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NEW ROLES OF PHYTOCHROMES DURING SEED GERMINATION

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Among the most important environmental sensors in plants are the phytochromes, members of a duplicated-gene family of photoreceptors. We characterized the functional diversification of this gene family with respect to a single ecologically important and highly environmentally sensitive process—germination—and thereby addressed the consequence of gene duplication in the evolution of environmental sensitivity. We exposed single and multiple mutants of different phytochromes of *Arabidopsis thaliana* to dormancy-breaking or dormancy-inducing treatments that resemble different seasonal environmental conditions, and we recorded their germination responses. Much redundancy of function occurred, but an impressive degree of functional diversification was also apparent. Specifically, PHYB was an important contributor to germination when seeds did not receive low-temperature imbibition. It interacted nonadditively with PHYA, whose contribution to germination was redundant to that of PHYB and most apparent when seeds experienced high followed by low temperatures. We also determined that PHYD was necessary for full germination when seeds were exposed to a high temperature during imbibition. PHYE appears to contribute redundantly to germination, except when seeds experienced high followed by low temperatures. Finally, we found new evidence that PHYA and possibly PHYD redundantly contribute to the suppression of germination. The diversification of activities of all these phytochromes during this one process of germination suggests that they all have a combined function in the regulation of germination responses to complex seasonal conditions and that gene duplication can contribute to the evolution of precise responses to variable environments.

Keywords: dormancy, duplicated genes, germination, phenotypic plasticity, phytochrome.

Seed germination is one of the most important developmental switches in a plant's life history, as the seasonal timing of germination has major effects on total lifetime fitness (Finch-Savage and Leubner-Metzger 2006). In one experimental population of *Arabidopsis thaliana*, for example, variation in germination timing was associated with up to 72% of the variation in fitness among recombinant inbred genotypes (Donohue et al. 2005). To germinate at the appropriate time of year, seeds need to process complex environmental cues that accurately predict seasonal conditions. Seasonal germination cueing can be impressively precise because some environmental factors are necessary to break dormancy and additional factors are necessary to permit germination after dormancy is broken (Bewley 1997; Foley 2001). The precise physiological and genetic mechanisms regulating germination timing in response to seasonal environmental factors are largely unknown.

Among the most important environmental sensors in plants are the phytochromes—a duplicated-gene family of photoreceptors that perceive red-light and far-red-light wavelengths (Koornneef et al. 1980; Reed et al. 1994; Shinomura et al. 1994; Poppe and Schaefer 1997; Shinomura 1997; Terry 1997; Whitelam and Devlin 1997; Casal and Sanchez 1998). Phytochromes are photoreversible biliproteins, composed of an apoprotein and a chromophore. They are synthesized in the red-absorbing form (Pr), and on exposure to red light, they switch isomeric conformation into the far-red-absorbing form (Pfr), which is the primary bioactive form. In the Brassicaceae, which includes *A. thaliana*, the apoproteins are encoded by five genes that have arisen through a series of gene duplications (Sharrock and Quail 1989; Clack et al. 1994; Mathews and Sharrock 1997). An early duplication led to two phytochrome clades, one of which now comprises PHYA and PHYC and one that comprises PHYB, PHYD, and PHYE. PHYD is a recent duplicated copy, most closely related to PHYB, and it occurs only in the Brassicaceae. Determining the functional diversity of these phytochromes is an area of intense research that has contributed to our understanding of the evolution of duplicated-gene families and the origin of functional novelty (reviewed in Mathews 2006). Here we investigate how functional diversification of the duplicated phytochromes contributes to the precision of environmental sensing during germination.

It has long been known that PHYA and PHYB regulate germination responses to light (Shinomura et al. 1994; Poppe and Schaefer 1997; Shinomura 1997; Ritchie and Gilroy 1998; Koornneef et al. 2002), with PHYB being important

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under red light and PHYA being important under far-red light. Much evidence exists for the role of phytochromes in promoting the synthesis of gibberellins (GAs), which are important stimulants for germination (Derkx et al. 1994; Toyomasu et al. 1998; Yamaguchi et al. 1998; Yamaguchi and Kamiya 2000; Garcia-Martinez and Gil 2002; Koornneef et al. 2002; Ogawa et al. 2003). Phytochromes also play a role in regulating the sensitivity to GAs (Hillhorst and Karssen 1988; Yang et al. 1995). Recently, phytochromes have also been shown to be involved in the degradation of abscisic acid, the major plant hormone that maintains dormancy (Seo et al. 2006). PHYE has been shown to contribute to germination in continuous far-red light (Hennig et al. 2002). In addition, PHYD, in combination with PHYB, has been shown to have a role in inhibiting the PHYA-mediated very low-fluence response in far-red light (Hennig et al. 2001). Thus, different phytochromes are involved in diverse aspects of light responses to germination, in part through hormonal pathways.

