Diversification of phytochrome contributions to germination as a function of seed-maturation environment

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Summary

• Environmental conditions during seed maturation influence germination, but the genetic basis of maternal environmental effects on germination is virtually unknown.
• Using single and multiple mutants of phytochromes, it is shown here that different phytochromes contributed to germination differently, depending on seed-maturation conditions.
• *Arabidopsis thaliana* wild-type seeds that were matured under cool temperatures were intensely dormant compared with seeds matured at warmer temperature, and this dormancy was broken only after warm seed-stratification followed by cold seed-stratification. The warm–cold stratification broke dormancy in fresh seeds but not in dry after-ripened seeds. Functional *PHYB* and *PHYD* were necessary to break cool-induced dormancy, which indicates a previously unknown and ecologically important function for *PHYD*. Disruption of *PHYA* in combination with *PHYD* (but not *PHYB*) restored germination to near wild-type levels, indicating that *PHYA* contributes to the maintenance of cool-induced dormancy on a *phyD* background. Effects of seed-maturation temperature were much stronger than effects of seed-maturation photoperiod. *PHYB* contributed to germination somewhat more strongly in seeds matured under short days, whereas *PHYD* contributed to germination somewhat more strongly in seeds matured under long days.
• The variable contributions of different phytochromes to germination as a function of seed-maturation conditions reveal further functional diversification of the phytochromes during the process of germination. This study identifies among the first genes to be associated with maternal environmental effects on germination.

Key words: dormancy, duplicated genes, germination, life history, maternal effects, phenotypic plasticity, phytochrome, seasonal cues.

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Introduction

Many weedy plants exhibit variation in basic life history, with some combinations of biennial, winter-, spring-, or autumn-annual, or rapid cycling life histories being expressed within a single species or even within a single population. Which life history is expressed depends critically on two phenological transitions: germination and reproduction (Galloway, 2001; Donohue, 2005). The season of seed germination determines a plant's exposure to major environmental cues, such as temperature and photoperiod, which regulate the transition to reproduction (Koornneef *et al.*, 1991; Nordborg & Bergelson,

1999; Mouradov *et al.*, 2002; Simpson & Dean, 2002). In this manner, seasonal germination timing can influence reproductive phenology. The seasonal timing of reproduction, in turn, influences germination phenology by determining the environmental conditions a seed experiences during maturation and immediately after dispersal.

The season of seed germination depends on the timing of dormancy loss and the seasonal conditions that permit the germination of nondormant seeds (Baskin & Baskin, 1998). 'Dormancy' is the temporary failure of a viable seed to germinate under environmental conditions that later evoke germination, following some environmental ('dormancy-breaking') treatment (Simpson, 1990), such as exposure to cold. Seeds can be shed in a nondormant state or in a dormant state, in which case they exhibit 'primary dormancy'. A nondormant seed may germinate if permissible environmental conditions exist, or it may remain an ungerminated seed until permissible conditions arrive. It may also enter 'secondary dormancy' and require a dormancy-breaking treatment before it can germinate under permissible conditions. A seed that is shed with primary dormancy, in contrast, may gradually lose this dormancy through a process of 'dry after-ripening' (Evanari, 1965; Baskin & Baskin, 1998). With prolonged dry conditions after dispersal, some seeds cycle in and out of dormancy (Baskin & Baskin, 1983).

The seasonal timing of reproduction influences germination phenology because both the conditions during seed maturation and the sequence of seasonal conditions after dispersal significantly influence dormancy status and the conditions that permit the germination of nondormant seeds. Seasonal environmental factors during seed development, such as temperature and photoperiod, can vary widely in species with variable reproductive phenology, and both photoperiod and temperature during seed development are known to influence dormancy and germination (Roach & Wulff, 1987; Gutterman, 1992; Baskin & Baskin, 1998; Munir *et al.*, 2001). The seasonal timing of seed maturation also determines the environment experienced immediately after dispersal, including the duration of dry after-ripening and the probability that imbibed seeds experience hot temperatures of summer vs cold winter temperatures – conditions that are known to induce or break dormancy, respectively, in many species. Therefore, seeds matured under different seasonal conditions experience different predispersal and postdispersal conditions, have different degrees of dormancy, have different requirements for germination, and may thereby germinate under different seasonal conditions and times of year.

Arabidopsis thaliana exhibits extensive variation in life history (Ratcliffe, 1965; Effmertova, 1967; Evans & Ratcliffe, 1972; Napp-Zinn, 1976; Nordborg & Bergelson, 1999; Griffith *et al.*, 2004), and the season of flowering varies from early spring, through midsummer, with some populations even reproducing in late autumn (Thompson, 1994; Griffith *et al.*, 2004). In natural populations in New England, plants mature seeds in photoperiods ranging from 9 to > 14 h, and across a range of temperature from < 10 to > 25°C (K. Donohue & G. C. K. Chiang, pers. obs.). In this study, we examined how the photoperiod and temperature during seed maturation influence the germination of mutant genotypes of *A. thaliana*.

In a previous study using *A. thaliana*, we identified phytochrome as an important regulator of germination responses to seasonal environmental cues experienced during seed maturation and after dispersal (Donohue *et al.*, 2007). Phytochromes are red and far-red photoreceptors, and they have long been known to regulate germination responses to light (Borthwick *et al.*, 1952; Shinomura *et al.*, 1994; Poppe & Schäfer, 1997; Reed *et al.*, 1994; Shinomura, 1997; Whitelam & Devlin, 1997; Casal & Sanchez, 1998; Ritchie & Gilroy, 1998; Koornneef *et al.*, 2002). Phytochrome is a biliprotein, composed of an apoprotein and a chromophore, and exists in a dynamic equilibrium of two photoreversible forms: an inactive red-absorbing form (Pr) and a bioactive far-red-absorbing form (Pfr), the equilibrium ratio of which depends on the ratio of red light:far-red light. Recently, phytochromes have been shown to promote germination through the up-regulation of gibberellin oxidases that convert inactive gibberellins (GAs) into bioactive GAs, which are important stimulants for germination (Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998; Yamaguchi & Kamiya, 2000; Garcia-Martinez & Gil, 2002; Ogawa *et al.*, 2003). Phytochromes have also been implicated in the catabolism of bioactive abscisic acid, the major plant hormone associated with dormancy maintenance (Seo *et al.*, 2006).

In the Brassicaceae, which includes *A. thaliana*, the phytochrome apoproteins are encoded by five genes – *PHYA* through *PHYE* – that have arisen through a series of gene duplications (Sharrock & Quail, 1989; Clack *et al.*, 1994; Mathews & Sharrock, 1997). PhyA is light-labile and critical for germination responses to very low fluence light, while PhyB is light-stable and important for photoreversible germination at intermediate fluence levels (note that lower-case nonitalics refers to the gene product). More recently, *PHYE* has been shown to be necessary for germination under continuous far-red light (Hennig *et al.*, 2002), and *PHYD* has been shown to have a role in inhibiting the *PHYA*-mediated very-low-fluence-response in far-red light, in combination with *PHYB* (Hennig *et al.*, 2001). Thus, different phytochromes are involved in diverse aspects of germination responses to light experienced by imbibed seeds.

