2010; Baskin and Baskin, 2014). One environmental factor that is likely to influence seedling performance is neighbouring vegetation. Plants detect vegetation using irradiance and the ratio of red to far-red light (R:FR), both of which are reduced when sunlight is filtered through or reflected from photosynthetic tissue (Casal and Sánchez, 1998; Smith, 2000). Phytochromes are plant photoreceptors that sense R and FR light; the inactive conformation (Pr) is converted by R light into the bioactive conformation (Pfr) that stimulates germination and other developmental processes (Casal and Smith, 1989; Casal and Sánchez, 1998). Phytochromes therefore regulate responses to R:FR, which can predict competition even before it occurs (Smith, 2000), but other photoreceptors and resource-sensing mechanisms are involved in responses to total irradiance (Aphalo and Ballaré, 1995; Casal, 2013).

Conditions of low irradiance or R:FR, indicating nearby vegetation, often impede germination in taxa that require gaps for

establishment (King, 1975; Deregibus *et al.*, 1994; Gutterman, 2000; Pons, 2000; Dechaine *et al.*, 2009). In such taxa, preventing germination under competitive conditions will increase a seed's fitness; therefore, germination should be less likely to occur under light cues that indicate competition. The value of preventing germination under vegetation will then depend on the risk of mortality in seeds that do not germinate (Donohue *et al.*, 2010). However, light cues may interact with other seasonal cues to regulate germination. In particular, temperature varies seasonally, and seeds of most species require specific temperatures during imbibition in order to germinate (Baskin and Baskin, 2014). If temperature requirements are not met, seeds will not germinate even when other conditions, such as light, are favourable. Therefore, the hypothesized inhibitory effect of a vegetative canopy is more likely to be observed under temperatures that promote germination.

While light cues experienced during seed maturation can indicate competitive conditions in the maternal environment, vegetation varies through space and time and is unlikely to remain perfectly stable from the time of seed maturation to the time of seed germination. Consequently, vegetation cues experienced by dispersed seeds may be more accurate predictors of seedling competitive conditions and therefore performance than those same cues experienced during maturation. If so, germination responses to vegetation in the seed environment are expected to over-ride germination responses to vegetation in the parental environment.

Seed dormancy will further reduce the ability of vegetation cues in the maternal environment to predict vegetation present in the seedling environment. Dormancy prevents germination under ephemeral conditions that would stimulate germination in non-dormant seeds, thereby synchronizing non-dormancy with the appropriate germination season (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Baskin and Baskin, 2014; Footitt *et al.*, 2014). Seeds that must lose dormancy over time (after-ripen) would require more 'lag time' between the perception of cues during maturation and responses to those cues during germination (DeWitt *et al.*, 1998), which could reduce the accuracy of pre-dispersal cues in predicting seedling performance. Consequently, the effects of vegetation cues during maturation on germination are hypothesized to be weaker in species or genotypes with stronger dormancy.

In species with non-deep physiological dormancy, seeds that lose dormancy during after-ripening may be induced into secondary dormancy if germination requirements are not met, which allows seeds to delay germination until the next favourable season (Bewley and Black, 1994; Baskin and Baskin, 2014; Footitt *et al.*, 2014; Auge *et al.*, 2015). Such additional delays of germination through secondary dormancy induction may further reduce the accuracy with which the maturation environment can predict the seedling environment. As a consequence, after-ripened seeds that are induced into secondary dormancy are expected to respond to cues experienced during imbibition, but not to cues in the maternal environment.

For the reasons discussed above, it is hypothesized that a dispersed seed's environment is a more accurate predictor of its potential to experience competition than the environment of the maternal parent. Therefore, if cues are in conflict between maternal and offspring environments, one would expect that seeds will respond to their own environment more strongly than to

that of their maternal parent. However, the maternal environment may provide cues of future competition that are not yet present in a dispersed seed's immediate environment, in which case germination responses to the maternal environment may supersede responses to the seed's environment. Further, parent-offspring conflict and selection acting directly on maternal plants for their regulation of offspring phenotypes may cause offspring to produce phenotypes that reduce their individual performance (Marshall and Uller, 2007; Uller, 2008).

In this study, we tested germination responses to light cues in pre- vs. post-dispersal environments in genotypes of *Arabidopsis thaliana* (Brassicaceae) that differ in dormancy. Specifically, we tested whether: (1) germination proportions are lower in seeds matured or imbibed under a vegetative canopy compared with those matured or imbibed in white light; (2) germination responses to a canopy experienced during imbibition are stronger than responses to a canopy experienced during maturation; (3) seeds with greater dormancy, as determined by genotype or the induction of secondary dormancy, are less likely to respond to a vegetative canopy experienced during seed maturation; and (4) germination responses to vegetative canopies are mediated by the detection of R and FR light.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana is an annual plant in the Brassicaceae with physiological seed dormancy (Baskin and Baskin, 1983). Dormancy varies among natural populations of *A. thaliana* and has been shown to be under natural selection and to contribute to local adaptation (Bentsink *et al.*, 2010; Huang *et al.*, 2010; Kronholm *et al.*, 2012; Montesinos-Navarro *et al.*, 2012; Debieu *et al.*, 2013; Postma *et al.*, 2016). *Arabidopsis thaliana* is usually a winter annual, germinating in the autumn and flowering in the spring, but it can exhibit a spring-annual life history – germinating and flowering in spring – or a rapid-cycling life history – germinating and flowering multiple times per year (Ratcliffe, 1965, 1976; Baskin and Baskin, 1972, 1983; Thompson, 1994; Donohue, 2009). Variation in germination contributes to this life-history variation.

To test the hypothesis that germination responses to a canopy experienced during maturation and imbibition depend on seed dormancy, we used three genotypes of *A. thaliana* that differ in dormancy and represent a sub-set of the natural variation in germination behaviour in this species (for a schematic overview of our experimental design, see Supplementary Data Fig. S1). Our most dormant genotype was the standard accession Landsberg *erecta* (*Ler*). We also used a near isogenic line ('NIL') that has a quantitative trait locus (QTL) associated with germination on chromosome 5 from the Cape Verde Islands (Cvi) ecotype introgressed onto the *Ler* background (Alonso-Blanco *et al.*, 1998). The NIL has lower dormancy than *Ler*, manifest as a wider range of temperatures that elicit germination in non-after-ripened seeds (Chiang *et al.*, 2009). Finally, we used the Columbia (Col) accession to compare germination responses to vegetative canopies in the two standard lab strains of *A. thaliana* (*Ler* and Col) that are known to differ in germination behaviour. Col is less dormant than *Ler* under most conditions (Chiang *et al.*, 2009; Burghardt *et al.*, 2016).