Our recent work indicates that phytochromes influence germination responses not only to light but also to multiple seasonal cues, including seed maturation conditions, dormancy-breaking and dormancy-inducing temperatures, and poststratification temperature (Donohue et al. 2007). Characterizing the functional diversification within this duplicated-gene family with respect to germination, a single ecologically important process, will contribute to our understanding of the consequence of gene duplication in the evolution of environmental sensitivity.

In this study, we investigated the contributions of different phytochromes to germination after different dormancy-breaking and dormancy-inducing temperature treatments. Dormancy is defined as the temporary failure of a viable seed to germinate under a set of environmental conditions that later evoke germination following some environmental treatment (Simpson 1990). We exposed single and multiple loss-of-function mutants of different phytochromes to dark, wet imbibition treatments that resembled different seasonal conditions and recorded their germination responses in white light. We identified novel contributions of phytochromes to germination and dormancy and found a diversification of phytochrome contribution under different conditions.

Methods

We compared germination responses of several phytochrome mutants of *Arabidopsis thaliana* to those of their background genotype, Landsberg *erecta* (Ler hereafter; table 1). The Ler background genotype is not dormant under standard culturing conditions, possibly because of artificial selection for convenience of culturing over many generations in the lab, although it may have lacked dormancy before extensive culturing. However, seeds of Ler can be induced into dormancy under some conditions. Other nondormant natural ecotypes have been identified under standard growth conditions (K. Donohue, unpublished data), so Ler is not anomalous in regard to dormancy. Most physiological mutants of *A. thaliana* are derived from this Ler background. Mutants with loss of phytochrome function that restore dormancy under conditions in which Ler is nondormant can identify pathways that are likely to be downregulated during dormancy maintenance. Mutants with loss of phytochrome function that do not break dormancy under conditions that break dormancy in the Ler genotype can identify pathways whose expression is necessary to overcome dormancy. Had multiple phytochrome mutants been available on a background more dormant than Ler, loss-of-function mutants that had high germination could have identified loci required for primary dormancy maintenance. Such mutants are

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
$phyA_1B_5$		*phyA-201/phyB5*	ABRC-CS6224
$phyA_1B_{1S}$		*phyA-201/phyB-1(S)*	RAS
phyD	Natural null (introgressed allele from the Wassilewskija ecotype)	*phyD-1*	RAS
$phyB_{1S}D$		*phyB-1(S)/phyD-1*	RAS
$phyA_1D$		*phyA-201/phyD-1*	RAS
$phyE_1$		*phyE-1*	GW
$phyA_2E$		*phyA-202/phyE-1*	GW
$phyB_1E$		*phyB-1/phyE-1*	GW
$phyB_1DE$		*phyB-1/phyD-1/phyE-1*	GW

Note. "Reference name" is the name given to the line in this article. ABRC = Arabidopsis Biological Resource Center at Ohio State University. RAS = R. A. Sharrock. GW = G. Whitelam.

being developed, but they were not available at the time of this study.

With the exception of *phyD-1*, all *phy* 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 a 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 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 using a PCR assay as described by Aukerman et al. (1997). Homozygous *phyD* BC7 F_2 plants were identified and were used to construct the further multiple mutant lines. The corresponding backcrossed Ler line, into which the PHYD allele was introgressed and which contains the wild-type Ler PHYD allele, was used for all comparisons of mutants containing *phyD*. Multiple *phyE* mutants were constructed as described by Devlin et al. (1998).

Replicates of each genotype were planted in Conviron E7/2 growth chambers (Controlled Environment, Winnipeg, MB), with a 14L : 10D cycle of full-spectrum (fluorescent plus incandescent) light at 22°C. These conditions resemble seasonal conditions during a typical seed maturation season in late spring in temperate climates. Plants were grown in four temporal blocks, with two chamber compartments per block and three plants per genotype per compartment. Plantings were conducted so that seeds of all genotypes matured simultaneously. Seeds were harvested during 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 imposed four seed imbibition treatments to assess dormancy breakage and dormancy induction. We conducted the germination assays on seeds within 7 d after harvesting them. 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 the four temporal blocks, were used for each genotype in each imbibition treatment (144 seeds per genotype per treatment). Four dark imbibition treatments were imposed: "neutral," which consisted of 5 d at 22°C; "low-temperature," which consisted of 5 d at 4°C; "high-temperature," which consisted of 7 d at 31°C; and "high-low," which consisted of 7 d at 31°C, followed by 5 d at 4°C. All plates were placed at 22°C in a Percival germination incubator (Percival Scientific, Perry, IA) with a 12-h photoperiod of white fluorescent light (with photon flux density of 90 μmol m^{-2} s^{-1}) after their period of imbibition. Treatments were staggered so that all plates would be transferred to light simultaneously. Preliminary studies of Ler and other ecotypes showed that seeds tended to germinate to high frequencies after five or fewer days at 4°C, while 7 d or longer at 31°C induced dormancy in natural ecotypes (K. Donohue, unpublished data). Therefore, the neutral treatment assesses primary dormancy, the low-temperature treatment assesses the ability of 4°C to overcome primary dormancy, the high-temperature treatment assesses the ability of 31°C to induce dormancy, and the high-low treatment assesses the ability of 4°C to break primary plus warm (31°C)-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 and Baskin 1998).

