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Phytochrome mediates germination responses to multiple seasonal cues

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ABSTRACT

We identified a new role of phytochrome in mediating germination responses to seasonal cues and thereby identified for the first time a gene involved in maternal environmental effects on germination. We examined the germination responses of a mutant, *hy2-1*, which is deficient in the phytochrome chromophore. The background genotype, Landsberg *erecta* (Ler), lacked dormancy in most treatments, while *hy2-1* required cold stratification for germination in a manner that resembled a more dormant ecotype, Columbia (Col). Unlike Col, *hy2-1* was not induced into dormancy by warm stratification. Therefore, the down-regulation of phytochrome-mediated germination pathways results in sensitivity to cold, but we found no evidence that reduced phytochrome activity enables the warm-induction of dormancy. Cool temperatures during seed maturation induced dormancy. The *hy2-1* mutants did not overcome this dormancy, indicating that phytochrome-mediated pathways are required to break cold-induced dormancy. Ler did not respond to post-stratification temperature, but *hy2-1* did respond, suggesting phytochrome pathways are involved in germination responses to temperature. In summary, phytochromes mediate dormancy and germination responses to seasonal cues experienced both during seed maturation and after dispersal. Phytochromes therefore appear to be involved in mediating seasonal germination timing, a trait of great ecological importance and one that is under strong natural selection.

Key-words: dormancy; life history; maternal effects; phenology; phenotypic plasticity.

INTRODUCTION

Seeds need to sense seasonal environmental conditions to ensure germination at an appropriate time of year. The seasonal timing of germination is a major determinant of total lifetime fitness in plants (e.g. Biere 1991; Gross & Smith 1991; Simons & Johnston 2000), and it has explained up to 72% of the variance in fitness among genotypes in an experimental population of *Arabidopsis thaliana* (Donohue *et al*. 2005b). The timing of germination depends on environmental conditions experienced both during seed maturation and after dispersal, and the conditions during seed maturation frequently alter germination responses to post-dispersal environmental factors (Karssen 1970; Junttila 1973; Gutterman 1992; Baskin & Baskin 1998; Munir *et al*. 2001). Seasonal cues experienced before and after dispersal can be accurate predictors of time of year, and appropriate responses to these cues result in adaptive germination phenology (Vleeshouwers, Bouwmeester & Karssen 1995; Baskin & Baskin 1998).

In the field, the seasonal conditions of seed maturation and dispersal can vary greatly even within a species. In many spring-flowering species, the duration of seed maturation can span a range of temperature and photoperiod. Many winter annuals begin flowering quite early in the spring and continue maturing seeds through early summer. Some species vary even more extremely in flowering season. For example, some populations of *A. thaliana* flower and set fruit in both autumn and spring (Thompson 1994; Griffith, Kim & Donohue 2004), such that seasonal conditions during seed maturation and the season experienced immediately after dispersal (winter versus summer) are very different. The seasonal timing of seed maturation and dispersal can in turn determine the season of seed germination and overall life history. For example, in *Campanula americana* (Galloway 2001, 2002), seeds from early flowering plants tended to germinate in the autumn instead of spring and subsequently expressed an annual instead of biennial life history. Thus, seasonal seed maturation conditions and consequent germination phenology can strongly influence the basic life history of plants.

Germination phenology depends on two processes: dormancy breakage and germination after dormancy is broken. A seed is said to be dormant if it does not germinate under conditions that would otherwise permit germination – that is, had the seed first experienced a dormancy-breaking treatment (Simpson 1990). Dormancy can be broken by some environmental stimuli, such as a cold treatment during imbibition, or seeds can gradually lose dormancy over a period of dry afterripening before imbibition (Baskin & Baskin 1998). Dry afterripening can be especially important for plants that experience a dry season shortly after seed dispersal. Seeds dispersed in a non-dormant state may germinate immediately after dispersal,

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but if environmental conditions are not permissive, a non-dormant seed will be unable to germinate. Thus, germination timing depends on, firstly, the timing of dormancy breakage and secondly, the arrival of permissive conditions for germination.

Numerous genetic and physiological studies have identified pathways required for or involved with dormancy maintenance and germination. Germination proceeds as a consequence of embryonic growth and degradation of the seed coat – processes that are regulated by multiple hormonal and metabolic pathways (Koornneef & Karssen 1994; Bewley 1997; Debeaujon & Koornneef 2000; Debeaujon, Leon-Kloosterziel & Koornneef 2000; Foley 2001). For example, it is well documented that abscisic acid (ABA) is required to maintain dormancy after imbibition, and that active gibberellin (GA) overcomes ABA-induced dormancy (Karssen *et al*. 1983, 1989; Koornneef & Karssen 1994; Leon-Kloosterziel *et al*. 1996). The manner in which these processes vary with environmental conditions regulates the seasonal conditions under which germination will occur. Despite this knowledge of basic pathways required for dormancy and germination, the genetic basis of germination responses to seasonal environmental factors is as yet largely unknown – especially seasonal factors experienced during seed maturation.