To test whether a canopy during seed maturation alters germination, we matured seeds of the three genotypes under unfiltered white light ('white light' maturation) and a green filter ('canopy' maturation; LEE colour effect filters, #089 'moss green'; LEE Filters, Andover, Hampshire, UK). The green filter reduced R:FR from 1.4 to 0.3 and total irradiance by 50 %, simulating dense vegetation. While the green filter cannot distinguish the effects of total irradiance, R:FR and changes in other spectral qualities such as blue light and UVA/B, the filter realistically mimics the effect of a vegetative canopy by reducing both R:FR and total irradiance.

Seeds of the three genotypes were induced to germinate in pots filled with Metromix 360 soil (Scotts Sierra, Maysville, OH, USA) and vernalized for 4 weeks at 4–5 °C to initiate and synchronize flowering. Maternal plants were then transferred to GCW-30 growth chambers (Environmental Growth Chambers, Chagrin Falls, OH, USA) at 22 °C under a 12 h light cycle. After bolting, maternal plants were transferred to 15 °C in 8 h light into the two maturation environments (white light and canopy) in the same chambers, experiencing different light treatments only after bolting. The temperature and daylength during seed maturation were chosen to differentiate the dormancy levels of the three genotypes – specifically to increase dormancy in the less dormant genotypes (Col and the NIL) without inducing deep dormancy in the more dormant genotype (*Ler*). Six plants per genotype were matured in each treatment, with three plants each in two chambers, and fertilized every 2 weeks with a 300 ppm nitrogen solution of Blossom Booster Fertilizer (JR Peters, Allentown, PA, USA). Mature seeds were synchronously harvested and stored dry at room temperature prior to germination assays.

Germination assays

Fresh seeds (primary dormancy). We performed germination assays using fresh seeds (<1 week of after-ripening) to assess the effects of seed-maturation light on the temperature and light requirements for germination immediately after seeds are shed. We used three imbibition light treatments – white light (12 h photoperiod), green filter (12 h photoperiod, 'canopy' hereafter) and dark – and two imbibition temperatures – 10 and 22 °C. Comparisons between the white light and dark treatments test whether light is required for germination, while comparisons between the white light and canopy treatments test for germination responses to a canopy during imbibition. Comparisons between temperatures (10 and 22 °C) test whether the effects of genotype, maturation light and imbibition light are more likely to occur when seeds experience temperatures that promote germination (10 °C) as opposed to temperatures that are less conducive to germination (Auge *et al.*, 2015; Burghardt *et al.*, 2016).

Seeds from six maternal plants (biological replicates) of each genotype in each maturation-light treatment were used for germination assays, for a total of six sowing replicates per combination of genotype, maturation light and imbibition. Twenty seeds per replicate were sown in Petri plates on 0.7 % (w/v) agar. Plates were wrapped with parafilm to prevent desiccation and immediately transferred to their treatments in GC-82 growth chambers (Environmental Growth Chambers). Total

irradiance [photosynthetically active radiation (PAR)] was 240 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in 10 °C and 160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in 22 °C. For the dark treatment, plates were placed in cardboard boxes wrapped in two layers of aluminium foil. For the white light and canopy treatments, plates were randomly arranged on plastic trays with transparent lids. For the canopy treatment, the same green filters used for the maturation treatment were fitted under each tray lid. We censused plates in the white light and canopy imbibition treatments for germination every 2–5 d until day 14, at which point germination had plateaued. Exposure of plates in these treatments to light in the laboratory during censuses was minimal. Plates in the dark treatment were censused on day 14. The criterion for germination was the emergence of the radicle from the seed coat. Germination proportion was scored as the number of germinated seeds per total number of viable seeds in each plate; thus, the plate is our unit of analysis. Seeds were considered viable if they were firm after germination had plateaued.

Hot-stratified, after-ripened seeds (secondary dormancy). We performed a second germination assay to test whether the effects of a seed-maturation canopy persist after seeds are induced into secondary dormancy. In addition, to examine potential physiological mechanisms of germination responses to a maturation canopy, we exposed after-ripened seeds that were induced into secondary dormancy to light pulses of different wavelengths (R and FR) during imbibition.

Seeds were first after-ripened to allow them to lose primary dormancy, then re-induced into secondary dormancy using hot stratification (Auge *et al.*, 2015). Specifically, seeds were stored dry at room temperature for 6 weeks to allow them to after-ripen. Then, after-ripened seeds were sown into Petri plates filled with 0.7 % (w/v) agar, wrapped with parafilm, placed in aluminium-wrapped cardboard boxes and imbibed in the dark at 35 °C for 2 d. Although we did not test germination of after-ripened seeds to confirm that they were non-dormant before stratification, these genotypes lose most dormancy after 6 weeks of after-ripening under the conditions of this experiment. Previous studies have shown that seeds of the NIL and Col that were matured under a similar temperature (14 °C) and after-ripened for a comparable period (7 weeks) germinate to 100 % at 8 °C and to 75 % at 22 °C, while *Ler* germinated to 80 % at 8 °C and to 60 % at 22 °C (Burghardt *et al.*, 2016). Thus, some residual primary dormancy may have remained in *Ler* at the time of hot stratification. Regardless, differences in germination between the seed-maturation treatments in our experiment would indicate that the effect of a maternal canopy persists as seeds lose and re-gain dormancy.

Following hot stratification, seeds were imbibed in the dark or given pulses of FR and/or R light using light-emitting diode chambers (Percival Scientific, Perry, IA, USA) then transferred to darkness. Active phytochrome (Pfr) is involved in light-stimulated germination (Whitelam and Devlin, 1997; Heschel *et al.*, 2007). FR light converts phytochrome from its active (Pfr) to inactive (Pr) form, and R light converts Pr to bioactive Pfr. Seeds given the FR and R treatments were first exposed to a 30 min 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ FR pulse to convert Pfr to inactive Pr, then either moved immediately to darkness (FR treatment) or exposed to a 30 min 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ R pulse (to convert Pr to active Pfr) and then moved to darkness (R treatment).

Following the light pulses, plates in all three treatments (dark, R and FR) were placed in cardboard boxes double-wrapped in aluminium foil and then imbibed in the dark at either 10 or 22 °C for 14 d. After 14 d, plates were assayed for germination as the number of germinated seeds out of the total number of viable seeds per plate, as in the first experiment.