To test for significant differences in responses to experimental treatments among the genotypes, we conducted ANOVAs and verified significance with nonparametric Kruskal-Wallis tests. We first conducted analyses with all lines in a full model with block, genotype, and imbibition treatment and saw highly significant interactions (results not shown). We then tested for significant differences between each mutant and the Ler background in their responses to the imbibition treatments by conducting a series of separate ANOVAs for each mutant-background pair. A significant treatment-by-genotype interaction from the ANOVA indicates that the genotype differed from Ler in response to imbibition and that the loss of the functional phytochrome caused a significant alteration of germination response to imbibition. We conducted similar tests for significant differences between specific mutant pairs in their response to imbibition. Significance levels were Bonferroni corrected according to the total number of analyses run to test for genotype-environment interactions over the whole experiment. We next compared, in each imbibition treatment separately, the germination of each mutant to the background, Ler, and conducted a priori contrasts of specific mutant pairs in a model with all lines by using the "contrast" statement in SAS (1990). Because data were not always normally distributed, we conducted a series of nonparametric Kruskal-Wallis tests to verify the significance of these results. In most cases, the significance did not differ between the analyses, and if it did, it changed from marginally significant to nonsignificant or vice versa. Differences are indicated in the tables. Bonferroni significance, based on the total number of comparisons tested within each imbibition treatment, is also indicated in the tables. Block was included in all models as a fixed factor.

Finally, we tested for nonadditive interactions between allelic states in multiple mutants using ANOVA in which functional versus nonfunctional alleles were coded as dummy variables. We tested for nonadditive interactions between PHYA and PHYB by using a model that included Ler, *phyA*, *phyB*, and the *phyA/phyB* double mutants (model: germination = $phyA + phyB + phyA \times phyB$; with *phyA* and *phyB* scored as 0 [mutant] or 1 [wild-type]). Similarly, we also tested for interactions between PHYB and PHYD by using a model that included Ler, *phyB*, *phyD*, and the *phyB/phyD* double mutant, and we tested for interactions between PHYA and PHYD by using a model that included Ler, *phyA*, *phyD*, and the *phyA/phyD* double mutant.

Results

PHYA and PHYB

The background genotype, Ler, exhibited a lack of dormancy in all treatments. Disruption of PHYA alone did not significantly alter germination responses to our dormancy-breaking or dormancy-inducing imbibition treatments, as the monogenic *phyA* mutants resembled the wild type by being nondormant and unresponsive to the imbibition treatments (fig. 1; table 2). In contrast, monogenic *phyB* mutants had