Among the most important environmental sensors in plants are the phytochromes, which regulate germination (and several other) responses to light (Reed *et al*. 1994; Shinomura *et al*. 1994; Poppe & Schäfer 1997; Shinomura 1997; Whitelam & Devlin 1997; Casal & Sanchez 1998). Phytochromes are photoreversible biliproteins that are synthesized in the red-absorbing (Pr) isomer and, upon absorbing red light, are converted to the far-red absorbing (Pfr) isomer. Pfr is thought to be the bioactive isomer that mediates light-induced responses, such as germination, although some evidence suggests that the Pr-Pfr heterodimers or short-term intermediates may also have bioactivity (Shinomura, Uchida & Furuya 2000). Active phytochrome regulates the synthesis of GA, which promotes germination (Hilhorst, Smitt & Karssen 1986; Karssen & Lacka 1986; Hilhorst & Karssen 1988; Yang *et al*. 1995; Ritchie & Gilroy 1998; Toyomasu *et al*. 1998; Yamaguchi *et al*. 1998, 2004; Yamaguchi & Kamiya 2000; Garcia-Martinez & Gil 2002; Ogawa *et al*. 2003). While the role of phytochromes in sensing light conditions is well established, it is relatively unknown how phytochromes respond to temperature. Phytochromes do exhibit temperature-dependent activities during the regulation of flowering time in *A. thaliana* (Halliday & Whitelam 2003; Halliday *et al*. 2003; Franklin & Whitelam 2004). Because temperature has been shown to influence endogenous GA concentration in seeds (Derkx, Vermeer & Karssen 1994; Yamaguchi *et al*. 2004), seeds' sensitivity to GA (Derkx & Karssen 1993; Yamaguchi *et al*. 2004), rates of dark reversion of Pfr to Pr (Kristie & Fielding 1994; Hennig & Schäfer 2001) and possibly the rate of phytochrome photoconversion (Pons 1986), phytochromes may also have temperature-dependent effects on germination. We tested the possible role of phytochrome in regulating germination responses to temperature experienced in both the light and dark by comparing germination of a wild-type genotype of *A. thaliana* to a chromophore-deficient and thereby phytochrome-dysfunctional mutant.

In *A. thaliana*, the genes *PHYA-PHYE* encode five different phytochrome apoproteins (Clack, Mathews & Sharrock 1994; Mathews & Sharrock 1997). *PHYA* and *PHYB* have well-characterized roles in germination responses to red and far-red light at different fluence rates (Shinomura *et al*. 1994; Poppe & Schäfer 1997; Shinomura 1997). More recently, *PHYE* has been shown to be involved in light responses to germination as well (Hennig *et al*. 2002). As a first step in testing the role of phytochrome in regulating germination responses to the seasonal cues of photoperiod and temperature, we used a mutant that is deficient in all active phytochromes. The *hy2-1* mutant lacks a functional chromophore that is common to all five phytochromes, and thus the activity of all five phytochromes is impaired (Koornneef, Rolff & Spruit 1980; Parks & Quail 1991; Terry 1997). This study demonstrates that phytochrome is involved in germination responses to seasonal cues of light and temperature that are experienced both during seed maturation and after dispersal.

METHODS

We conducted two experiments to compare germination responses of the *hy2-1* mutant of *A. thaliana* (Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA, stock number CS68) and the background ecotype, Landsberg *erecta* (Ler hereafter; stock number CS20). The Ler genotype is non-dormant under many conditions most likely in part because of selection for convenience of culturing over many generations in the lab, although it is possible that it was non-dormant before extensive culturing. Most physiological mutants of *A. thaliana* are derived from this background. Loss-of-function mutants that restore dormancy under conditions in which Ler is non-dormant can identify pathways that are likely to be down-regulated during dormancy induction or maintenance. In addition, when Ler does exhibit dormancy, loss-of-function mutants that do not break dormancy can identify pathways whose expression is necessary to overcome dormancy. Thus, these comparisons can identify pathways that are both down-regulated during dormancy and up-regulated during dormancy breakage and/or germination.

In both experiments, seeds were matured under three treatments in Conviron E7/2 growth chambers (Controlled Environment Ltd., Winnipeg, Manitoba, Canada). The first treatment, 'long-day', imposed a 14 h light/10 h dark cycle of full-spectrum light at 22 °C, and resembles seasonal conditions during a typical seed-maturation season in late spring in temperate climates. The second treatment, 'short-day', imposed a 10 h light/14 h dark cycle at 22 °C. The third treatment, 'short-cold', imposed a 10 h light/14 h dark cycle at 10 °C, and resembles the seasonal conditions during seed maturation when plants mature seeds in the autumn or

very early spring. Comparing the long-day to short-day treatment reveals the effect of maternal photoperiod during seed maturation. Comparing the short-day to short-cold treatment reveals the effect of maternal temperature. Plants were grown in four temporal blocks, with two chamber compartments per block and two replicates of each genotype 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 for each block, and seeds were pooled over both compartments in each block for germination assays.