Seeds imbibed in the dark without any light pulse have Pfr levels established during maturation. Dark imbibition therefore assesses whether seeds can germinate without light, and a difference in germination in the dark between the maturation treatments would indicate that the effect of a maturation canopy persists as seeds lose and re-gain dormancy. The comparison between dark and FR treatments tests whether Pfr levels established during maturation are adequate to induce germination in seeds with secondary dormancy, compared with a treatment in which most Pfr was abolished by exposure to FR light. Thus, the comparison of dark vs. FR across maturation light treatments reveals whether a maturation canopy influences germination via differences in Pfr established during maturation. The R treatment increases bioactive Pfr during imbibition; therefore, the comparison between FR and R treatments quantifies the degree to which germination increases in response to increased *de novo* Pfr. A comparison of R vs. FR across maturation light treatments, moreover, would test whether seed maturation under a canopy affects germination responses to *de novo* Pfr induced by exposure to R light during imbibition.

Statistical analysis

All analyses were performed using R v. 3.1.3 (R Core Team, 2015). *P*-values were adjusted for multiple comparisons using the Holm procedure in ‘p.adjust’. Final germination proportions were analysed using generalized linear models (glms) fit with a logit link function using ‘glm’ in the ‘stats’ package, and likelihood ratio (LR) tests were performed using ‘lrtest’ in ‘lmerTest’ (Zeileis and Hothorn, 2002). When treatments had little germination and data separation prevented glms from converging, we instead used bias-reduced logistic regression (‘brglm’ package; Kosmidis, 2013) followed by LR tests. The magnitudes of effects were determined using coefficients from bias-reduced logistic regression models. In cases where no germination occurred in any of the treatments being compared, we did not perform statistical tests. For both experiments, maturation light (‘Maturation’), genotype (‘Genotype’) and imbibition temperature (‘Temperature’) were treated as fixed factors, and reference levels were white light for maturation, *Ler* for genotype and 10 °C for temperature. For the experiment with fresh seeds, imbibition light (‘Imbibition’) was treated as a fixed factor and white light was used as the reference level. For the experiment with hot-stratified seeds induced into secondary dormancy, imbibition pulse (‘Imbibition Pulse’) was treated as a fixed factor and FR was used as the reference level.

In fresh seeds, we first tested the effects of Maturation, Genotype, Imbibition and Temperature on germination probability. Temperature frequently interacted with other factors (Supplementary Data Table S1), so we subsequently analysed each temperature separately to interpret these interactions. To interpret Maturation × Imbibition, we tested whether Maturation altered light requirements for germination (Maturation × Imbibition) in dark vs. white light imbibition

for each genotype. We also tested Maturation × Imbibition in white light vs. canopy imbibition to test whether Maturation altered germination responses to a canopy during imbibition. To interpret Maturation × Genotype, we tested for differences between genotypes (*Ler* vs. NIL and *Ler* vs. Col) in their sensitivity to Maturation (Maturation × Genotype) within each combination of Imbibition and Temperature. To interpret Maturation × Imbibition and Maturation × Genotype further, we tested Maturation within each combination of Imbibition, Genotype and Temperature. We estimated the magnitudes of Maturation effects in each combination of Imbibition, Genotype and Temperature, and of Imbibition effects in each combination of Maturation, Genotype and Temperature. Finally, we tested Genotype in each combination of Maturation, Imbibition and Temperature.

To examine effects on dormancy induction in after-ripened seeds that were hot stratified and induced into secondary dormancy, we first tested the effects of Maturation, Genotype and Temperature on the depth of dormancy induction measured as germination proportion of hot-stratified, dark-imbibed seeds only. To interpret Maturation × Temperature × Genotype interactions, we next tested Maturation in each Genotype and Temperature separately, and estimated the magnitude of the Maturation effect on dark germination.

To determine whether the effect of canopy maturation on germination after secondary dormancy induction may be mediated by phytochromes, we tested the effects of Maturation, Genotype and Temperature on germination responses to R and FR light during imbibition. First, we tested whether Pfr established during seed maturation (dark imbibition) induced more germination than in seeds without Pfr (FR treatment), and whether those effects depended on genotype and temperature (Imbibition Pulse interactions with Maturation, Genotype and Temperature in dark vs. FR treatments). To interpret these interactions, we next tested Maturation on germination responses to dark vs. FR imbibition (Maturation × Imbibition Pulse) in each combination of Genotype and Temperature. Finally, we determined the magnitude of germination responses to FR light compared with the dark treatment for each combination of Maturation, Genotype and Temperature.

To test whether a maturation canopy alters Pfr requirements (i.e. R light requirements) for germination in seeds with secondary dormancy, we tested the effects of maturation and genotype on germination responses to FR vs. R imbibition in each temperature (Imbibition Pulse interactions with Maturation, Genotype and Temperature). We interpreted these interactions by testing the effect of a maturation canopy on germination responses to FR vs. R treatments (Maturation × Imbibition Pulse) in each combination of Genotype and Temperature. Finally, we quantified the magnitude of germination responses to R light compared with FR.

RESULTS

Vegetative canopies experienced during seed maturation and seed imbibition have opposing effects on germination of fresh seeds

Seed maturation under a canopy almost always increased germination proportions, and those germination proportions were

usually greater at 10 °C than at 22 °C (Figs 1 and 2; Supplementary Data Table S2). At both temperatures, a maturation canopy increased germination more in some imbibition light treatments and genotypes than others (Genotype × Maturation × Imbibition in Table 1).

At 10 °C, the temperature that elicited greater germination, seed maturation under a canopy relaxed the light requirement for germination by allowing seeds to germinate in the dark (Fig. 3A; Table 2; Supplementary Data Table S3). In Col, seeds did not have a strong light requirement for germination even when matured under white light, so the effect of canopy maturation was not significant (Table 2).

At 10 °C, germination did not respond to imbibition under a canopy due to high germination proportions in both white light and canopy imbibition treatments (Figs 1 and 3B; Table S3). Germination of canopy-matured seeds of Col was lower when imbibed under a canopy than when imbibed in white light, but this weak response is unlikely to be biologically significant (Fig. 1B).

At 22 °C, the temperature that was less conducive to germination, seed maturation under a canopy did not allow seeds to germinate in the dark as it did at 10 °C (Figs 1C, D and 3C; Table 2; Table S3). Seeds from the most dormant genotype (*Ler*) could only germinate if matured under a canopy, and then did so in white light but not in the dark. Thus, when imbibition temperature restricted germination, seeds were able to respond

to imbibition light if they had been matured under a canopy, even though a seed-maturation canopy did not allow germination in the dark.

At 22 °C, imbibition under a canopy reduced germination compared with white light in the less dormant genotypes (*Col* and the NIL), although in the NIL the effect was weaker if seeds had been matured under a canopy (Figs 1 and 3D; Table 2; Table S3). In contrast, seeds of the most dormant genotype (*Ler*) were able to germinate only if they were matured under a canopy, and then germinated less when imbibed in the canopy treatment than when imbibed in white light.