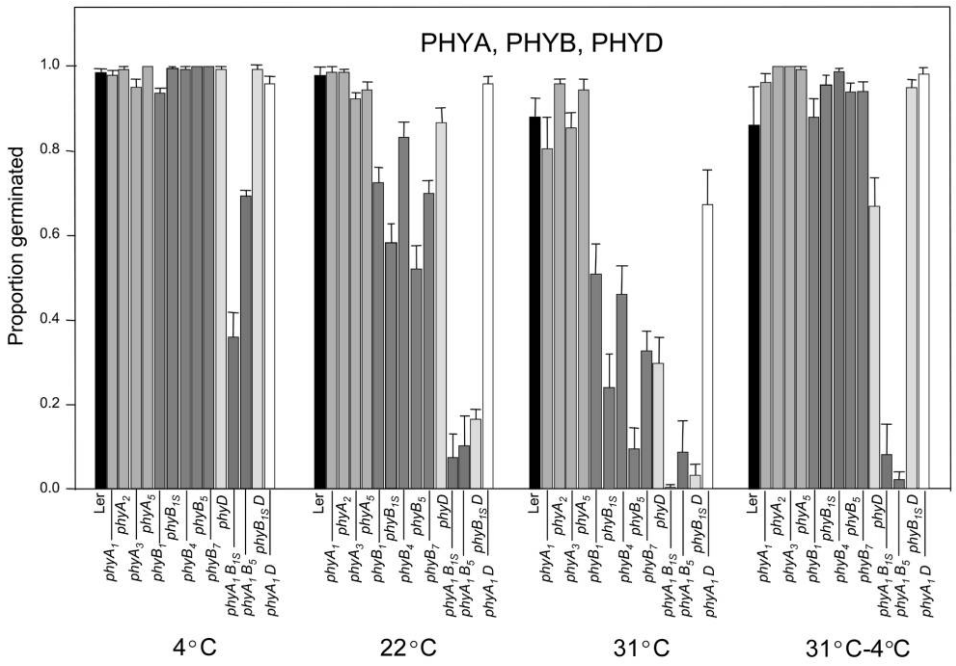

Fig. 1 Proportion of seeds germinated of the background genotype, Ler (black bars), and mutants of PHYA, PHYB, and PHYD. Means and standard errors are shown.

much-reduced germination in imbibition treatments that did not include a low-temperature treatment (that is, the 22° and 31°C treatments), and the difference was particularly pronounced in the 31°C imbibition treatment. Both double mutants of *phyA/phyB*, unlike the single *phyB* mutant, were not able to germinate well even after a low-temperature treatment (4° or 31°–4°C treatment). Both *phyA/phyB* mutants had germination that was reduced below that of the single *phyB* mutant in all treatments except the 31°C imbibition treatment (table 3). The effect of disrupting PHYA was smallest in 31°C imbibition because the *phyB* mutant already had very low germination. The effect of disrupting PHYA (on the *phyB* background) was most pronounced in the high-low imbibition treatment. Thus, phyA did contribute to germination under the experimental conditions, but it did so redundantly and to a lesser extent than phyB. The effects of the *phyA* and *phyB* mutations were nonadditive in all but the 31°C imbibition treatment, indicating a nonlinear dosage response of germination to contributions by these two phytochromes (table 4).

PHYB and PHYD

Disruption of PHYD alone significantly reduced germination in treatments that had a high-temperature (31°C) imbibition, even if that high-temperature imbibition was followed by low temperature (31°–4°C; fig. 1; table 2). Thus, phyD is necessary for full germination when seeds are exposed to high-temperature imbibition—a function not previously known. Like *phyA*, the effect of the *phyD* mutation was strongly apparent on the *phyB* background even under the neutral (22°C) imbibition treatment (table 3), with nearly significant nonadditivity between allelic effects in neutral and high-temperature imbibition (table 4), indicating a nonlinear dosage response of germination to contributions by phyB and phyD.

PHYA and PHYD

In addition to contributing to germination, as shown above, phyA and phyD apparently redundantly suppress germination. The double *phyA/phyD* mutant exhibited high germination across all treatments but with slightly reduced germination compared to that of Ler after imbibition at 31°C (fig. 1; table 2). Importantly, compared to the *phyD* mutant, the *phyA/phyD* mutant actually had higher germination in all but the low-temperature (4°C) imbibition treatment (only marginally significant in the 31°C imbibition treatment after Bonferroni correction), indicating that phyA suppresses germination under these conditions (table 3). However, the suppression of germination by phyA was apparent only with disruption of PHYD (figs. 1, 2; table 2), suggesting that phyA and phyD simultaneously suppress germination. Significant nonadditivity of the two phytochromes was detected in the 31°C imbibition treatment, in which the single *phyD* mutant had the greatest effect on germination (table 4).

PHYE

Disruption of PHYE alone did not influence germination significantly in any treatment (fig. 2; table 2), and none of the multiple mutants in PHYE had reduced germination in the high-low (31°–4°C) imbibition treatment. Disrupting PHYE on a $phyA_2$ background (*phyA* vs. *phyA/phyE*) reduced germination in all but the high-low imbibition treatment (marginally significant after Bonferroni correction in the 22°C imbibition treatment). Disruption of PHYE on a *phyB* background (*phyB*

Table 2

Tests for Significant Differences between Mutant and Background (Ler) Genotype

Genotype vs. Ler	4°C	22°C	31°C	31°–4°C	Genotype × stratification
Mutants in PHYA:					
phyA-2	.01	.01	.76	2.36	1.24
phyA-3	.38	.44	.10	1.96	1.77
phyA-5	.06	.18	.51	2.35	1.38
phyA-201	.02	.01	.76	1.32	.84
Mutants in PHYB:					
phyB-1	.75	9.07**	18.09***	.05	4.91**
phyB-1S	.02	22.18***	65.45***	.30	23.95***
phyB-4	.02	3.03	22.99***	2.17	9.11***
phyB-5	.06	29.34***	80.33***	.84	28.61***
phyB-7	.0	29.26***	95.18***	.51	13.45***
Double mutants in PHYA and PHYB:					
phyA201-/phyB-5	27.02***	107.23***	81.87***	94.85***	7.88***
phyA-201/phyB-1	129.64***	114.15***	116.49***	119.95***	4.41**
Single and multiple mutants in PHYD:					
phyD-1	.02	1.17	55.48***	15.51***	14.81***
phyB-1/phyD-1	.02	92.46***	109.62***	.35	166.27***
phyA-201/phyD-1	.55	.06	10.07**	.05	5.05**
Single and multiple mutants in PHYE:					
phyE-1	3.92[a,*]	.34	.10	.79	.99
phyA-2/phyE-1	14.57***	8.80**	13.52***	2.39	3.64*
phyB-1/phyE-1	.02	35.37***	89.09***	1.56	43.75***
phyB-1/phyD-1/phyE-1	19.85***	75.37***	99.88***	.03	33.80***