Experiment 1: effect of temperature during dark stratification

The first experiment compared germination responses to different seed stratification treatments in the dark. We used seeds of the *hy2-1* mutant, Ler and the ecotype, Columbia (Col hereafter; stock number 3176). We used the Col ecotype to determine responses to our treatments that were more likely to resemble the responses of more somewhat dormant natural ecotypes than those of Ler. We conducted the germination assays on 'fresh' seeds (seeds that were harvested 7 to 10 d previously) and on seeds that had been dry afterripened at 22 °C for 3 months. Germination assays were conducted using 12 seeds of a given genotype in a single Petri plate (50 × 9 mm) containing 0.5% agar dissolved in distilled water. Twelve Petri plates, distributed over four temporal blocks, were used for each genotype in each maternal and stratification treatment (144 seeds per genotype per treatment). Four dark, wet stratification treatments were imposed after the seeds were placed on the agar: 'neutral', which consisted of 5 d at 22 °C; 'cold', which consisted of 5 d at 4 °C; 'warm', 7 d at 31 °C; 'warm-cold' 7 d at 31 °C followed by 5 d at 4 °C. In the last three blocks of the warm-cold treatment, the plates were given a warm stratification, then transferred to light at 22 °C for 3 d, after which time all germinants were removed. The seeds were counted, and the plates with only dormant seeds were then placed into a cold stratification treatment. After their period of stratification, all plates were placed at 22 °C in a 12 h photoperiod of white light in a single Percival germination incubator (Percival Scientific, Inc., Perry, IA, USA) and were randomized across treatments. Treatments were staggered so that all plates would be transferred to the light simultaneously. Preliminary studies of Ler and other ecotypes showed that seeds tended to germinate to high frequencies after five or fewer days in the cold, while 7 d in the warm induced dormancy in natural ecotypes (unpublished data). Therefore, the neutral treatment assessed primary dormancy, the cold treatment assessed the ability of cold to overcome primary dormancy, the warm treatment assessed the ability of warm to induce dormancy, and the warm-cold treatment assessed the ability of cold to break primary plus warm-induced dormancy. We estimated germination proportion as the total number of germinants after 10 d in the light at 22 °C, divided by the total number of viable seeds. Seed viability was assessed by testing firmness to touch (Baskin & Baskin 1998). For the first block of the warm-cold treatment, we corrected germination proportions to be comparable to the other three blocks, in which non-dormant seeds were removed before placing into the cold, by subtracting from each plate the mean number of germinants of the genotype in the warm treatment from the total number of germinants in that plate, and dividing by the total number of viable seeds minus the mean number of germinants in the warm treatment. That gives the estimated number of germinants derived from dormant seeds (after warm stratification), divided by the number of seeds that were dormant before being placed in the cold. Thus, all four blocks had comparable measurements of dormancy breakage after warm stratification.

Experiment 2: effect of temperature in the light

The second experiment compared germination of cold-stratified and non-cold-stratified seeds at two temperatures: 10 and 22 °C. Twelve to 14 (depending on block) seeds of *hy2-1* or Ler, from a given maternal treatment, were placed on agar in Petri plates, as described earlier. In the first germination treatment, '10 to 10 °C', seeds on Petri plates were placed in the dark at 10 °C for 5 d and then transferred to 10 °C in the light (12 h photoperiod, full-spectrum light). In the '4 to 10 °C' treatment, the seeds were placed in the dark at 4 °C for 5 d and then transferred to 10 °C in the light. In the '4 to 22 °C' treatment (the same as the cold treatment in experiment 1), the seeds were placed in the dark at 4 °C for 5 d and then transferred to 22 °C in the light. In the '22 to 22 °C' treatment (the same as the neutral treatment in experiment 1), the seeds were placed in the dark at 22 °C for 5 d and then transferred to 22 °C in the light. These assays were conducted on fresh seeds, seeds dry-afterripened for 4 months and seeds dry-afterripened for 12 months. Fresh seeds and seeds afterripened for 4 months had 15 replicate plates (188 total seeds distributed over three blocks and 182 total seeds distributed over two blocks, respectively) per treatment per genotype. Seeds afterripened for 12 months had six replicates (84 seeds in one block) per treatment per genotype. Germination proportions and seed viability were calculated as described earlier.

Analysis

To test for significant differences in responses to experimental treatments among the different genotypes, we conducted analyses of variance. A significant treatment by genotype interaction indicates that the genotypes differ in response to treatments, and in the case of Ler versus *hy2-1*, indicates that the loss of functional chromophore caused a significant alteration of germination response to the experimental treatment. We tested for significant differences in response to seed maturation conditions (maternal conditions) in each seed treatment separately, and we tested for significant differences in response to seed treatment in each maternal treatment separately. We also tested for significant

differences in response to afterripening within each combination of maternal and seed treatments. Because the data were not normally distributed, probability values from these analyses are only approximate. We therefore conducted a series of non-parametric Kruskal–Wallis tests. Within each treatment, we tested for significant differences between genotypes. Next, we tested for significant differences between maternal treatments for each genotype in each stratification and afterripening treatment, and we then tested for significant differences between seed treatments for each genotype in each maternal and afterripening treatment. Finally, we tested for significant effects of afterripening for each genotype in each combination of maternal and seed treatments.

RESULTS

Effect of temperature during dark stratification

The *hy2-1* and Ler seeds differed significantly in their responses to seed stratification treatment ($P < 0.05$ for all except afterripened seeds matured under short-cold conditions) and seed maturation conditions on the maternal plant ($P < 0.001$ except for afterripened seeds given a warm-cold stratification; Fig. 1). Ler exhibited a lack of dormancy under all conditions when seeds were matured at 22 °C. While Ler exhibited little response to maternal photoperiod (marginally significant greater germination of seeds matured under short days than long days in the warm stratification treatment in fresh seeds; $\chi^2 = 5.96$, $P = 0.015$), maternal temperature significantly altered its germination, with cool temperatures during seed maturation inducing dormancy ($P < 0.005$ for all treatments except the warm-cold stratification treatment in fresh seeds). This dormancy was broken only by stratification in warm followed by cold in fresh seeds. Afterripened seeds that matured at 10 °C did not respond to this temperature cycle and remained dormant (effect of afterripening: $\chi^2 = 8.63$, $P = 0.003$). Three months of afterripening did not influence the germination of Ler in any other treatment ($P > 0.05$) because even fresh Ler seeds were not dormant.