Differences among genotypes depended on the combination of seed maturation and imbibition canopy treatments. In both temperatures, the most dormant genotype (*Ler*; see Figs 1 and 2) was also the most sensitive to maturation under a canopy. Because maturation under a canopy increased germination in the most dormant genotype, genetic differences in germination responses to imbibition treatments depended on the maturation-light environment (Fig. 1; Table 3; Supplementary Data Table S4). When matured under a canopy as opposed to white light, differences between *Ler* and the NIL were reduced or completely masked in all imbibition conditions, except when imbibed at 22 °C in the dark or under a canopy; in these cases, maturation under a canopy slightly increased the difference between the two genotypes (Fig. 1D). Similarly, seed maturation under a canopy modified differences in germination between

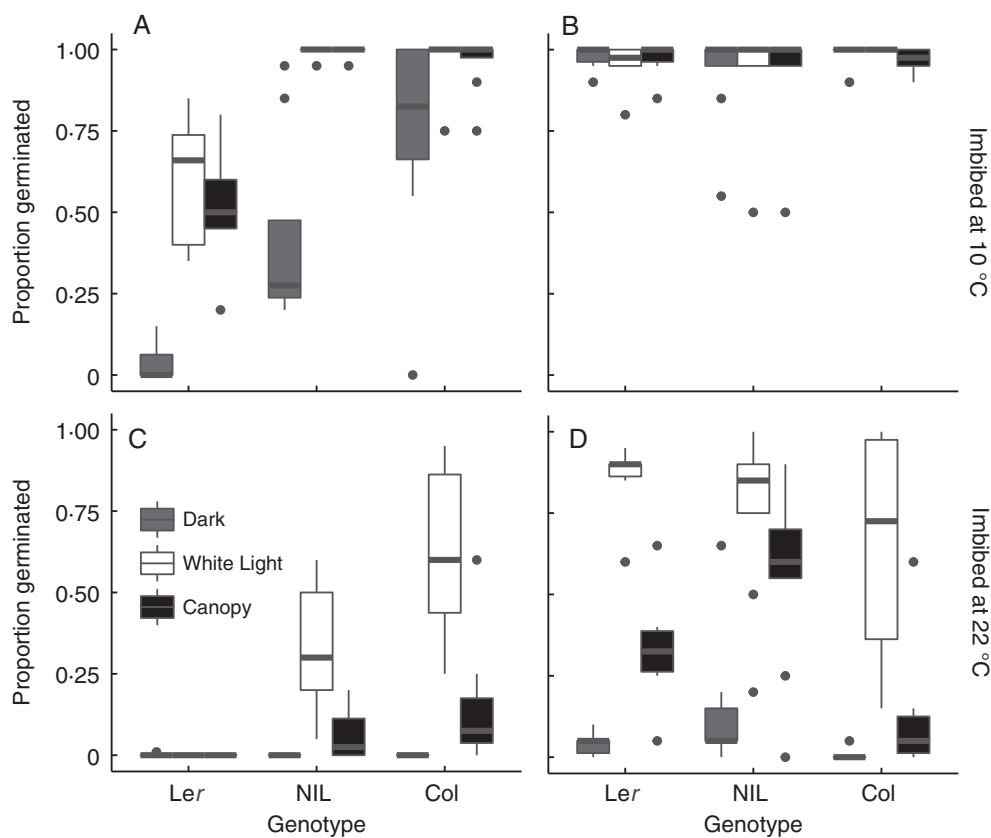


Fig. 1. Germination of fresh seeds of each genotype matured under white light (A, C) or a canopy (B, D) and then imbibed in the dark, white light or canopy at either 10 °C (A, B) or 22 °C (C, D). Black horizontal lines within boxes represent median germination proportion. Box hinges indicate 75th and 25th percentiles. Whiskers span 1.5 times the interquartile range, and black points are observations that fall outside these values.

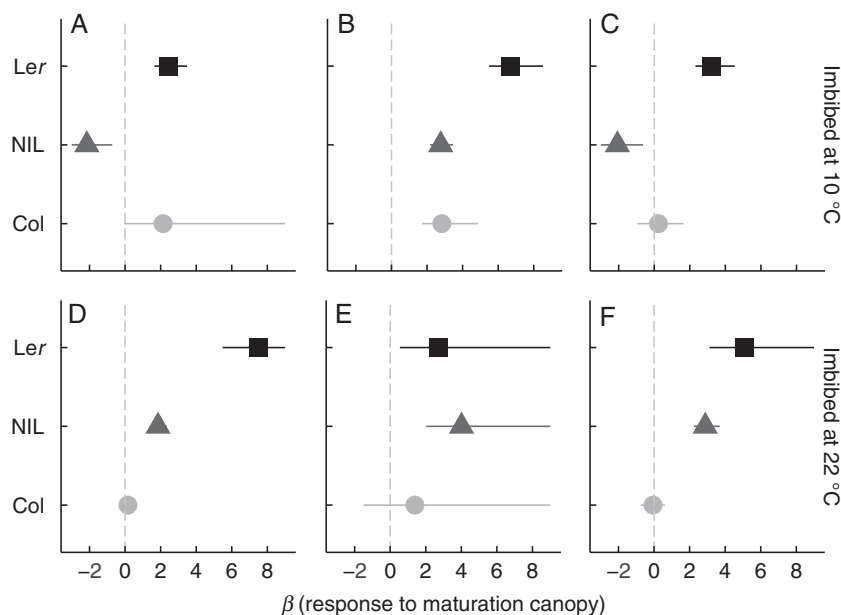


FIG. 2. Direction and strength of the effect of seed maturation under a canopy on germination of fresh seeds imbibed in white light (A, D), darkness (B, E) or canopy (C, F) at either 10 °C (A, B, C) or 22 °C (D, E, F). Colours and shapes indicate the genotype. Each estimate (β) is the change in log odds with 95 % confidence intervals of germination caused by canopy maturation: positive values indicate an increase in germination compared with white light maturation. Confidence intervals crossing zero (vertical grey line) indicate that there was no effect of canopy maturation.

TABLE 1. Analysis of germination proportions of fresh seeds at 10 and 22 °C

Source	d.f.	Imbibition at 10 °C		Imbibition at 22 °C	
		LR χ^2	P-value	LR χ^2	P-value
Genotype	2	123.39	<0.001	52.09	<0.001
Maturation	1	50.09	<0.001	269.30	<0.001
Imbibition	2	153.49	<0.001	618.20	<0.001
Genotype \times Maturation	2	42.39	<0.001	200.20	<0.001
Genotype \times Imbibition	4	23.64	<0.001	23.48	<0.001
Maturation \times Imbibition	2	33.57	<0.001	7.79	0.020
Genotype \times Maturation \times Imbibition	4	10.54	0.032	1.80	0.773

Effects of genotype, seed maturation treatment ('Maturation'), seed imbibition light ('Imbibition') and their interactions on germination proportions, based on logit-linked generalized linear models. Likelihood ratios (LRs) were tested based on χ^2 . Reference levels were white light for maturation, *Ler* for genotype and white light for imbibition light. The residual d.f. in each imbibition temperature = 117. To aid in interpretation of significant effects, see Tables 2 and 3; Tables S2–S4.