Note. *F* ratios are given from ANOVAs that test for significant differences between mutant and background genotypes in each imbibition treatment. The last column gives the *F* ratios for genotype × stratification interaction to test for significant differences between mutants and background in response to imbibition. Bonferroni significance is indicated by underlining. The block effect was significant in all imbibition treatments except the 31°–4°C treatment (4°C: $F = 34.18$, $P < 0.001$; 22°C: $F = 15.34$, $P < 0.001$; 31°C: $F = 10.83$, $P < 0.001$; 31°–4°C: $F = 1.01$). All results are consistent with nonparametric Kruskal-Wallis tests unless otherwise indicated.

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

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

vs. *phyB/phyE*) reduced germination when seeds did not receive low-temperature treatment (marginally significant for the 22°C imbibition after Bonferroni correction and significant for the 31°C imbibition). Disruption of PHYE on a *phyB/phyD* background (*phyB/phyD* vs. *phyB/phyD/phyE*) significantly reduced germination after imbibition at 4°C (table 3) but not in the treatments without low temperature, in large part because the *phyB/phyD* mutant already had low germination in these treatments. These combined results suggest a contribution of phyE to germination under our treatments, except when seeds experience high followed by low temperatures (31°–4°C).

Discussion

We found evidence that PHYA, PHYB, PHYD, and PHYE all are involved in germination in *Arabidopsis thaliana*. PhyB was an important contributor to germination when seeds did not receive a low-temperature (4°C) imbibition. It interacted nonadditively with phyA, whose contribution to germination was redundant to that of phyB and whose contribution was most apparent when seeds experienced high followed by low temperatures (31°–4°C). We also determined that phyD was necessary for full germination when seeds were exposed to a high-temperature (31°C) imbibition—a previously unknown function. PhyE also appears to contribute redundantly to germination, except when seeds experience high-temperature followed by low-temperature imbibition. Finally, we found new evidence that phyA and possibly phyD simultaneously contribute to the suppression of germination or dormancy. Thus, phytochromes exhibit functional redundancy as well as a diversity of behaviors and potential for specialized functions during germination.

Confirmed Roles of PHYA and PHYB

This study confirmed the contribution of phyA and phyB to germination (Shinomura et al. 1994; Poppe and Schaefer 1997; Shinomura 1997; Ritchie and Gilroy 1998; Koornneef et al. 2002) and established an essential role of phyB for germination when seeds do not receive a low-temperature (4°C) treatment. Thus, downregulation of phyB-mediated pathways may be essential for germination to exhibit sensitivity to cold. A cold requirement for germination is likely to be adaptive, by preventing premature germination during late spring or summer, when drought conditions might exist.

The observation that monogenic *phyA* and *phyB* mutants can germinate after low-temperature imbibition accords with

Table 3

Tests for Significant Differences between Specific Mutant Pairs

Mutant contrasts	4°C	22°C	31°C	31°–4°C	Genotype × stratification
Mutants of PHYA, PHYB, and PHYD:					
phyB-1 vs phyA-201/phyB-1	126.84***	35.69***	7.31**	113.24***	7.87***
phyB-5 vs phyA-201/phyB-5	29.65***	24.39***	.01	125.25***	16.90***
phyB-1 vs phyB-1/phyD-1	.00	24.07***	5.66*	.00	8.13***
phyD-1 vs phyA-201/phyD-1	.00	68.65***	9.13**	11.69***	4.42**
Multiple mutants of PHYE:					
phyE-1 vs phyA-2/phyE-1	3.38	5.68*	11.29***	.48	2.27+
phyA-2 vs phyA-2/phyE-1	15.25***	9.25**	20.70***	.00	1.75
phyE-1 vs phyB-1/phyE-1	3.45	28.78***	83.20***	.12	45.84***
phyB-1 vs B-1/phyE-1	.55	8.62**	26.88***	1.16	1.51
phyB-1/phyD-1 vs phyB-1/phyD-1/phyE-1	20.97***	.89	.10	1.59	7.80***

Note. *F* ratios are given from ANOVAs that test for significant differences between the two mutants indicated in the first column in each imbibition treatment. The last column gives the *F* ratios for genotype × stratification interaction to test for significant differences between mutants in response to imbibition. Bonferroni significance is indicated by underlining. All results are consistent with nonparametric Kruskal-Wallis tests.