Phytochromes have also been shown to be involved in germination responses to the red:far red ratio of light during seed maturation (McCullough & Shropshire, 1970; Hayes & Klein, 1974). More recently, two Dof zinc-finger genes, *DAG1* and *DAG2*, have been implicated in maternal effects on dormancy (Gualberti *et al.*, 2002; Papi *et al.*, 2002) that are also potentially mediated by phytochrome. *DAG2* is a germination-promoting factor, whereas *DAG1* suppresses a germination factor that appears to be regulated in a photoreversible manner by phytochrome. Thus, phytochromes regulate germination responses to light experienced not only after dispersal but also to light experienced during seed development.

Table 1 List of mutants and their sources

Reference name	Type of mutation	Allele	Source-stock number
Ler	'Wild type'	Landsberg *erecta*	ABRC-CS20
$phyA_1$	Deficient (null)	*phyA-201*	ABRC-CS6219
$phyA_2$	Deficient (null)	*phyA-202*	ABRC-CS6220
$phyA_3$	Deficient (null)	*phyA-203*	ABRC-CS6221
$phyA_5$	Deficient (null)	*phyA-205*	ABRC-CS6222
$phyB_1$	Null	*phyB-1*	ABRC-CS69
$phyB_{1S}$	Null	*phyB-1*	RAS
$phyB_4$	Missense	*phyB-4*	ABRC-CS6212
$phyB_5$	Null	*phyB-5*	ABRC-CS6213
$phyB_7$	Deficient (null)	*phyB-7*	ABRC-CS6215
phyD	Natural null (Ws ecotype)	*phyD-1*	RAS
phyE	Null	*phyE-1*	GCW
$phyA_1B_5$		*phyA-201/phyB5*	ABRC-CS6224
$phyA_1B_{1S}$		*phyA-201/phyB-1(S)*	RAS
$phyA_1D$		*phyA-201/phyD-1*	RAS
$phyB_{1S}D$		*phyB-1(S)/phyD-1*	RAS
$phyB_1E$		*phyB-1/phyE-1*	GCW
$phyB_1DE$		*phyB-1/phyD-1/phyE-1*	GCW

'Reference name' is the name given to the line in this paper. ABRC, Arabidopsis Biological Resource Center at Ohio State University; RAS, R. A. Sharrock; GCW, G. C. Whitelam.

We have recently shown that phytochromes are involved in germination responses not only to light quality but also to environmental factors such as photoperiod and temperature during seed development (Donohue *et al.*, 2007), based on a study of an *A. thaliana* mutant deficient in the phytochrome chromophore (*hy2-1*) and thus deficient in the activity of all five phytochromes (Parks & Quail, 1991; Terry, 1997). We also found that specific phytochromes contributed to germination differently depending on the postdispersal temperature experienced by seeds (Heschel *et al.*, 2007). To test whether seasonal conditions during seed maturation influence phytochrome-mediated germination, in the present study we examined whether the contributions of specific phytochromes to germination and dormancy changed depending on the photoperiod or temperature of seed maturation. We grew single, double and multiple phytochrome mutants in three treatments that manipulated the photoperiod and temperature during seed maturation, and then examined the germination of fresh and dry after-ripened seeds after different seed-stratification treatments that represent different seasonal cues.

Materials and Methods

The germination responses of several phytochrome mutants of *A. thaliana* (L.) Heynh. were compared with those of their background ecotype, Landsberg *erecta* (referred to hereafter as Ler). The Ler genotype is not dormant under standard culture conditions, possibly because of artificial selection for convenience of culturing over many generations in the laboratory, although it may have lacked dormancy before extensive culturing. Seeds of Ler can be induced into dormancy under some conditions, however. Other nondormant natural ecotypes have been identified under standard growth conditions, so Ler is not anomalous in that regard. Most characterized physiological mutants of *A. thaliana* are derived from this Ler background. Mutants with loss of phytochrome function, and which restore dormancy under conditions in which Ler is nondormant, can be used to identify pathways that are likely to be down-regulated during dormancy maintenance. Mutants with loss of phytochrome function, and which do not germinate under conditions that break dormancy in dormant seeds of the Ler genotype, can be used to identify pathways whose expression is necessary to break dormancy and/or permit germination.

Table 1 lists the mutants used in this study and their original sources. With the exception of *phyD-1*, all phytochrome mutations in Table 1 were isolated in the Ler genetic background and were combined into multiple mutants by crossing the mutant lines and following either the morphological phenotype caused by the individual mutation or by conducting a polymerase chain reaction (PCR) assay that distinguishes the mutant and wild-type alleles (Devlin *et al.*, 1998). The *phyD-1* mutation was identified in the Wassilewskija (Ws) ecotype (Aukerman *et al.*, 1997). The Ws *phyD-1* line was crossed to the Ler wild type, Ler *phyA-201* and Ler *phyB-1*, and was then backcrossed seven times (BC7) to those parents. The *phyD-1* allele was identified in each generation by a PCR assay, as described in Aukerman *et al.* (1997). Homozygous *phyD* BC7 F_2 plants were identified and used to construct the additional multiple mutant lines. The corresponding backcrossed Ler line, containing the wild-type Ler *PHYD* allele, was used for all comparisons of mutants containing *phyD*. Multiple *phyE* mutants were constructed as described in Devlin *et al.* (1998).

Homozygous, inbred lines were used in these experiments. Therefore, the maternal and progeny genotypes were identical,

precluding the resolution of maternal genotypic vs progeny genotypic determination of phenotypic effects. In highly selfing species such as *A. thaliana*, maternal and progeny genotypes are frequently nearly identical because of inbreeding, so genetic conflicts across generations are naturally diminished. The use of inbred lines in these experiments is therefore ecologically realistic, although future studies that dissect the genetic control of environmental effects during seed maturation would be interesting.

Seeds were matured under three treatments in Conviron E7/2 growth chambers (Controlled Environment, Ltd, Winnipeg, Manitoba, Canada): 'long-warm', 'short-warm', and 'short-cold'. The 'long-warm' treatment imposed a 14 h light: 10 h dark cycle of full-spectrum light (fluorescent plus incandescent) at 22°C and resembles seasonal conditions during a typical seed-maturation season in late spring in temperate climates. The 'short-warm' treatment imposed a 10 h light: 14 h dark cycle at 22°C. The 'short-cold' treatment imposed a 10 h light/14 h dark cycle at 10°C and resembles the seasonal conditions during seed maturation when plants mature seeds in the autumn or very early spring. The 'short-warm' treatment is included for comparative purposes but does not represent common natural conditions in temperate climates. Comparing the 'long-warm' with the 'short-warm' treatment reveals the effect of maternal photoperiod during seed maturation. Comparing the 'short-warm' with the '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 of all genotypes in all treatments within a temporal block matured 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. Seeds were kept dry at room temperature during this processing time.