Ler and *Col* in nearly all the imbibition conditions, but the effect depended on those conditions. Maturation under a canopy reduced germination differences in these genotypes if seeds were imbibed at 10 °C, but reversed the rank order of the genotypes if seeds were imbibed at 22 °C in white light or under a canopy (Fig. 1; Table S4).

In summary, a vegetative canopy increased germination of fresh seeds if experienced during seed maturation, but decreased or had no effect on germination if experienced during imbibition. The magnitude of these effects depended on the

temperature of imbibition. In particular, when seeds were matured under a canopy, responses to imbibition canopy were only apparent at the temperature that was less conducive to germination (22 °C). Moreover, maturation under a canopy eliminated light requirements for germination when the imbibition temperature was in the range under which germination could proceed (10 °C), but allowed seeds to respond to imbibition light at the temperature which restricted germination (22 °C). The effects of canopy maturation were strongest in the most dormant genotype, which led to changes in the expression of genotypic differences in germination across most of the imbibition conditions we tested.

Maternal canopy effects on germination persist into secondary dormancy

Maturation under a canopy increased the germination of seeds that were induced into secondary dormancy in *Ler* and the NIL (Figs 4 and 5). Although the effect of seed maturation under a canopy did not differ statistically between imbibition temperatures (Maturation \times Temperature in Table 4), it tended to be stronger and to increase germination in more genotypes at 10 °C than at 22 °C.

In two of the genotypes (*Ler* and the NIL), some germination occurred even in dark-imbibed seeds when they had matured under a canopy, indicating that seed maturation under a canopy can weaken light requirements for germination in seeds with secondary dormancy. The effect of seed maturation under a canopy was most pronounced for *Ler* and the NIL (Genotype \times Maturation in Table 4), as no effect was observed in *Col* seeds (Fig. 4).

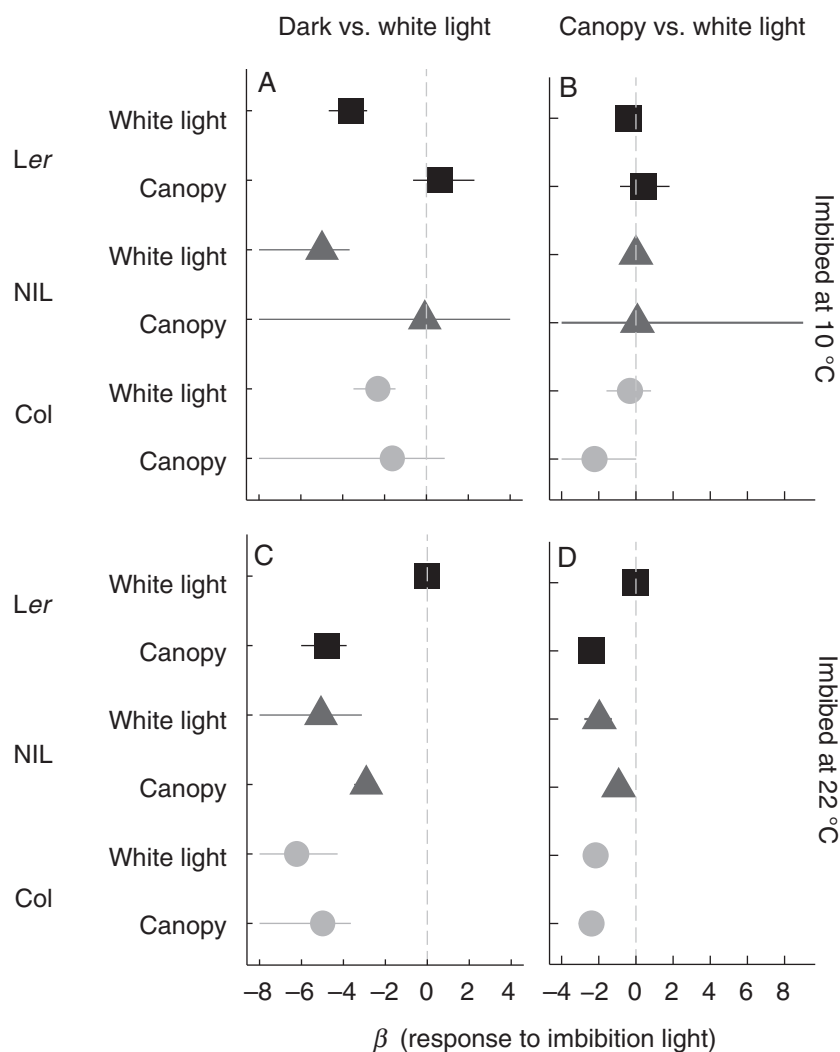


FIG. 3. Direction and strength of the effect of imbibition light on germination of fresh seeds matured under white light or canopy conditions (left axis) and imbibed at either 10 °C (A, B) or 22 °C (C, D). Colours and shapes indicate the genotype. A light requirement for germination was assessed as the difference in germination between dark and white light imbibition (A, C); negative values indicate seeds germinated less in white light. Germination response to an imbibition canopy was assessed as the difference in germination between white light and canopy imbibition (B, D); negative values indicate a decrease in germination compared with white light imbibition. Each estimate (β) is the change in log odds with 95 % confidence intervals of germination caused by dark (A, C) or canopy imbibition (B, D); negative values indicate a decrease in germination compared with white light imbibition. Confidence intervals crossing zero (vertical grey line) indicate that there was no effect of imbibition light.

In summary, the effects of maturation under a canopy persisted as seeds were induced into secondary dormancy, and they weakened light requirements for germination.

Maternal canopy effects do not appear to be mediated by Pfr levels

The comparison of seeds imbibed in the dark vs. the FR treatment showed that Pfr acquired during seed maturation did enhance germination slightly in some treatments, but that its effect did not differ between seed-maturation treatments. Although the FR treatment (white bars in Fig. 4) slightly reduced germination compared with dark imbibition, maturation under a canopy did not modify this effect in either temperature, except in Col (Maturation \times Imbibition in Table 4; Fig. 4;

Supplementary Data Table S5; Fig. S2). Canopy-matured seeds of Col did not respond to FR, but the effect of canopy maturation on this response was extremely small.