In contrast, *hy2-1* seeds exhibited pronounced dormancy (neutral and warm stratification treatments), even when matured at 22 °C. Dormancy was broken by cold stratification (cold and warm-cold stratification treatments). Like Ler, *hy2-1* was induced into dormancy by cool temperatures during seed maturation ($P < 0.01$ for all treatments). This effect was especially pronounced in seeds that had been given a cold or warm-cold stratification; seeds matured under warmer temperatures lost dormancy after cold stratification whereas seeds matured under cool temperatures did not. Unlike Ler, the dormancy induced by cool maturation temperature was not broken by a stratification cycle of warm followed by cold. Despite being more dormant than Ler, *hy2-1* did not lose dormancy with 3 months of afterripening ($P > 0.05$ in all treatments, with a marginally significant effect of afterripening in the short-cold maternal treatment with warm-cold stratification; $\chi^2 = 3.75$, $P = 0.053$). Like Ler, *hy2-1* seeds did not respond to maternal photoperiod.

In general, the Col ecotype exhibited germination that was between that of Ler and *hy2-1* (Fig. 1). Like *hy2-1* instead of Ler, fresh seeds required cold for maximal germination. As in *hy2-1*, cold stratification also increased germination of afterripened seeds matured at 22 °C. Like both Ler and *hy2-1*, Col was induced into dormancy by cool temperatures during seed maturation ($P \leq 0.02$ for all treatments in both fresh and afterripened seeds). In fresh seeds, both a cold and a warm-cold stratification treatment overcame this cold-induced dormancy. In afterripened seeds, only the warm-cold stratification significantly overcame dormancy. Fresh seeds of Col alone were induced into dormancy by warm stratification when they were matured at 22 °C. Afterripened seeds were also induced into dormancy by warm stratification when they were matured under short days at either temperature, but not under long days. Despite the photoperiod-dependent effect of stratification, the photoperiod effect itself was not significant in any treatment. Cold overcame the warm-induced dormancy in fresh seeds (warm versus warm-cold: $P < 0.05$ for all maturation treatments) and in afterripened seeds that were matured under short days, whereas long-day seeds were already mostly non-dormant. Col seeds did not lose dormancy with afterripening except when seeds were matured under long days and given a warm stratification ($\chi^2 = 15.07$, $P < 0.001$) or when seeds were matured under short days and given a neutral stratification treatment ($\chi^2 = 5.75$, $P = 0.017$).

Effect of temperature in the light

Ler seeds were not as responsive to post-stratification temperature as *hy2-1* (Fig. 2; $P < 0.01$ for genotype–seed treatment interactions for all except fresh and 4-month-old afterripened seeds matured in cool conditions). Cold-stratified (at 4 °C) Ler seeds had higher germination at 22 °C than 10 °C in only two treatments: when matured under short days at 22 °C and afterripened for 4 months, and when matured under short days at 10 °C and afterripened for 12 months. Cold stratification (4 °C) sometimes actually inhibited germination of afterripened seeds compared to non-cold-stratified seeds (at 10 °C: in the long-day and short-day, 4-month-old afterripened treatments; and in the short-cold, 12-month-old afterripened treatment. At 22 °C: short-cold, 4-month-old afterripened treatment). Ler seeds that did not receive cold stratification germinated just as well at 10 °C as at 22 °C.

The *hy2-1* seeds that were matured at 22 °C were much more responsive to cold stratification than Ler seeds when transferred to light at 22 °C, as in experiment 1 (Fig. 2). When transferred to light at 10 °C, cold stratification promoted germination only in 12-month-old afterripened seeds matured under long days, and cold stratification actually inhibited germination of seeds that were matured under short days and afterripened for 4 months.