We conducted the germination assays on 'fresh' seeds (seeds that were harvested approx. 1 wk previously) and on seeds that had been dry after-ripened at 22°C for 3 months. Thus, the experimental design was factorial with respect to seed maturation conditions, stratification treatments and after-ripening duration (three maternal treatments × four stratification treatments × two after-ripening treatments). Germination assays were conducted using 12 seeds of a given genotype in a single Petri plate (50 × 9 mm) containing 0.5% agar. Twelve Petri plates, distributed over four temporal blocks (three replicates per block), were used for each genotype in each maternal and stratification treatment (144 seeds per genotype per treatment). Four dark, wet stratification treatments were imposed: 'neutral', which consisted of 5 d at 22°C; 'cold', which consisted of 5 d at 4°C; 'warm', which consisted of 7 d at 31°C; 'warm–cold', which consisted of 7 d at 31°C followed by 3 d in the light to remove any nondormant seeds, and then 5 d in the dark at 4°C. Preliminary studies of Ler and other ecotypes showed that seeds tended to germinate to high frequencies after 5 or fewer days in the cold, whereas ≥ 7 d in the warm induced dormancy in natural ecotypes (K. Donohue and M. S. Heschel, 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 warmth to induce dormancy, and the 'warm–cold' treatment assessed the ability of cold to break primary plus warm-induced dormancy. All plates were placed in Percival germination incubators (Percival Scientific, Inc., Perry, IA, USA) at 22°C in a 12-h photoperiod of white fluorescent light (with photon flux density of 90 μmol m^{-2} s^{-1}) after their period of stratification. Treatments were staggered so that all plates would be transferred to light simultaneously. We estimated the 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).

To test for significant differences in response to experimental treatments among the genotypes, we first conducted analyses with all lines in a full model, with block, line, maternal treatment, stratification treatment and after-ripening as fixed factors, and saw highly significant interactions (results not shown). To test for effects of specific phytochromes, we compared the germination response of each mutant with that of the wild-type background, Ler. A significant treatment-by-genotype interaction, as indicated from the ANOVA, suggests that that the loss of the functional phytochrome caused a significant alteration of the germination response to the experimental treatment. To interpret the interactions among genotype, maternal treatment, stratification and after-ripening, we tested for differences in response to the maternal treatments within each stratification and after-ripening treatment separately, and we tested for differences in their response to stratification within each maternal and after-ripening treatment. We also tested for significant differences between Ler and each mutant in their response to after-ripening within each maternal and stratification treatment separately. To interpret differences in these responses (interactions between genotype and treatment), we tested for significant differences between each mutant and Ler in each treatment, as well as differences between specific mutant pairs, using *a priori* contrasts in a model that included all lines. Because the data were not always normally distributed, we conducted a series of nonparametric Kruskal–Wallis tests to verify the significance of these results. Significance levels of comparisons between the mutants and wild-type background within each treatment were Bonferroni corrected based on the number of comparisons to each wild-type line plus the number of comparisons between mutant pairs within a given background (plus one, to account for the *phyB*/*phyD* vs *phyB*/*phyD*/*phyE* comparison, which used both backgrounds). Corrections were implemented separately for each treatment, because different seed samples were used in each treatment. Bonferroni corrections for the significance levels of line × treatment interactions were implemented based on the total number of each interaction term tested within each wild-type background (plus one, as above).

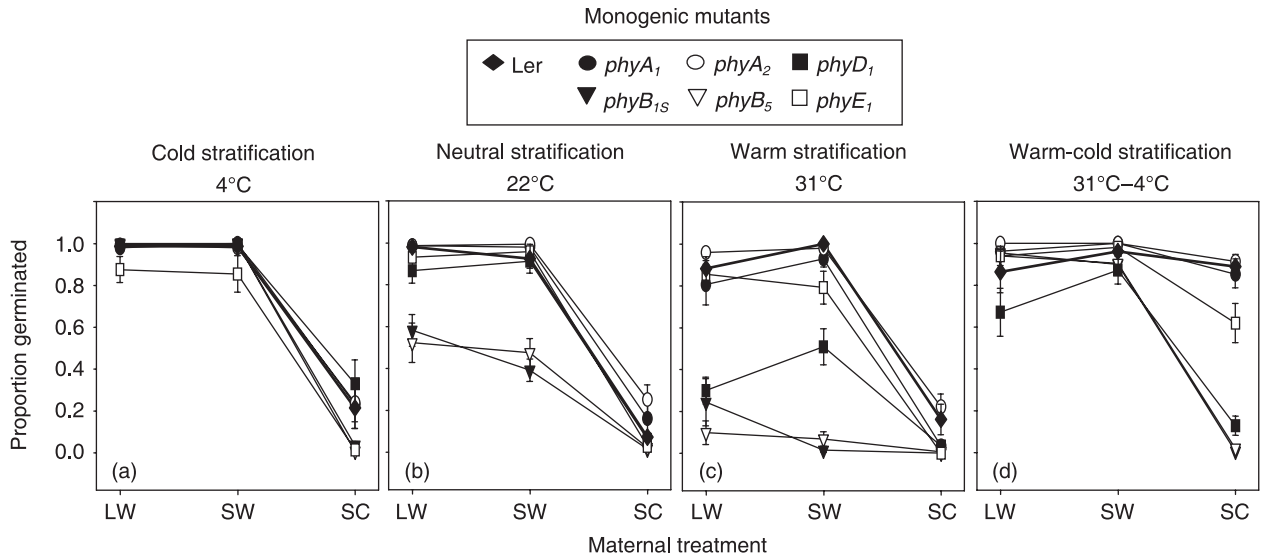

Fig. 1 Monogenic mutants. Means (± standard error) of the proportion of fresh seeds that germinated. The Ler background and monogenic mutants are shown. The results are for fresh seeds matured under different maternal conditions ('Maternal treatment') and given different stratification treatments. LW, 'long-warm' maternal treatment; SW, 'short-warm' maternal treatment; SC, 'short-cold' maternal treatment. Because the different *phyA* mutants and the different *phyB* mutants behaved consistently, only those alleles that are contained in the multiple mutants are shown. Ler is shown with a thick line.

Results

Response to maternal temperature ('short-warm' vs 'short-cold')

The Ler background genotype was induced into strong dormancy by a cool (10°C: 'short-cold') seed-maturation temperature compared with seeds matured at a warmer (22°C: 'short-warm') temperature (Fig. 1). This dormancy was broken in fresh seeds only by a sequence of warm (31°C) seed-stratification followed by cold (4°C) seed-stratification (Fig. 1d). Seeds dry after-ripened for 3 months did not lose cool-induced dormancy with this warm–cold seed stratification (Fig. 2c; lower, far right panel), but after-ripened, cool-matured Ler seeds germinated to slightly, but not significantly, higher proportions in all other stratification treatments (Fig. 2c).