The comparison of seeds imbibed in the R treatment vs. the FR treatment indicated that Pfr increases germination, but seed maturation under a canopy did not significantly affect these responses. The R treatment (red bars in Fig. 4) increased germination compared with the FR treatment, indicating that Pfr promotes germination, but was independent of seed-maturation light (Fig. 4; Supplementary Data Fig. S2B, D; Imbibition in Table 4; Maturation \times Imbibition in Supplementary Data Table S6).

To summarize, the canopy during seed maturation influenced the germination of seeds even with secondary dormancy, but the effect of a seed-maturation canopy does not appear to be the consequence of increasing Pfr levels in seeds during maturation. Further, maturation under a canopy did not alter the

TABLE 2. The effect of seed-maturation light on germination responses to imbibition light in fresh seeds of each genotype imbibed at 10 and 22 °C

Genotype	Type of imbibition response	Imbibition at 10 °C			Imbibition at 22 °C		
		LR χ^2	Effect	P-value	LR χ^2	Effect	P-value
<i>Ler</i>	White light vs. dark	27.78	↓	<0.001	–	–	–
	White light vs. canopy	1.40	–	0.237	–	–	–
NIL	White light vs. dark	52.54	↓	<0.001	7.01	↑	0.008
	White light vs. canopy	0.01	–	0.954	6.59	↓	0.020
<i>Col</i>	White light vs. dark	0.35	–	0.557	1.50	–	0.220
	White light vs. canopy	3.58	–	0.118	0.29	–	0.591

We tested whether maturation light modified two types of germination responses to imbibition light (Type of imbibition response): a light requirement for germination (White light vs. dark imbibition light) and germination response to an imbibition canopy (White light vs. canopy imbibition light). Germination proportions were analysed with logit-linked generalized linear models, and likelihood ratio (LR) tests were used to compare full models with reduced models that lacked the interaction between Maturation and Imbibition. Reference levels were white light for maturation and white light for imbibition. For each test, d.f. = 1 with 24 residual d.f. Arrows in the Effect column indicate that a maturation canopy significantly increased or decreased germination responses to imbibition light, as determined by the LR tests. Data separation prevented tests for *Ler* seeds at 22 °C, but these seeds responded to imbibition light treatments only if they were canopy matured (see Table S2).

TABLE 3. The effect of seed-maturation light on genotype differences in germination of fresh seeds, within each combination of imbibition light and temperature

Imbibition light	Imbibition at 10 °C			Imbibition at 22 °C		
	LR χ^2	Effect	P-value	LR χ^2	Effect	P-value
<i>Ler</i> vs. NIL						
Dark	37.93	↓	<0.001	–	–	–
White light	40.41	↓	<0.001	69.90	↓	<0.001
Canopy	49.69	↓	<0.001	6.54	↑	0.011
<i>Ler</i> vs. <i>Col</i>						
Dark	9.73	↓	0.002	–	–	–
White light	0.596	–	0.440	143.78	↓	<0.001
Canopy	11.81	↓	<0.001	47.65	↓	<0.001

Germination proportions were analysed with logit-linked generalized linear models, and likelihood ratio (LR) tests were used to compare full models with reduced models that lacked the interaction between Maturation and Genotype. Reference levels were *Ler* for genotype and white light for maturation. For each test d.f. = 1 with 24 residual d.f. Arrows in the Effect column indicate that a maturation canopy significantly increased or decreased differences between genotypes, based on LR tests. Data separation prevented tests in the dark at 22 °C. However, germination of *Ler* and the NIL differed in these imbibition conditions only if canopy matured (see Table S4).

ability of seeds with secondary dormancy to respond positively to the specific wavelength of light (R) that promotes germination.

DISCUSSION

Reduced germination in response to vegetation or vegetation cues has been documented in many taxa, and is interpreted as a mechanism for avoiding competition (King, 1975; Deregibus *et al.*, 1994; Gutterman, 2000; Pons, 2000; Dechaine *et al.*,

2009). Negative germination responses to a vegetative canopy were generally absent in our study, except in a few cases in which seeds were exposed to a canopy during imbibition (post-dispersal). Surprisingly, we observed strong, positive germination responses to a vegetative canopy in the maturation (pre-dispersal) environment that prevented responses to offspring environments and persisted as seeds were induced into secondary dormancy.

Potential ecological and evolutionary consequences of maternal and offspring canopy effects

A canopy in the maternal environment increased germination in nearly all combinations of imbibition light and temperature, consequently reducing the ability of seeds to respond to cues in their immediate environments. This over-riding effect of the maternal environment seems unlikely to be favourable for seedlings, since offspring environments are likely to be better predictors of performance, and germination cueing is crucial to seedling survival (DeWitt *et al.*, 1998; Donohue *et al.*, 2010; Baskin and Baskin, 2014). One way that this over-riding maternal effect may increase offspring performance, however, is if it predicts competition in later stages of the life cycle, even though dispersed seeds do not yet detect competition. The effects of neighbours on plant performance are known to change over the course of an individual's lifetime (Goldberg *et al.*, 2001; Miriti, 2006; Wright *et al.*, 2014). In such cases, expanding the conditions that permit germination could enable seeds to germinate more quickly after dispersal. Earlier germination could then allow emergence before competitive conditions develop or intensify, and provide an advantage to seedlings over their future competitors (Geber and Griffen, 2003; Mercer *et al.*, 2011; Weis *et al.*, 2015). Further, if neighbours increase seedling fitness, as documented in some studies of *A. thaliana* (Thompson, 1994; Callahan and Pigliucci, 2002; L. D. Leverett and K. Donohue, unpubl. res.), increased germination following canopy maturation could be beneficial for offspring.

An increase in germination following maturation under a canopy could be favoured by selection at the lineage level, even if that maternal effect comes at a cost of reduced offspring fitness (Kirkpatrick and Lande, 1989; Marshall and Uller, 2007; Uller, 2008). Specifically, if maternal plants benefit from increased germination of offspring, for example because of potential costs of inducing dormancy (Cohen, 1966; Baskin and Baskin, 2014), and that benefit outweighs the risk of offspring emerging under competitive or other sub-optimal conditions, the increase in germination following maturation under a canopy we observed would be advantageous. Field studies that manipulate germination time and neighbour presence at seedling and adult stages and that measure fitness at the maternal and offspring levels could test these adaptive hypotheses concerning the observed responses to maternal and offspring canopy.