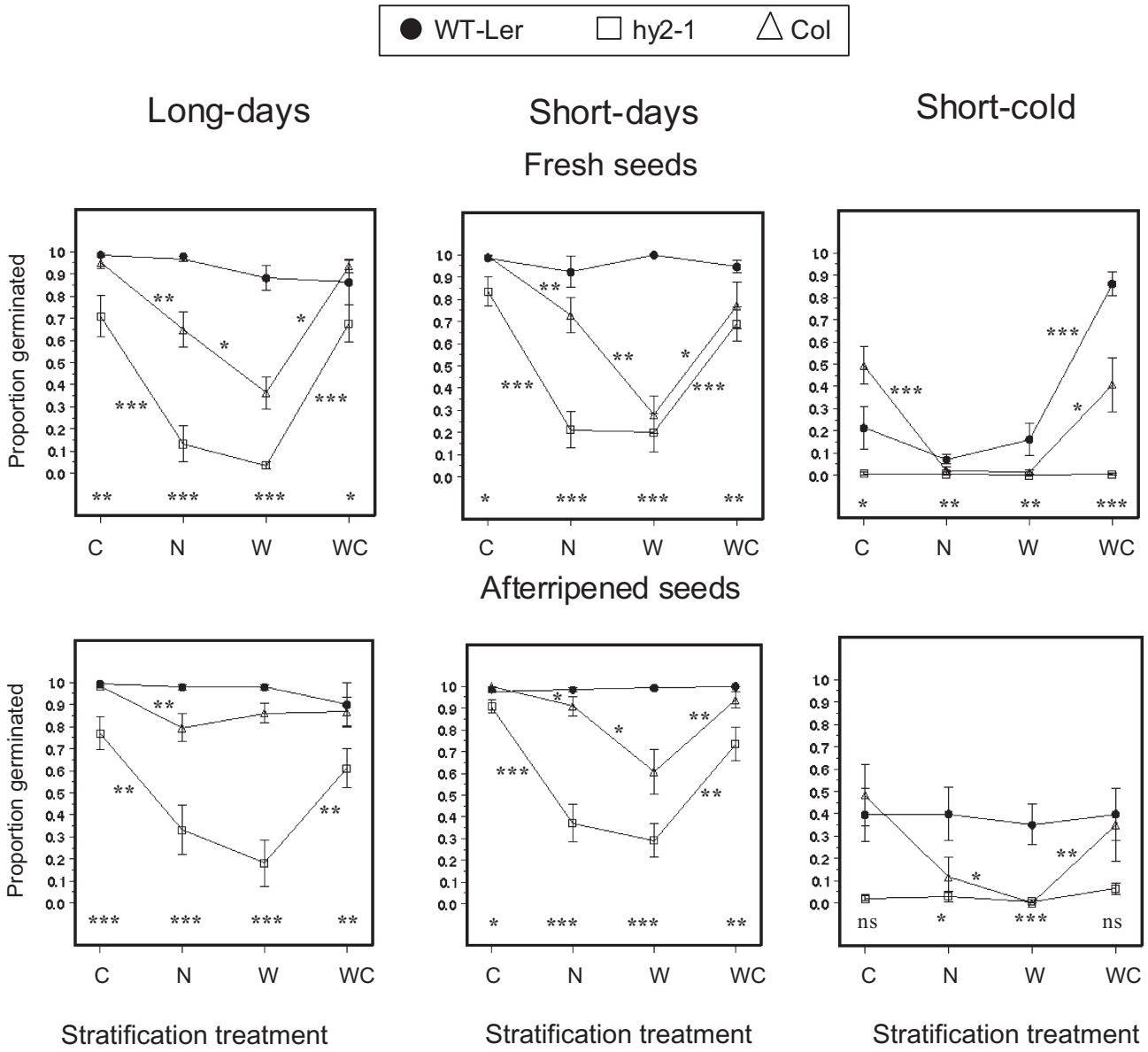

Figure 1. Means and SEs of germination proportions in different seed maturation and stratification treatments. Significant differences between adjacent stratification treatments are indicated for each genotype above [Landsberg *erecta* (Ler) and Columbia (Col)] or below (*hy2-1*) the line connecting the means in two stratification treatments. Results are shown separately for seeds matured in each of three treatments (long-days, short-days and short-cold), and for each afterripening duration (fresh and 3 months of dry afterripening). Significance levels for differences between the *hy2-1* mutant and background line, Ler, are indicated at the bottom of the graph for each stratification treatment in each panel. ns, not significant; C, cold; N, neutral; W, warm; WC, warm-cold; WT, wild type; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

The *hy2-1* seeds frequently responded to post-stratification temperature, unlike seeds of Ler (Fig. 2). In seeds matured at 22 °C, non-cold-stratified seeds germinated to higher percentages when transferred to light at 10 °C than at 22 °C, and cold-stratified seeds germinated to higher percentages when transferred to light at 22 °C than at 10 °C. Strongly afterripened seeds (for 12 months) that were cold-stratified germinated just as well at both temperatures. As in experiment 1, *hy2-1* seeds were not able to overcome dormancy induced by cold temperatures during seed maturation in any treatment.

The *hy2-1* seeds were also much more responsive to maternal photoperiod than Ler seeds (Fig. 2, Table 1). Afterripened *hy2-1* seeds exhibited a fairly consistent effect of maternal photoperiod, with seeds matured under short days germinating to higher percentages than seeds matured under long days, but fresh seeds in the '10 to 10 °C' treatment germinated to higher percentages if they were