Fresh seeds of all mutants, except for monogenic *phyA* and *phyE* mutants and the *phyA*/*phyD* mutant, had altered responses to seed-maturation temperature in at least one stratification treatment (Table 2, Figs 1,3). Disruption of *PHYA* and *PHYE* did not significantly decrease germination under the conditions of this study. Their monogenic mutants did not significantly differ from Ler (Tables 2–4), and their disruption did not significantly decrease germination in other mutant backgrounds (Table 5).

Fresh seeds of the single and multiple mutants of *PHYB* showed reduced responses to seed-maturation temperature compared with Ler, especially in seed-stratification treatments that lacked cold (Fig. 1b,c and 3b,c; Table 2). Unlike Ler, mutants deficient in *PHYB* exhibited dormancy even when matured under warmer temperatures (Table 3), so the difference between warm and cool maturation conditions was less for these mutants than for Ler. This effect was consistent for fresh and for dry after-ripened seeds (Supplementary material Table S1). In the cool seed-maturation temperature, disruption of *PHYB* was less apparent in all but the warm–cold stratification treatment, because Ler seeds were also induced into strong dormancy when matured under cool temperature (Table 4). However, cool-matured seeds of some *phyB* single and multiple mutants did have significantly lower germination than Ler (Table 4).

Mutants deficient in *PHYD* also had reduced responses to seed-maturation temperature (Table 2) because they were more dormant than Ler when matured in the warm temperature, although this effect was apparent in the warm stratification treatment but not in the neutral stratification treatment (Table 3, Fig. 1c). This increase in dormancy of warm-matured *phyD* seeds was consistent for fresh and dry after-ripened seeds (Supplementary material Table S1). In the cool seed-maturation temperature, dry after-ripened seeds of mutants deficient in *PHYD* were more dormant than Ler in treatments that lacked cold, because Ler lost some dormancy with after-ripening, but the *phyD* mutants lost less dormancy with after-ripening than Ler (Fig. 2c, middle panels; Supplementary material Table S2).

A striking result was that mutants deficient in either *PHYB* or *PHYD* were not able to break the dormancy that was induced by cool maturation temperature, even in the warm–cold seed-stratification treatment that broke the dormancy of Ler seeds (Figs 1d and 3d; Tables 2 and 4). Fresh seeds of the monogenic *phyE* mutant also had a slightly reduced ability to break cool-induced dormancy after warm–cold stratification, but not as severely as in monogenic *phyB* and *phyD* mutants (Fig. 1d, Table 4; not significant after Bonferroni correction).

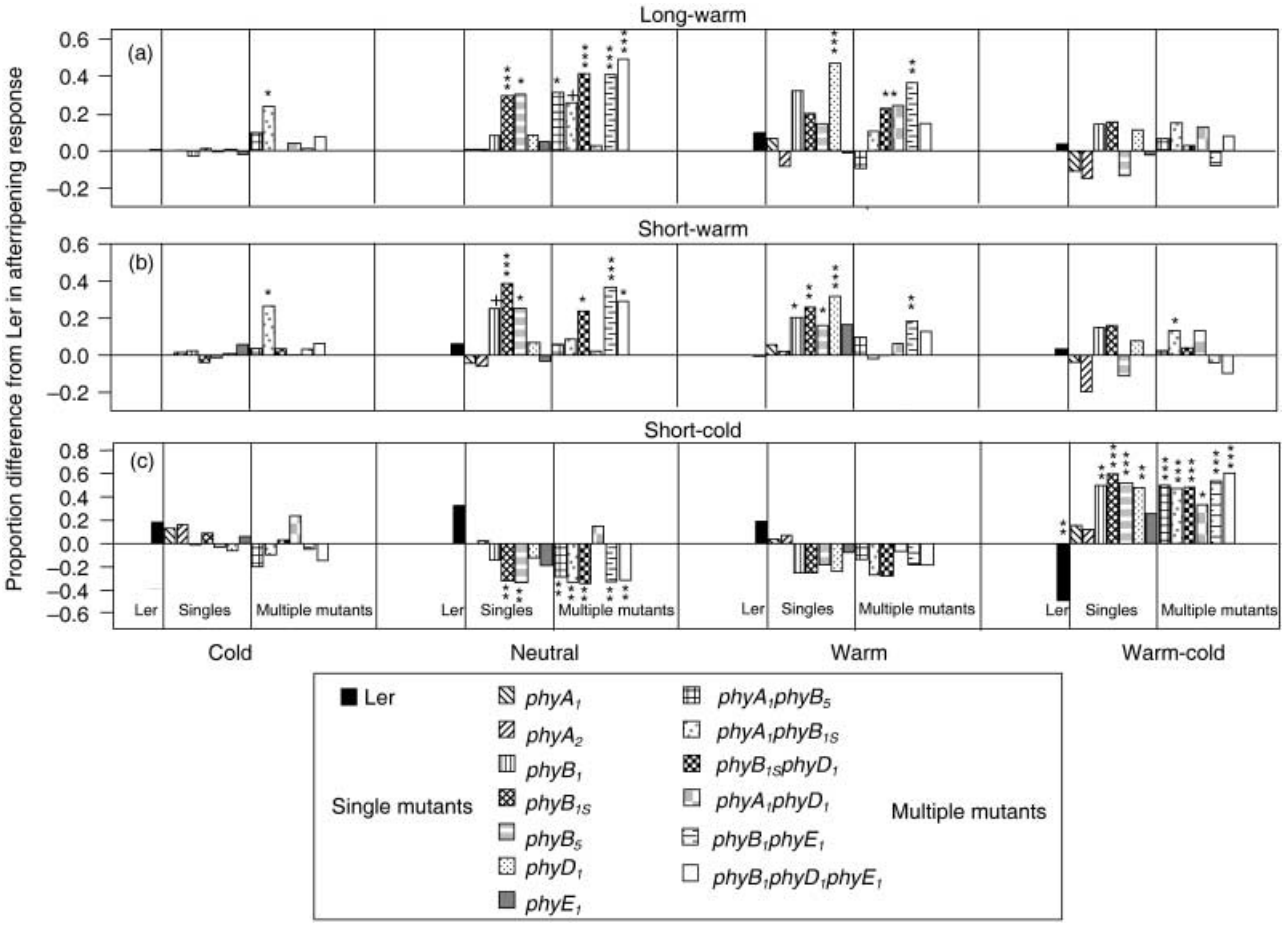

Fig. 2 Response to after-ripening of Ler and mutant seeds matured under long-warm (a), short-warm (b) and short-cold (c) treatments and given four seed-stratification treatments (left to right). The black bar shows the proportion of after-ripened seeds of the Ler genotype that germinated, compared with fresh seeds (proportion of after-ripened seeds that germinated minus the proportion of fresh seeds that germinated); an increase in the proportion of seeds that germinated (above the zero line) indicates a loss of dormancy in after-ripened seeds compared with fresh seeds, and a decrease in the proportion of seeds that germinated (below the zero line) indicates increased dormancy with dry seed storage. All other bars show the effect of after-ripening of each mutant compared with Ler. An increase (above the zero line) in the proportion of seeds that germinated indicates that after-ripening during dry seed storage increased germination more in the mutant than in the Ler background (greater loss of dormancy with after-ripening than in Ler). A decrease (below the zero line) in the proportion of seeds that germinated indicates that dry seed storage decreased germination more in the mutant than in Ler (greater increase in dormancy with dry seed storage than in Ler). Asterisks above the black bars indicate a significant effect of after-ripening in the Ler background. Asterisks above bars representing mutants indicate a significant difference between the mutant and Ler in response to after-ripening. +, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Bonferroni-corrected significance requires a P-value of < 0.002.