Because seeds matured under a canopy had greater germination propensities, genotypes with higher dormancy exhibited stronger germination responses to a seed-maturation canopy. The difference between *Ler* and the NIL in dormancy and in turn the effect of a maturation canopy could be due to *FLOWERING LOCUS C (FLC)*, which is located in the introgressed chromosomal segment in the NIL. Previous work on

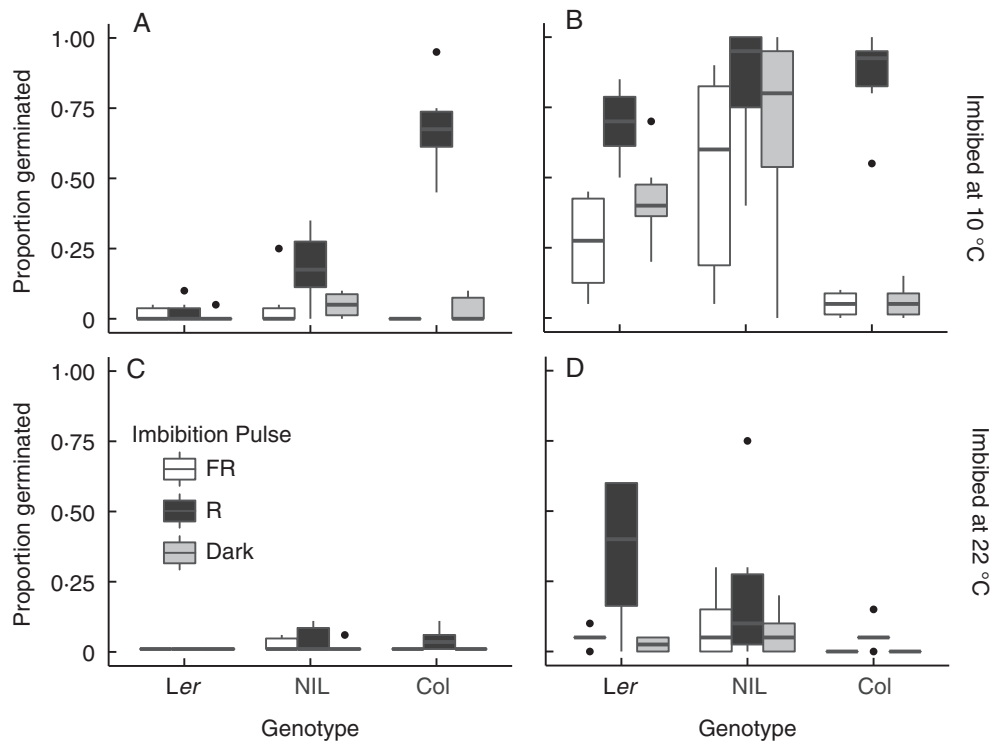


FIG. 4. Germination of seeds following after-ripening and hot stratification to induce secondary dormancy. Seeds were matured under white light (A, C) or a canopy (B, D) then imbibed at either 10 °C (A, B) or 22 °C (C, D). Box colours indicate whether seeds were imbibed directly into the dark, pre-treated with an FR pulse prior to imbibition in the dark or pre-treated with FR and R pulses prior to imbibition in the dark. Black horizontal lines within boxes represent median germination proportion. Box hinges indicate 75th and 25th percentiles. Whiskers span 1.5 times the interquartile range, and black points are observations that fall outside these values.

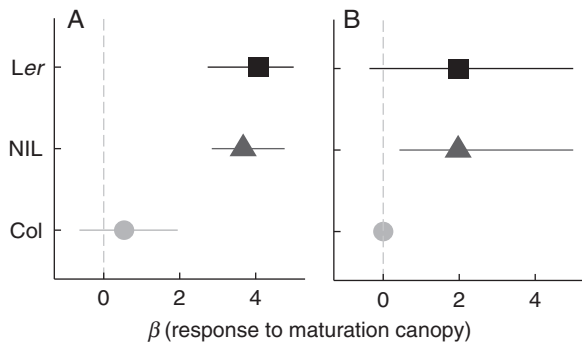


FIG. 5. Direction and strength of the effect of seed maturation under a canopy on germination in the dark at 10 °C (A) or 22 °C (B) following after-ripening and hot stratification to induce dormancy. Colours and shapes indicate the genotype. Each estimate (β) is the change in log odds with associated 95 % confidence intervals of germination caused by canopy maturation: positive values indicate that canopy maturation increases germination compared with white light maturation. Confidence intervals crossing zero (vertical grey line) indicate that there was no effect of canopy maturation.

Ler and the NIL demonstrated that *FLC* promotes germination in white light (Chiang *et al.*, 2009). Increased *FLC* expression may also have led to the weaker maturation-canopy effect that we observed in the NIL by reducing dormancy. However, because *Ler* and the NIL may differ at other causative loci within the introgressed region, we cannot directly implicate *FLC*.

Regardless, our results do imply that allelic variation in dormancy is associated with differences in the effects of a canopy during maturation, and that the expression of allelic variation in germination can be reduced by maturation under a canopy.

By reducing germination cueing to post-dispersal environments as well as differences among genotypes in such cueing, seed maturation under a canopy could alter the action and outcome of natural selection on germination. First, increased germination in response to vegetation in the maternal environment may lead to a more rapid depletion of below-ground populations. Given the importance of seed banks as temporal sources of gene flow in *A. thaliana* (Falahati-Anbaran *et al.*, 2014), changes in the size of below-ground populations could influence effective population size and in turn the efficacy of natural selection. Secondly, the reduction or complete masking of differences in germination cueing among genotypes by a maturation canopy could increase the synchrony of germination among genotypes and preclude responses to selection on germination (Falconer and Mackay, 1996).

Germination responses to canopies in the maternal and offspring environments, and in turn the ecological and evolutionary consequences of these environmental effects, are likely to vary among dispersal cohorts. Reduced germination in response to a canopy in the seed environment was only observed in the warmer imbibition temperature (22 °C). Thus, any competitive advantage of avoiding germination under neighbouring vegetation may only be realized in cohorts that are dispersed into

TABLE 4. Effects of genotype, maturation light and temperature on germination in the dark, germination responses to a Pfr-reducing treatment (interactions with 'Imbibition Pulse') and germination responses to a Pfr-increasing treatment (interactions with 'Imbibition Pulse') for seeds that had been hot stratified to induce secondary dormancy