+ $P < 0.1$.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

studies that document the upregulation of genes involved in GA synthesis by both phytochrome and low temperature (Toyomasu et al. 1998; Yamaguchi et al. 1998; Hedden and Phillips 2000; Yamaguchi and Kamiya 2000; Garcia-Martinez and Gil 2002; Ogawa et al. 2003; Yamauchi et al. 2004; Penfield et al. 2005). Phytochrome is thought to repress PIL5, which in turn represses AtGA3ox1 and AtGa3ox2 (Oh et al. 2004; Penfield et al. 2005), two genes whose products convert inactive GA_9 into bioactive GA_4, which stimulates germination. Another gene, SPT, represses (in part via PIL5) AtGA3ox2 and especially AtGA3ox1. However, low temperature suppresses SPT and thereby indirectly upregulates AtGA3ox1 and AtGA3ox2, enabling the conversion of inactive to active GA. Thus, low temperature can increase the concentration of active GA_4, even without functional phytochromes.

The contributions of PHYA and PHYB (and of PHYB and PHYD) were strongly nonadditive, with the effect of PHYA disruption being apparent only on a PHYB-deficient background. One possible mechanism for the nonlinear response of germination to phytochrome activity is a nonlinear dose response of germination to GAs, whose synthesis is regulated by phytochromes (Toyomasu et al. 1998; Yamaguchi et al. 1998; Yamaguchi and Kamiya 2000; Ogawa et al. 2003). Both phytochromes may contribute to GA synthesis independently, with phyB contributing more than phyA to GA synthesis. When GA concentrations are saturating for germination, disruption of PHYA may not reduce GA concentrations below that required for germination, but if PHYB is also disrupted and GA concentrations are already low, disruption of PHYA could further reduce germination. This interpretation is consistent with the finding that phyB regulates AtGA3ox2, whose product catalyzes the final conversion from inactive to active GA and which is not subjected to negative regulation by active GA. In contrast, AtGA3ox1, which serves the same function, is regulated by other phytochromes (although probably not phyA) and is negatively regulated by bioactive GA. Therefore, the phyB-mediated synthesis of bioactive GA continues even at high bioactive GA levels, whereas GA synthesis mediated by other phytochromes does not proceed as well when bioactive GA levels are high. The contribution of other phytochromes to GA synthesis may be apparent only after the phyB-mediated pathway is disrupted and bioactive GA levels are low enough for the contribution of other phytochromes to be apparent. The difference in the effect of phytochrome disruption as a function of imbibition treatment could be due either to temperature-dependent allelic effects on GA synthesis (as mentioned above) or to temperature-dependent GA dose-response curves (change in GA sensitivity).

Alternative mechanisms for nonadditivity between phytochromes, including those seen between PHYB and PHYA and between PHYB and PHYD, could be antagonistic interactions between the phytochromes themselves or between components of specific phytochrome-mediated germination pathways. For example, if phyB acts antagonistically to phyA or its germination pathway, then disrupting PHYA when phyB is actively antagonizing the action of phyA may not have much phenotypic effect. If phyB antagonism of phyA is abolished, however,

Table 4

Tests for Interactions between Alleles in Each Imbibition Treatment

Interaction	4°C	22°C	31°C	31°–4°C
$phyA_1 \times phyB_5$	7.93**	12.96***	.23	103.11***
$phyA_1 \times phyB_{1S}$	75.14***	24.91***	1.15	53.32***
phyB × *phyD*	.30	9.36**	7.56**	1.72
phyA × *phyD*	1.06	2.30	8.58**	1.85

Note. Presence of a functional versus nonfunctional allele was coded as a dummy variable in an ANOVA. *F* ratios are given for the interaction between two allelic states from the analysis of double mutants (*phyA/phyB* and *phyB/phyD*). Bonferroni significance is indicated by underlining.