Interestingly, the germination of cool-matured *phyD* seeds was restored to wild-type levels in the warm–cold stratification by the simultaneous disruption of *PHYA*. This restoration of germination is indicated by the significantly higher germination of *phyA*/*phyD* seeds compared with *phyD* seeds (Table 5) and the unaltered germination of *phyA*/*phyD* seeds compared with Ler (Fig. 3d, Table 3) in the warm–cold stratification treatment. This result was consistent for fresh and dry after-ripened seeds (Supplementary material Table S3). Disruption of *PHYA* in combination with *PHYB*, in contrast, did not restore germination (Fig. 3d). Thus, functional *PHYA* appears to impede the germination of cool-matured seeds, but only on a *phyD* background.

Like Ler, 3 months of dry after-ripening reduced the ability of most genotypes to break cool-induced dormancy in response to warm–cold seed stratification (Table 2, compare the columns of fresh vs after-ripened seeds; Supplementary material Table S2). However, most mutants did not gain as much dormancy with dry after-ripening as Ler Fig. 2(c), far right), largely because they were already more dormant as fresh seeds.

Response to maternal photoperiod ('long-warm' vs 'short-warm')

Ler exhibited no response to maternal photoperiod (Fig. 1). In fresh seeds matured at 22°C that were given seed-stratification

Table 2 Response to maternal temperature: short-warm vs short-cold maternal treatments

	Fresh				After-ripened			
Genotype vs Ler	Cold	Neutral	Warm	Warm-cold	Cold	Neutral	Warm	Warm-cold
Single mutants								
phyA-2	0.10	1.33	0.74	0.08	1.08	1.23	0.89	0.98
phyA-3	0.10	0.02	0.21	2.21	0.61	0.11	0.30	0.57
phyA-5	0.67	2.08	0.27	0.99	0.77	0.66	0.57	4.16*
phyA-201	0.00	0.10	0.52	1.03	0.66	0.18	0.36	0.29
phyB-1	0.95	4.32*	**21.62*****	**14.33*****	0.51	0.43	0.38	0.01
phyB-1S	7.15*	**96.90*****	**102.25*****	**112.50*****	0.25	0.94	4.24*	**11.62****
phyB-4	2.92	0.29	7.92**	**57.30*****	0.47	3.69	0.19	2.84
phyB-5	6.37*	**16.19*****	**98.23*****	**232.39*****	5.14*	1.87	**18.06*****	3.18
phyB-7	3.14	**12.01****	**31.71*****	**100.78*****	0.41	1.79	7.46**	0.04
phyD-1	0.99	3.56	**8.56****	**35.50*****	0.03	1.92	4.91*	2.84
phyE-1	0.34	1.05	0.24	7.67**	0.30	4.36*	3.30	0.01
Multiple mutants								
phyA201-/phyB-5	0.51	**105.75*****	**131.20*****	1.44	2.36	**14.28*****	**27.48*****	**7.80****
phyA-201/phyB-1	**10.85****	**289.67*****	**107.20*****	4.88*	0.00	**12.66*****	**24.44*****	**13.60*****
phyA-201/phyD-1	1.07	0.17	0.24	0.44	1.23	1.43	1.85	2.70
phyB-1/phyD-1	4.26*	**201.12*****	**81.82*****	**51.65*****	1.36	1.31	**15.25*****	6.11*
phyB-1/phyE-1	1.05	**40.62*****	**120.79*****	**179.61*****	2.71	1.89	**20.38*****	5.89*
phyB-1/phyD-1/phyE-1	1.18	**67.62*****	**138.57*****	**180.75*****	0.49	0.51	**21.79*****	1.23

Tests for significant differences between mutant and background genotype in response to maternal temperature for fresh (left) and after-ripened (right) seeds. *F*-ratios for the genotype–maternal treatment interaction (d.f. = 2 for all tests) are given from analyses of variance that test for significant differences between mutant and background genotypes in each stratification treatment. Tests were conducted separately for each mutant, and Bonferroni significance is indicated by boldface. All results are consistent with nonparametric Kruskal–Wallis tests.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

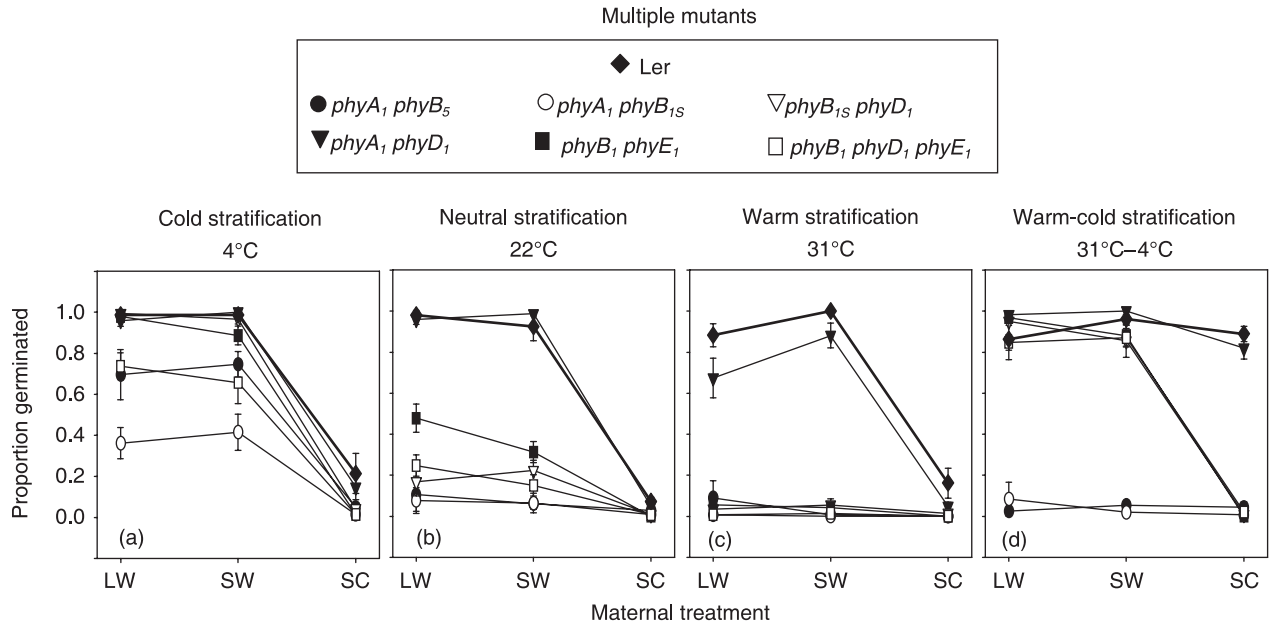

Fig. 3 Multiple mutants. Means (± standard error) of the proportion of fresh seeds that germinated. The Ler background and multiple mutants deficient in *PHYA*, *PHYB*, *PHYD* and *PHYE* are shown. The results are for fresh seeds matured under different maternal conditions ('Maternal treatment') and given different stratification treatments. LW, 'long-warm' maternal treatment; SW, 'short-warm' maternal treatment; SC, 'short-cold' maternal treatment. Ler is shown with a thick line.