Source	Dark imbibition			Dark vs. FR treatment			R vs. FR treatment		
	LR χ^2	d.f.	<i>P</i> -value	LR χ^2	d.f.	<i>P</i> -value	LR χ^2	d.f.	<i>P</i> -value
Genotype	114.39	2	<0.001	218.94	2	<0.001	21.63	2	<0.001
Maturation	188.35	1	<0.001	325.40	1	<0.001	375.65	1	<0.001
Imbibition Pulse				5.78	1	0.016	352.48	1	<0.001
Temperature	167.02	1	<0.001	240.09	1	<0.001	458.03	1	<0.001
Genotype × Maturation	15.15	2	<0.001	9.96	2	0.007	45.90	2	<0.001
Genotype × Imbibition Pulse				0.04	2	0.978	87.72	2	<0.001
Maturation × Imbibition Pulse				0.17	1	0.682	0.80	1	0.798
Genotype × Temperature	0.25	2	0.885	1.71	2	0.426	51.95	2	<0.001
Maturation × Temperature	0.66	1	0.416	1.69	1	0.194	1.71	1	0.191
Imbibition Pulse × Temperature				6.07	1	0.014	0.02	1	0.883
Genotype × Maturation × Imbibition Pulse				4.55	2	0.103	4.00	2	0.136
Genotype × Maturation × Temperature	1.56	2	0.459	0.67	2	0.714	2.47	2	0.291
Genotype × Imbibition Pulse × Temperature				0.98	2	0.611	0.89	2	0.643
Maturation × Imbibition Pulse × Temperature				0.37	1	0.542	0.30	1	0.587

The four-way interaction did not improve the model (LR $\chi^2 = 3.23$, d.f. = 4, $P = 0.521$) and was dropped to increase power. Germination proportions were analysed with logit-linked generalized linear models, and likelihood ratios (LRs) were tested based on χ^2 . Residual d.f. = 59 for Dark imbibition and 122 for Dark vs. FR treatment and Dark vs. FR treatment. Reference levels were white light for maturation, Ler for genotype and 10 °C for temperature. Because the effects of Imbibition Pulse are not tested in Dark imbibition, the rows for associated terms are left blank. For models that included the effects of imbibition pulses, reference levels were dark (dark vs. FR) or FR (R vs. FR). To aid in interpretation of significant effects, see Tables S5 and S6.

warm conditions, such as those associated with late spring or early autumn. Further, because a canopy in the maternal environment did not mask genotypic differences at the warmer imbibition temperature, cohorts that are dispersed in late spring or early autumn may have a greater capacity to respond to natural selection.

Mechanisms of canopy maturation effects on germination

Germination in *A. thaliana* is known to respond to the ratio of R to FR light during seed maturation. Seeds matured in light enriched in R have been shown to germinate more in the dark than those matured in light enriched in FR (McCullough and Shropshire, 1970; Hayes and Klein, 1974). Similarly, seeds matured under reduced R:FR had stronger light requirements than those matured under higher R:FR, although this effect varied among accessions (Dechaine *et al.*, 2009). The results of these studies are in contrast to our finding that maturation under a canopy increased germination, even in the dark. However, our maturation conditions differed from these other studies in that we manipulated not only wavelength, but also irradiance. Additionally, seeds in the other studies were stored or otherwise treated to release dormancy prior to germination, whereas our seeds had some dormancy. Thus, the disparity between this and previous studies may be due to differences in dormancy and total irradiance.

The canopy effects we observed do not appear to be mediated by phytochromes. Phytochromes were demonstrated to regulate germination responses to R:FR during maturation in the *Ler* background (Dechaine *et al.*, 2009). However, in that study, germination was tested only under neutral shade, so it is unclear whether phytochromes mediated germination through light requirements in seeds. In our study, seed maturation under

a canopy did not alter germination responses to FR light in seeds that had been induced into secondary dormancy through hot stratification; thus, Pfr levels set during maturation cannot fully explain the maternal effects we observed in these seeds. However, it is possible that Pfr reverted to Pr after 2 d in the dark at 35 °C while seeds underwent hot stratification (Heschel *et al.*, 2007; Casal, 2013). If so, we would not expect differences between dark and FR treatments. Although exposing hot-stratified seeds to a pulse of R light increased germination, this did not depend on maturation treatment. Thus, Pfr set during seed maturation and photoconversion by light during imbibition do not appear to mediate the effects of a canopy on germination that we observed.

The effects of a maturation canopy observed here are probably due to light properties other than R and FR. Our experimental design reduces both R:FR and irradiance, as in natural vegetative canopies. Total irradiance under the canopy was lower than under white light, and low irradiance during seed maturation has been shown to increase germination in other species (Schmitt *et al.*, 1992; Galloway, 2001). Further, reduced irradiance combined with a short photoperiod may have simulated autumn conditions, in which case the increased germination of seeds matured under a canopy could be related to seasonal cueing. Our green filter also reduced transmission of blue and UV light, which could have been detected by cryptochromes and UVA or UVB photoreceptors, respectively. In barley, blue light increases dormancy (Gubler *et al.*, 2008), an effect that is consistent with our observation that maturation under a canopy (with reduced blue light) reduced dormancy in fresh seeds.

Finally, the strong maternal effect we observed could be due to limited light during seed development. In *Polygonum persicaria* (Polygonaceae), seeds matured under severe light limitation

(8 % of total light) had thinner seed coats and greater germination than those matured under full light (Sultan, 1996). In our study, canopy-matured seeds experienced 50 % less PAR, which may have reduced resources available during seed development and maturation. If so, the thinner coats of canopy-matured seeds could increase the risk of mortality, in turn lowering the potential longevity of seeds in the seed bank (reviewed in Long et al., 2015). Future experiments could manipulate different wavelengths of light as well as total irradiance to determine the exact mechanisms of canopy effects that we detected.

CONCLUSION

A maturation canopy increased germination, but an imbibition canopy either had no effect or reduced germination. This stronger effect of the maternal environment prevented offspring from responding to temperature and light cues in their immediate environments. Further, the effects of a maturation canopy on germination persisted as seeds were induced into secondary dormancy. Such trans-generational effects could influence germination behaviour, maternal and offspring fitness, and patterns of natural selection in annual plants.

SUPPLEMENTARY DATA

Supplementary data are available online at www.oxfordjournals.org and consist of the following. Table S1: analysis of germination proportions of fresh seeds. Effects of genotype, maturation treatment, imbibition light and temperature, and their interactions on germination proportions, based on logit-linked generalized linear models. Table S2: effect of seed-maturation light on germination proportions of fresh seeds of each genotype in each combination of imbibition light and temperature. Table S3: effect of light treatments during imbibition on germination proportions of fresh seeds, in each combination of seed-maturation light and imbibition temperature. Table S4: genotype differences in germination proportions of fresh seeds in each combination of maturation light, imbibition light and temperature. Table S5: effect of seed-maturation light on Pfr-mediated germination of after-ripened and hot-stratified seeds at each imbibition temperature. Table S6: effect of seed-maturation light on enhancement of germination of after-ripened and hot-stratified seeds by *de novo* Pfr in each imbibition temperature. Figure S1: overview of the experimental design for this study. Figure S2: direction and strength of the effect of imbibition pulse treatment on germination of after-ripened, hot-stratified seeds matured under white light or canopy conditions and imbibed at either 10 or 22 °C.

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