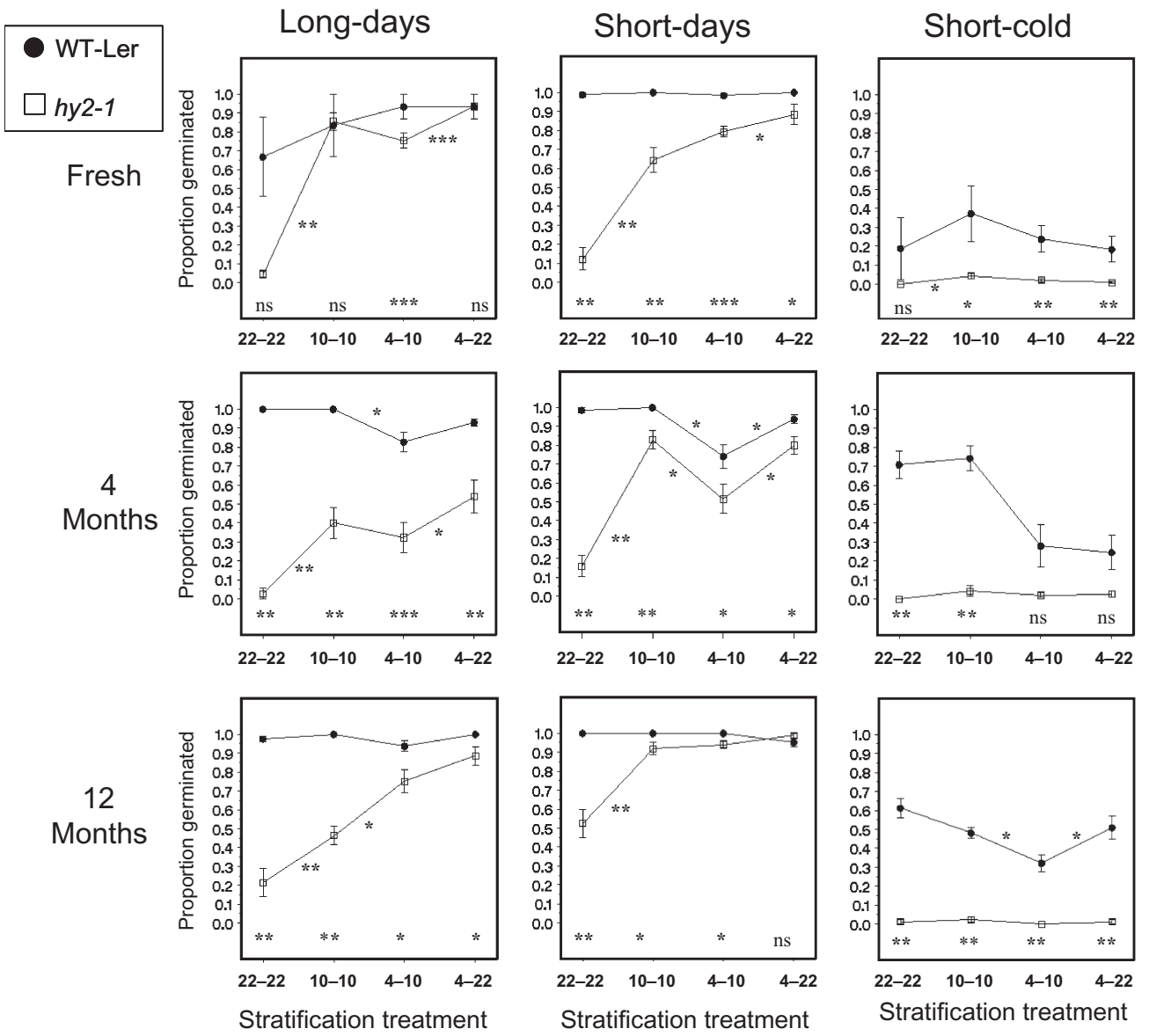

Figure 2. Means and SEs of germination proportions in different post-stratification temperatures for cold-stratified and non-cold-stratified seeds. Significant differences between adjacent treatments are indicated for each genotype above [Landsberg *erecta* (Ler)] or below (*hy2-1*) the line connecting the means in two treatments. Results are shown separately for seeds matured in each of three treatments (long-days, short-days and short-cold), and for each afterripening duration (fresh, 4 months of dry afterripening, 12 months of dry afterripening). Significance levels for differences between the *hy2-1* mutant and background line, Ler, are indicated at the bottom of the graph for each stratification treatment in each panel. ns, not significant; WT, wild type; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

matured under long days. A comparison to experiment 1 suggests that maternal photoperiod effects become more pronounced with afterripening.

The *hy2-1* seeds also exhibited a stronger effect of short-term afterripening than Ler seeds (Fig. 2, Table 2). For seeds matured at 22 °C, *hy2-1* seeds matured under long days, in particular, showed a pronounced increase in dormancy with 4 months of afterripening in most treatments (10 to 10 °C: $\chi^2 = 6.62$, $P = 0.01$; 4 to 10 °C: $\chi^2 = 12.34$, $P < 0.001$; 4 to 22 C: $\chi^2 = 8.32$, $P = 0.004$; 22 to 22 °C: $\chi^2 = 0.40$, $P > 0.05$), and cold-stratified seeds matured under short days also exhibited increased dormancy after 4 months of afterripening ('4 to 10 °C': $\chi^2 = 7.48$, $P = 0.004$; '4 to 22 C': $\chi^2 = 3.36$, $P = 0.067$; $P > 0.05$ in the other stratification treatments). Prolonged afterripening (12 versus 4 months) was generally associated with a loss of dormancy in *hy2-1* seeds matured at 22 °C ($P < 0.05$ for all stratification treatments except for the 'long-day/4 to 22 C' treatment and all 10 to 10 °C stratification treatments). Afterripening had no effect on *hy2-1* seeds matured under cool temperatures. Ler seeds that were matured at 22 °C exhibited a slight induction of dor-

Table 1. Response to maternal photoperiod (left four columns) and maternal temperature (right four columns) in each stratification and afterripening treatment

	Short versus long				Short versus short-cold			
Afterripening (°C)	22–22	10–10	4–10	4–22	22–22	10–10	4–10	4–22
Landsberg *erecta*								
Fresh	0.71	1.00	0.90	1.00	6.24*	7.17**	20.13***	22.72***
4 months	1.00	0.00	0.89	0.36	6.73**	7.76**	8.21**	20.66***
12 months	2.20	0.00	3.58+	3.60+	9.50**	9.62**	9.54**	8.49**
hy2-1								
Fresh	0.72	4.44*	0.04	0.28	5.25*	8.67**	23.00***	24.68***
4 months	3.69+	6.03*	3.53+	3.96*	5.58*	7.08**	21.17***	21.65***
12 months	5.06*	8.43**	6.04*	3.55+	8.97**	8.70**	9.66**	9.66**

χ^2 values based on Kruskal–Wallis tests are shown. +$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mancy with short-term afterripening, which was apparent in cold-stratified seeds ($P < 0.01$), but this effect was not as strong as in the *hy2-1* seeds. Long-term afterripening decreased dormancy in the 4 to 10 °C treatment for Ler seeds matured under short days ($\chi^2 = 5.95$, $P = 0.01$), but it increased dormancy in the 10 to 10 °C treatment for seeds matured at 10 °C ($\chi^2 = 7.17$, $P = 0.007$).