** $P < 0.01$.

*** $P < 0.001$.

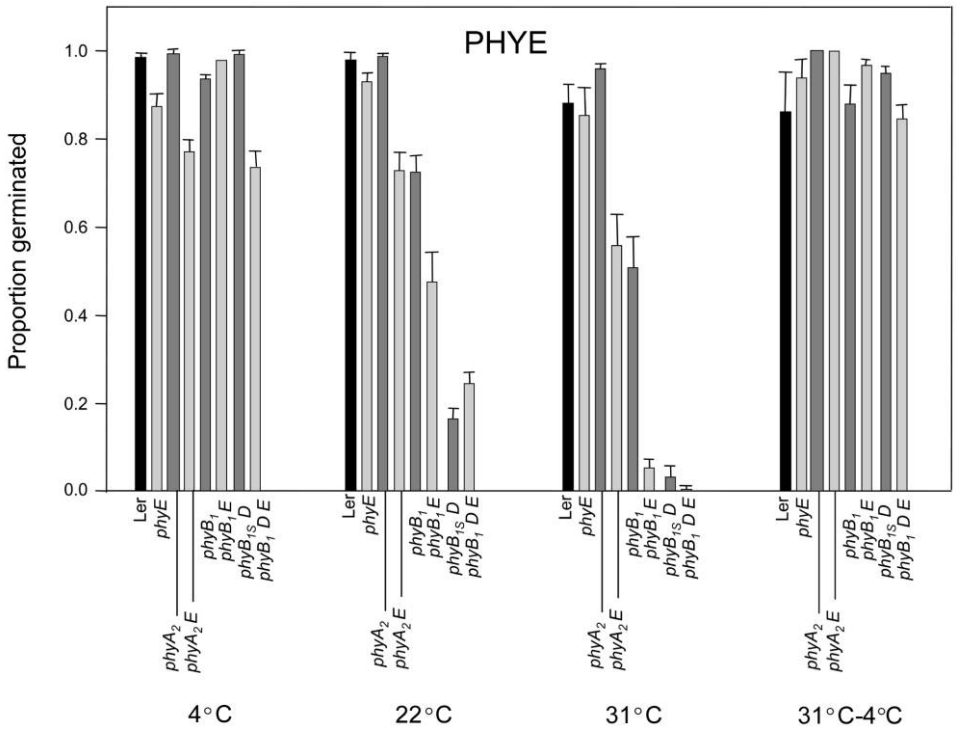

Fig. 2 Proportion of seeds germinated of the background genotype, Ler (black bars), and single and multiple mutants in PHYE. Means and standard errors are shown. Mutants with functional PHYE are shown by dark gray bars, and the comparable mutant lacking PHYE function is adjacent and is shown by light gray bars.

then disruption of PHYA could have observable phenotypic effects. Many mechanisms of antagonism are possible, including direct or indirect regulation of phytochrome gene expression, antagonism of phytochrome signal transduction, competition for binding partners (Sakamoto and Nagatani 1996; Shinomura 1997; Hennig et al. 2001), or even competitive heterodimerization of phytochrome apoproteins that may create nonfunctional complexes (although heterodimerization with phyA has not been documented; Sharrock and Clack 2004). In the high-irradiance response of de-etiolation, the antagonistic activities of phyA and phyB can be explained by their different photoactivities, with phyB inducing de-etiolation in red light and phyA inducing de-etiolation in far-red light (reviewed in Mathews 2006)—either through very low amounts of Pfr, even in far-red light, or possibly through some bioactivity of phyA's short-term intermediate isomers (Shinomura et al. 2000; Sharrock and Clack 2004).

New Roles for PHYD and PHYE

PHYD was shown to contribute strongly to germination after imbibition at high temperature (31°C). Imbibition at high temperature is likely to occur soon after seed dispersal when seeds are shed in early summer. Imbibition at high temperature has been shown to induce dormancy in some genotypes of *A. thaliana* (Donohue et al. 2008), and such warm induction of dormancy could prevent germination of seeds when drought conditions might be fatal. The observed contribution of phyD to germination after imbibition at high temperature suggests that the downregulation of phyD-mediated germination pathways is necessary for warm-induced dormancy or to prevent germination after exposure to high temperature. Like phyA, phyD also detectably contributed to germination under neutral (22°C) imbibition temperatures but only on a PHYB-deficient background.

PHYE acted redundantly to PHYA, PHYB, and PHYD during germination. Interestingly, in contrast to phyA, phyE appears to contribute to germination only when seeds do not experience high followed by low temperatures (31°–4°C). Hennig et al. (2002) also documented a role of phyE in germination and emphasized its role in far-red-light-dependent germination. Furthermore, Heschel et al. (2007) showed that functional phyE is necessary for germination at cool temperatures. While redundant to PHYA, PHYB, and PHYD under the conditions of this experiment, PHYE appears to have a distinct role in temperature-dependent germination.

PHYA-PHYD Dormancy

Perhaps the most surprising finding was that phyA and phyD not only contribute to germination but also appear to be involved in the repression of germination or the maintenance of dormancy. Disruption of PHYD decreased germination on the *phyB* background but not on the *phyA* background. More important, disruption of PHYA decreased germination significantly on the *phyB* and *phyE* backgrounds but not on the *phyD* background. Instead, the disruption of PHYA restored germination of *phyD* to wild-type levels in most treatments.