Table 3 Differences between wild-type and mutant genotypes in short-warm, fresh seeds

Genotype vs Ler	Cold	Neutral	Warm	Warm-cold	Genotype × stratification
Single mutants					
phyA-2	0.37	1.02	3.35	5.06*[a]	1.33
phyA-3	0.90	0.76	4.06[b]	3.73	2.77*
phyA-5	2.71	1.02	1.00	2.82	1.21
phyA-201	2.71	0.64	4.78*	7.06*[a]	1.87
phyB-1	0.62	**10.94****	**68.50*****	2.40	**11.49*****
phyB-1S	1.00	**137.29*****	**970.68*****	4.31	**1322.32*****
phyB-4	0.54	1.07[b]	**43.04*****	2.40	**10.58*****
phyB-5	2.71	**25.21*****	**821.19*****	2.10	**60.28*****
phyB-7	2.71	**17.23*****	**76.36*****	0.04	**34.27*****
phyD-1	0.00	6.68*[a]	**38.48*****	3.48	**9.44*****
phyE-1	2.36	0.21	8.19**	0.48	2.83*
Multiple mutants					
phyA201-/phyB-5	**16.26*****	**124.49*****	**1844.16*****	**849.42*****	**46.51*****
phyA-201/phyB-1	**54.03*****	**358.37*****	**1180.55*****	**1049.61*****	**12.59*****
phyA-201/phyD-1	1.00	0.37	0.55	0.00	0.54
phyB-1/phyD-1	1.73	**347.76*****	**412.92*****	4.01[b]	**74.09*****
phyB-1/phyE-1	8.18**	**63.72*****	**220.32*****	3.00	**68.04*****
phyB-1/phyD-1/phyE-1	**17.61*****	**94.20*****	**1064.21*****	4.15	**39.78*****

F-ratios are given for tests for significant differences between mutant and background genotype within each stratification treatment (d.f. = 1 for each contrast), based on *a priori* contrasts. *F*-ratios are given for tests of differences between mutant and background genotype for response to stratification treatment ('genotype × stratification': d.f. = 3 for each test). Bonferroni significance is indicated by boldface. All results are consistent with nonparametric Kruskal–Wallis tests unless otherwise indicated.
[a]Not significant based on Kruskal–Wallis test.
[b]Significant based on Kruskal–Wallis test.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 4 Differences between wild-type and mutant genotypes in the short-cold, fresh seeds

Genotype vs Ler	Cold	Neutral	Warm	Warm-cold	Genotype × stratification
Single mutants					
phyA-2	0.46	8.98**[a]	0.43	0.39	0.77
phyA-3	0.74	1.81	1.57	1.41	0.64
phyA-5	1.94	3.07	1.67	0.03	0.24
phyA-201	0.26	1.89	3.77	0.34	1.03
phyB-1	0.81	**10.80****	5.78*	**33.94*****	**13.15*****
phyB-1S	**10.88****	1.77	4.11[b]	**283.08*****	**34.38*****
phyB-4	6.58*[a]	5.43*[a]	4.84*[a]	**56.76*****	**15.92*****
phyB-5	7.90*	6.39*[a]	5.24*	**574.31*****	**39.98*****
phyB-7	5.22*[a]	**10.61****	5.10*	**113.41*****	**10.35*****
phyD-1	5.29*[a]	0.23	2.31	**96.69*****	**20.25*****
phyE-1	7.92*[a]	6.25*[a]	5.78*	10.02**	1.41
Multiple mutants					
phyA201-/phyB-5	5.26*[a]	6.56*[a]	5.78*	**395.60*****	**39.80*****
phyA-201/phyB-1	**12.25****	3.05	4.11[b]	**281.22*****	**34.40*****
phyA-201/phyD-1	3.64	0.04	1.68	0.96	0.35
phyB-1/phyD-1	**11.59****	3.05	3.26[b]	**278.89*****	**33.67*****
phyB-1/phyE-1	6.51*[a]	**17.93*****	5.78*	**639.57*****	**39.64*****
phyB-1/phyD-1/phyE-1	7.20*[a]	**15.80*****	5.78*	**521.43*****	**39.47*****

F-ratios are given for tests for significant differences between mutant and background genotype within each stratification treatment (d.f. = 1 for each contrast), based on *a priori* contrasts. *F*-ratios are given for tests of differences between mutant and background genotype for response to stratification treatment ('genotype × stratification': d.f. = 3 for each test). Bonferroni significance is indicated by boldface. All results are consistent with nonparametric Kruskal–Wallis tests unless otherwise indicated.
[a]Not significant based on Kruskal–Wallis test.
[b]Significant based on Kruskal–Wallis test.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 5 Significant differences between specific mutant pairs in short-cold, fresh seeds

Mutant contrasts	Cold	Neutral	Warm	Warm-col	Line × stratification
phyB-1 vs phyA-201/phyB-1	1.65	0.37	0.00	0.00	0.65
phyB-5 vs phyA-201/phyB-5	3.23	0.09	1.00	1.44	1.14
phyB-1 vs phyB-1/phyD-1	0.06	0.37	2.11	0.03	0.22
phyD-1 vs phyA-201/phyD-1	9.78**[a]	0.13	0.24	**91.48*****	**29.37*****
phyE-1 vs phyB-1/phyE-1	0.00	3.45[b]	0.00	**64.70*****	**41.77*****
phyB-1 vs B-1/phyE-1	0.82	2.71	0.00	1.00	0.51
phyB-1/phyD-1 vs phyB-1/phyD-1/phyE-1	1.15	0.00	2.11	0.99	1.09

F-ratios are given from analyses of variance that test for significant differences between the two mutants indicated in the first column in each stratification treatment, based on *a priori* contrasts (d.f. = 1 for each contrast). The last column gives the *F*-ratios for genotype–stratification interaction to test for significant differences between mutants in response to stratification. Tests were conducted separately for each mutant pair. All results are consistent with nonparametric Kruskal–Wallis tests unless otherwise indicated.

[a]Not significant based on Kruskal–Wallis test.

[b]Significant based on Kruskal–Wallis test.