DISCUSSION

Phytochrome-mediated germination pathways are important for the regulation of germination responses to seasonal environmental factors experienced in both maternal and progeny generations. The *hy2-1* mutant was more responsive to multiple environmental factors than the background Ler genotype, indicating that the down-regulation of phytochrome-mediated germination pathways is likely to be important for responding to seasonal cues. The Ler genotype, which has been cultured for convenience in the lab for many generations, exhibited an almost complete lack of primary dormancy under seed maturation conditions typical of both the laboratory environment and of seasonal conditions during fruit maturation in late spring in temperature climates. While naturally non-dormant ecotypes may also exist, and while Ler has exhibited some dormancy and some responsiveness to stratification temperature in other studies (Cone & Spruit 1983), in this experiment Ler was remarkably unresponsive to the photoperiod of seed maturation, the temperature of stratification and the post-stratification temperature. Such a pervasive lack of dormancy is almost certainly maladaptive in many natural conditions, especially those with summer drought conditions. Indeed, a study of recombinant inbred lines demonstrated that a lack of dormancy upon dispersal in the early summer was fatal in two geographic locations in North America (Donohue *et al.* 2005b). In contrast, both the *hy2-1* mutant and the Col ecotype exhibited greater primary dormancy and a requirement for cold stratification to break the dormancy. This requirement would prevent germination in the summer and promote germination in the autumn, the typical season of germination for *A. thaliana* in many natural populations (Effmertova 1967; Baskin and Baskin 1972, 1983; Evans & Ratcliffe 1972; Griffith *et al.* 2004). Thus, the down-regulation of phytochrome-mediated germination pathways permits adaptive germination responses to cold stratification.

Table 2. Response to afterripening

Maternal treatment	d.f.	Long	Short	Short-cold
10–10 °C	1	8.61**	7.72**	3.47*
4–10 °C	1	4.47*	0.97^{ns}	0.23^{ns}
4–22 °C	1	7.19**	1.95^{ns}	2.81+
22–22 °C	1	1.52^{ns}	11.23***	6.10**

Test for significant differences between *hy2-1* and wild type for response to afterripening. *F*-ratios are given for each maternal and stratification treatment separately. Results are based on Type III sums of squares. ns, not significant, +$P < 0.1$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Molecular-genetic evidence also exists for the interaction between phytochrome pathways and germination responses to cold stratification. In particular, phytochrome-B up-regulates the gene, *GA4H*, which encodes *GA3ox2*, which catalyses the final conversion of inactive GA to active GA, while another phytochrome up-regulates a second GA hydroxylase, *GA3ox1* (Yamaguchi *et al.* 1998; Yamaguchi & Kamiya 2000). *GA3ox1* is down-regulated by active GA, but up-regulated by cold via the repression of SPATULA (Penfield *et al.* 2005). Thus, phytochrome-mediated GA biosynthesis pathways interact with cold response pathways to influence germination. The results presented here, namely that disruption of the phytochrome pathway facilitates germination responses to cold, are consistent with the hypothesis that cold acts as a stimulant of GA biosynthesis primarily when phytochrome-stimulated GA biosynthesis is impaired, or that the additional stimulation of GA synthesis by cold is most apparent when GA levels are low due to disrupted phytochrome-stimulated pathways.

While disrupting phytochrome-mediated pathways increased responsiveness to cold stratification, it did not

alter responsiveness to warm stratification in this experiment. Both Ler and *hy2-1* exhibited a lack of induction of dormancy in response to warm stratification, whereas the Col ecotype was induced into dormancy by warm temperatures, like other natural ecotypes (unpublished data). Thus the background Ler genotype, shared by *hy2-1*, differs from Col in genes associated with warm-induction of dormancy, and we found no evidence that disruption of phytochrome pathways accounts for that response. It is possible that phytochromes may mediate the response in some manner, especially on more dormant backgrounds, because the magnitude of warm-induced dormancy in Col differed with maternal photoperiod. However, direct disruption of phytochrome function did not cause increased sensitivity to warm stratification in the same manner as it caused increased sensitivity to other environmental treatments.

In addition to responding to cold stratification, the *hy2-1* mutant also responded more to post-stratification temperature than did Ler. This is of ecological relevance because the timing of germination depends firstly on the dormancy status of seeds, and secondly on the conditions under which a non-dormant seed can germinate (Bewley 1997). That is, cold may break dormancy, but the non-dormant seed may still not germinate unless the temperature is permissive. In this study, Ler germinated regardless of stratification and post-stratification temperature. For *hy2-1*, non-cold-stratified seeds germinated to higher percentages at 10 °C than at 22 °C, but cold-stratified seeds germinated to higher percentages at 22 °C than at 10 °C. The first response would prevent germination until temperatures become cooler, as in autumn, and could prevent germination in summer when soil moisture levels are low. The second response could promote germination when temperatures fluctuate between cool and warm, as in early autumn, but inhibit germination when temperatures fluctuate between cold and cool, as in later autumn. This response would prevent germination late in the autumn when the risk of cold-induced mortality of young germinants could be high. Whether such responses are adaptive requires explicit field studies, but the completely unresponsive lack of dormancy in the Ler genotype is unlikely to be the optimal germination response to temperature in many locations within the range of *A. thaliana*. As before, the down-regulation of phytochrome-mediated germination pathways appears to be important for allowing appropriate germination responses to post-imbibition temperature.