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

treatments that lacked cold (i.e. the neutral and warm stratification treatments), some monogenic mutants of *PHYB* had slightly more germination of seeds matured under long days (14 h) than short days (10 h), suggesting that *PHYB* may contribute to germination somewhat more strongly in seeds matured under short days (Fig. 1b,c). Specifically, *phyB1S* and *phyB7* exhibited this trend in the neutral stratification treatment, and *phyB1S*, *phyB1*, *phyB4* and *phyB7* exhibited this effect in the warm stratification treatment. Despite being detected in several *phyB* mutants, the effect was quite weak, ranging from 10 to 25% germination at most, and the effects were not significant after Bonferroni correction ($0.03 < P < 0.07$ for all comparisons). Like the *phyB* mutants, the *phyB*/*phyE* mutant also had slightly more germination in long days (Fig. 3), but again, the effects were not significant after Bonferroni correction.

By contrast, the monogenic mutant of *PHYD* tended to have more germination of seeds matured under short days than long days when warm stratified (Fig. 1c; F (photoperiod × mutant vs Ler) = 5.25, $P < 0.05$), suggesting that *PHYD* may promote germination most strongly in seeds matured under long days. The *phyB*/*phyD* double mutants (and the *phyB*/*phyD*/*phyE* triple mutant) had low germination when matured in either photoperiod (Fig. 3b,c), suggesting that deficiency in *PHYD* evened out any effect of deficiency in *PHYB*.

In seeds after-ripened for 3 months, the *phyB1* mutant still had somewhat higher germination when matured in long days than in short days when warm stratified, although the effect was not significant after Bonferroni correction (F (photoperiod × mutant vs Ler) = 6.65, $P < 0.01$). The same was observed in the *phyB*/*phyE* mutant, as in fresh seeds, when given a neutral (F (photoperiod × mutant vs Ler) = 4.99, $P < 0.01$) or warm (F (photoperiod × mutant vs Ler) = 8.52, $P < 0.01$) stratification. However, deficiency in only *PHYD* no longer had any effect on the germination response to maternal photoperiod. Therefore, the direction of *phyB* mutational effects were consistent in after-ripened seeds, but the effect of deficiency in *PHYD* on germination was weaker.

Response to after-ripening

Ler seeds that matured at warmer temperatures (22°C: 'long-warm' and 'short-warm') exhibited no effect of dry after-ripening, because even fresh seeds were nondormant (Fig. 2a,b). Warm-matured single and multiple mutations lacking functional *PHYB* most consistently had altered responses to dry after-ripening, and this difference was most apparent in stratification treatments that did not include cold (Fig. 2a,b, middle two panels). Mutants in *PHYB* were the most dormant mutants as fresh seeds (Figs 1b,c and 3b,c), so they lost more dormancy with dry after-ripening than the other genotypes, increasing the magnitude of their response relative to Ler (Supplementary material Table S1). Monogenic *phyD* seeds were also more responsive to dry after-ripening than Ler when given a warm seed-stratification treatment (Fig. 2a,b, third panel). Fresh *phyD* seeds were induced into dormancy by warm stratification (Fig. 1c), unlike Ler, but dry after-ripening alleviated this dormancy, leading to a significant effect of after-ripening. Therefore, phytochromes – and especially *PHYB* – are important for the germination of fresh seeds that are not given cold stratification, but dry after-ripening reduced this requirement in the Ler background.

Ler seeds matured at cooler temperatures lost some (but not significant) dormancy with dry after-ripening (Fig. 2c), except when given the warm–cold seed-stratification treatment (Fig. 2c, far right). The warm–cold stratification treatment

broke dormancy in fresh Ler seeds, but not in after-ripened Ler seeds. In cool-matured seeds, mutants lacking functional *PHYB* did not lose dormancy with dry after-ripening in the neutral seed-stratification treatment (Fig. 2c, second panel), unlike Ler, indicating a potential role of *PHYB* in the after-ripening of cool-matured seeds, although this effect was not significant after Bonferroni correction. In the warm–cold seed-stratification treatment (Fig. 2, far right), all mutants except for monogenic *phyA* mutants and the monogenic *phyE* mutant differed from Ler in their response to after-ripening. These two mutants, like Ler, were nondormant as fresh seeds and acquired some dormancy with dry after-ripening. The other genotypes were dormant as fresh seeds and either remained dormant (e.g. *phyB*, *phyD*, *phyA*/*phyB*, *phyB*/*phyD*) or lost dormancy with dry after-ripening.

Discussion

In this study it was found that the environment during seed maturation strongly influenced germination and the role of different phytochromes during dormancy and germination. Maternal temperature effects on germination were much stronger than maternal photoperiod effects. Cool temperatures during seed maturation induced intense dormancy, and this dormancy was broken in the Ler background only by a sequence of warm seed-stratification followed by cold seed-stratification. Thus, seeds matured in early spring or autumn may not be able to germinate in their first spring, but they may have to first be exposed to warm (summer) temperatures and then to cool temperatures, and would probably germinate in the autumn. Therefore, these maternal temperature effects could promote a winter-annual rather than a rapid-cycling life history in this background. Interestingly, this response appears to vary among ecotypes, as an appreciable proportion of cool-matured seeds of the Columbia ecotype germinated after merely cold (as opposed to warm–cold) seed-stratification (Donohue *et al.*, 2007), suggesting that Columbia may express a rapid-cycling life history. These predictions need to be tested in the field with actual seasonal temperature fluctuations.

Importantly, both functional *PHYB* and functional *PHYD* were essential for the breakage of dormancy that was induced by cool seed-maturation temperatures. The pronounced ability of cool seed-maturation temperature to induce dormancy, and the complete dependence of functional *PHYB* and *PHYD* to break this dormancy, indicate a new ecologically important role of these phytochromes. Specifically, seeds matured in early spring or late autumn would not be able to germinate under a wide range of conditions unless both *PHYB* and *PHYD* were functional. Such a strong contribution of *PHYD* to germination has not been documented before.

Temperature-dependent phytochrome contributions to phenotypes have been documented previously. In particular, while *PHYB* had for many years been considered as the primary phytochrome to regulate flowering time, Halliday & Whitelam (2003) demonstrated that when plants are grown at cooler temperatures (16°C as opposed to 22°C), *PHYE* and *PHYD* are more important regulators of flowering. In addition, we previously showed that the contribution of particular phytochromes to germination depended on the seed imbibition temperature, with *PHYE* being particularly important for germination at low temperature (Heschel *et al.*, 2007). In this study we show that the contribution of particular phytochromes to germination also depends on the temperature of seed maturation. Thus, phytochromes now appear to be generally involved in phenological responses, not only to light conditions but also to temperature (Franklin & Whitelam, 2004).