Phytochrome-mediated germination pathways also appear to be important in germination responses to the conditions during seed maturation (see also McCullough & Shropshire 1970; Hayes & Klein 1974). Firstly, *hy2-1* seeds more frequently exhibited an effect of maternal photoperiod (see also Karssen 1970; Gutterman 1992), such that seeds that were matured under short days germinated to higher percentages than seeds matured under long days, and the effect was apparent especially when seeds experienced afterripening or cold temperatures. A study of recombinant inbred lines of *A. thaliana* (Munir *et al.* 2001) documented a maternal photoperiod effect in the same direction as that observed in *hy2-1* mutants, namely that seeds matured under short days were more responsive to dormancy-breaking cold stratification and germinated to higher percentages than seeds matured under long days. Interestingly, a recent field study showed a subtle effect of maternal photoperiod on germination timing, which was apparent only in seeds that had afterripened after dispersal in the field (Donohue *et al.* 2005a). Thus, these *hy2-1* mutants responded to maternal photoperiod in a manner similar to that observed in certain non-mutant lineages, suggesting that pathways that are involved with sensing maternal photoperiod in natural variants could be regulated by phytochrome. An increased responsiveness to cold of seeds matured under short days could increase the probability that seeds matured in autumn or early spring (i.e. under short days) germinate that same spring. These effects of maternal photoperiod could therefore promote a rapid-cycling life history.

Maternal temperature influenced germination much more strongly than maternal photoperiod in all genotypes, with cool temperature during seed maturation inducing dormancy (see also Junttila 1973). In Ler, this dormancy was overcome only by a cycle of warm followed by cold stratification. Interpreted in an ecological context, this suggests that seeds that are matured under the cool temperatures of early spring will not be able to germinate until autumn – after they experience first the hot temperatures of summer and then the cold nights of autumn. In contrast to the effects of maternal photoperiod, effects of maternal temperature would cause seeds to be less likely to germinate in the spring, just after dispersal, even if they experience cold stratification. This response could prevent germination at a time of year when the plants would not have adequate time to complete their life cycle due to conditions of temperature- or drought-stress. Likewise, seeds matured in autumn would not be able to germinate until the following autumn. Thus, this maternal temperature effect on germination would impose a winter annual life history. In short, whether germination phenology would result in a winter annual or a rapid-cycling life history may depend on the relative magnitude of maternal photoperiod versus maternal temperature effects on germination.

The two ecotypes, Ler and Col, differed in their response to cold seed maturation temperature, with Ler overcoming cold-induced dormancy only after a cycle of warm (as in summer) followed by cold (as in autumn). In contrast, Col overcame cold-induced dormancy with cold stratification alone, as would occur when autumn-matured seeds overwinter. Surprisingly, this result leads to the prediction that Col, not Ler, would be more likely to exhibit a rapid-cycling life history – a difference in life history due to differences in germination responses to maternal temperature.

The *hy2-1* seeds matured in cool temperatures had even less germination than Ler in almost all treatments. Unlike Ler, *hy2-1* seeds were not able to overcome the dormancy induced by cool seed-maturation temperature. Thus,

phytochrome-mediated pathways are necessary to overcome this particular sort of dormancy. This would be especially important for plants that mature seeds early in spring or autumn, and the lack of such a response could essentially prevent germination of these seeds indefinitely.

Finally, *hy2-1* seeds had a different pattern of afterripening from Ler (Evans & Ratcliffe 1972). In particular, *hy2-1* seeds matured under warm temperatures and afterripened for 4 months were less strongly stimulated to germinate by cold or cool temperatures. While Ler also exhibited this tendency, it was not as pronounced as in *hy2-1* mutants. Therefore, phytochrome-mediated germination pathways also appear to have some role in influencing the effects of afterripening on germination responses to temperature.

In conclusion, the down-regulation of phytochrome-mediated germination pathways appears to be important for enabling responses to seasonal cues, including cold stratification (but not warm stratification in this background), post-stratification temperature, the photoperiod and temperature during seed maturation, and afterripening duration. The activation of phytochrome-mediated germination pathways, in contrast, appears to be necessary for the breakage of dormancy that is induced by cool seed-maturation temperatures. Thus phytochrome, a photoreceptor, also plays an important role in mediating germination responses to temperature and afterripening. Phytochrome, moreover, has the potential to alter responses to seasonal cues experienced both during seed maturation on the maternal plant and after dispersal.

These results are among the first to identify a gene associated with maternal environmental effects on germination. Phytochrome pathways appear to be crucial sensors not only of light conditions but also of temperature conditions. This study demonstrates that pathways that are mediated by phytochrome have major effects on how seeds germinate in response to multiple seasonal cues. Given the extreme importance of germination timing to the lifetime fitness of plants, and given that plants must exhibit adaptive germination timing before they can even express adaptive life history traits at any subsequent life stage, the role of phytochrome in mediating germination responses to complex seasonal cues of light and temperature is likely to be a very important ecological function of phytochrome that has not been fully recognized.

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