It is notable that the *PHYD* mutant used in this experiment is a natural null allele (Aukerman *et al.*, 1997). Such natural variation in *PHYD* is especially interesting in light of these results. Natural selection on germination timing is intense in *A. thaliana* (Donohue *et al.* 2005). Natural selection on *PHYD* (and *PHYB*), operating through maternal temperature effects on germination, will probably be geographically variable because seasonal conditions during seed maturation are highly likely to vary geographically. One would expect strong natural selection to maintain the function of *PHYB* and *PHYD* in temperate regions, in which seeds are matured under cool conditions during early spring or autumn and thereby induced into strong dormancy. By contrast, in warmer climates or in summer annuals, in which seeds are not matured under cool temperatures, and therefore not induced into strong dormancy by cool maturation temperatures, natural selection on these phytochromes via germination could be relaxed. This would be so, especially for *PHYD*, which has a weaker influence on germination than *PHYB* in other conditions. Thus, selection on these phytochromes would depend both on geographic location and the phenology of reproduction. The WS ecotype, from which the natural null *PHYD* allele is derived, is thought to be a summer annual and would probably mature seeds under warmer temperatures, possibly avoiding the maternal effect of cool-induced dormancy. Thus, a null *PHYD* allele may not be disadvantageous in a summer-annual life history. As more information becomes available on the distribution of phytochrome alleles across geography and across ecotypes with different life-history expression, tests of these adaptive hypotheses will be possible.

PHYA also exhibited a novel effect that was most prominent when seeds were matured under cool temperature; namely, it appears to inhibit the germination of cool-matured seeds. Its effect was apparent only on the *phyD* background, and not on the *phyB* background. The release from cool-induced dormancy therefore was only apparent when both *PHYD* and *PHYA* function was disrupted, suggesting that these two phytochromes may redundantly impede the germination or dormancy breakage of cool-matured seeds. Such a contribution of these phytochromes to environment-dependent suppression of germination has not been reported previously, and the mechanism for this effect is completely unknown.

While maternal photoperiod effects on germination were much weaker than maternal temperature effects, we found tentative evidence that the contribution of particular phytochromes to germination may depend weakly on the photoperiod during seed maturation. At warm seed-maturation temperatures, functional *PHYB* contributed somewhat more strongly to the germination of seeds matured under short days, whereas functional *PHYD* contributed somewhat more strongly to the germination of seeds matured under long days. *PHYB* may therefore be an important regulator of the germination of seeds matured early in the spring in warm climates, whereas *PHYD* may be important for the germination of seeds matured later in spring and summer. These results, and the lack of response to photoperiod in the Ler background, in which all phytochromes are functional, suggests that the photoperiod-dependent down-regulation of particular phytochromes or phytochrome-specific pathways may contribute to the expression of maternal photoperiod effects on germination.

The dependence of germination on phytochromes was weaker for seeds that had been given dormancy-breaking treatments, such as cold stratification or dry after-ripening. Cold stratification is known to break dormancy in many species (Baskin & Baskin, 1998). Seeds given a cold stratification treatment were not as dependent upon functional phytochrome for germination. This observation accords with recent findings which show that the gibberellin oxidase gene (GA_4), whose protein metabolizes inactive GA_9 into bioactive GA_4, is up-regulated by both phytochrome, via the repression of the gene *PIL5*, and by cold, via the repression of the gene *SPATULA* (Toyomasu *et al.*, 1998; Yamaguchi *et al.*, 1998; Hedden & Phillips, 2000; Garcia-Martinez & Gil, 2002; Ogawa *et al.*, 2003; Oh *et al.*, 2004; Yamauchi *et al.*, 2004; Penfield *et al.*, 2005). Thus, if cold is present, phytochrome may no longer be required for GA_4 expression, and GA_4 can still be synthesized, leading to germination.

Likewise, in all cases, the dependence of germination responses on phytochromes was weaker in dry after-ripened seeds than in fresh seeds. After-ripening is the process of dormancy loss during a period of dry storage, and its physiological basis is largely unknown. Abscisic acid (ABA) is known to be associated with the induction and maintenance of dormancy (reviewed in Kucera *et al.*, 2005). Ali-Rachedi *et al.* (2004) showed that after-ripened nondormant seeds have slightly lower levels of ABA, ABA levels decreased more slowly in nonafter-ripened than in after-ripened imbibed seeds, and ABA levels increase again subsequently in nonafter-ripened seeds, indicating a key role of ABA in the loss of dormancy in after-ripened seeds. GA is known to oppose ABA-induced dormancy (Kucera *et al.*, 2005). GA is required for germination, but it has a reduced role when seeds lack ABA (Koornneef *et al.*, 1982, 2002; Karssen *et al.*, 1983, 1989). The reduced importance of phytochromes in promoting germination in after-ripened seeds is in accordance with its putative involvement in GA_4 synthesis; the requirement for active phytochrome could be lower in after-ripened seeds because the requirement for GA_4 is lower. In addition, phytochromes have recently been shown to be involved in ABA catabolism (Seo *et al.*, 2006). Several genes of unknown function are also involved in dormancy, including reduced dormancy genes (*RDO*) (Peeters *et al.*, 2002; Leon-Kloosterziel *et al.*, 1996) and delay of germination genes (*DOG*), genes that were identified from a quantitative trait locus (QTL) analysis of dormancy and after-ripening (Alonso-Blanco *et al.*, 2003), and one of which (*DOG1*) has just recently been cloned (Bentsink *et al.*, 2006). Because so little is known about the genetic and physiological basis of dormancy, it is difficult to know whether the phytochromes regulate germination solely through the physiological pathways of GA synthesis and ABA catabolism.

We found distinct effects of seed-maturation conditions on germination, and we determined that specific phytochromes contribute to germination most strongly under particular seed-maturation conditions. However, we were not able to determine the timing or anatomical location of phytochrome gene expression that is responsible for these maternal effects on germination. Reciprocal crosses would be necessary to determine whether the maternal or embryonic genotype controls these germination responses to seed-maturation conditions, and gene expression studies would be required to identify the timing and location of the expression of phytochrome genes and other genes in phytochrome-mediated pathways.

In conclusion, maternal environmental effects altered the relative contributions of different phytochromes to germination. Therefore, the relative importance of different phytochromes for promoting germination would depend on the seasonal timing of reproduction in natural populations. The down-regulation of particular phytochromes or phytochrome-specific germination pathways may be necessary for seeds to exhibit seasonal maternal effects on germination. Because flowering time varies both geographically and genetically, natural selection on phytochromes via their influence on germination is likely to vary as a function of geographic location and genetic background. In particular, we showed that a natural allelic variant of *PHYD* had lost its ability to germinate under conditions that break dormancy in the background genotype, when seeds were matured under one set of realistic (cool) environmental conditions. Thus, to examine the genetic basis of germination, we need to consider maternal environmental effects as major factors. Because of the direct link between flowering season and germination phenology via maternal effects on germination, considering maternal effects on germination also contributes to our understanding of the causes of variation in entire plant life cycles and generation time.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Differences between wild-type and mutant genotypes in short-warm, after-ripened seeds

Table S2 Differences between wild-type and mutant genotypes in short-cold, after-ripened seeds

Table S3 Significant differences between specific mutant pairs in short-cold, after-ripened seeds

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