Thus, while the disruption of either PHYA or PHYD singly caused reduced germination in some backgrounds, indicating their contribution to germination, simultaneous disruption of both PHYA and PHYD induced higher germination than the single *phyD* mutants. This result accords with another study (Donohue et al. 2008), which documented restored germination of *phyA/phyD* mutants compared to low-germinating *phyD* mutants under diverse conditions. The finding that both PHYA and PHYD had to be disrupted to relieve dormancy suggests that they contribute to dormancy redundantly.

These two phytochromes therefore simultaneously contribute to one function—germination—and also to the opposite function—dormancy. The contribution of phytochromes to the suppression of germination is not unprecedented, and one example is the hypothesized inhibitory effect of phyB during phyA-mediated germination in far-red light (Reed et al. 1994). Different possible mechanisms exist for the observed suppression of germination by phytochrome. First, phytochromes are known to regulate GA oxidases that metabolize and catabolize bioactive GA (Yamaguchi et al. 1998; Ogawa et al. 2003; Yamauchi et al. 2004). The contribution of phytochromes to germination is, in large part, through the conversion of inactive to active GA metabolites. Thus, their repression of germination could conceivably be through conversion of active to inactive metabolites. Some evidence for phytochrome-mediated GA catabolism via GA2ox during deetiolation exists in pea (Ait-Ali et al. 1999; Reid et al. 2002), but no such evidence has been found in *A. thaliana*.

Alternatively, phyA and phyD may suppress an entirely different germination pathway, or they may operate through a pathway of dormancy, such as the abscisic acid (ABA) pathway (Koornneef et al. 2002). Some recent evidence exists for interactions between phytochromes and ABA pathways, based on genome-wide expression studies. These studies found that ABA-regulated genes were upregulated in seedlings of *phyB* mutants, implicating phytochromes in their regulation, either directly or indirectly (Mazzella et al. 2005). Recently, Seo et al. (2006) showed that ABA levels in seeds decrease in red light, that this reduction in ABA depends on functional phyB, and that the expression of genes involved in ABA metabolism/catabolism is regulated by phytochrome in a photoreversible manner. Moreover, phytochrome-mediated ABA degradation has been documented in some taxa (Tillberg and Bjorkman 1993; Kraepiel et al. 1994), and GA (whose synthesis is stimulated by phytochrome) is associated with decreased levels of ABA in lettuce (Weatherwax et al. 1996; Ogawa et al. 2003; Gonai et al. 2004; Toyomasu et al. 2004). In *A. thaliana*, in contrast, addition of GA caused a transient increase in ABA (Ali-Rachedi et al. 2004). Thus, complex interactions between GAs and ABA biosynthesis and degradation pathways may contribute to the observation of phytochrome inhibition of germination. Specifically, if phytochrome-mediated GA synthesis does cause an increase in ABA, then the net balance could possibly result in an association between phytochrome function and ABA-induced dormancy. Further physiological studies are necessary to elucidate the mechanism of phytochrome-associated dormancy.

Finally, in other species, the high irradiance response (HIR) is in the form of germination inhibition in continuous far-red light, suggesting a role of phyA in germination inhibition (Hartman 1966; Hendricks et al. 1968; Schopfer and Placy 1993). While such a HIR inhibition of germination has not been documented in *A. thaliana* (Cone and Kendrick 1985; McCormac et al. 1993), it is conceivable that the mechanism of HIR suppression of germination observed in other species may be related to the suppression of germination by phyA (and perhaps phyD) in *A. thaliana* shown here. Moreover, in some monocots, red light inhibits germination (Hilton 1982; Hou and Simpson 1993), suggesting an evolutionarily flexible contribution of phytochromes to germination promotion and inhibition.

Functional Redundancy and Diversification

We found a great deal of redundancy of contributions of the different phytochromes to germination. In particular, the contribution of phyA was not apparent unless PHYB was dysfunctional, and the contribution of phyD was also much more apparent on a *phyB* background. Contributions of phyE to germination were not apparent, except on a *phyA*, *phyB*, or *phyB/phyD* background. In general, under the conditions of this experiment, it appears that the contribution of phytochromes to germination are roughly in the order of phyB > phyD > phyA > phyE.

Despite the redundant contributions of phytochromes to germination, much diversity of phytochrome function during germination exists, such that the relative importance of different phytochromes depends on the particular environmental conditions. Specifically, phyA appears to contribute to germination least strongly after high-temperature (31°C) imbibition but most strongly after imbibition at high followed by low temperatures (31°–4°C). PhyB is important primarily when seeds do not receive low-temperature (4°C) imbibition, while phyD is important primarily after high-temperature (31°C) imbibition. PhyE contributed to germination only if seeds did not experience high followed by low temperatures, and it is important for germination at low temperature (Heschel et al. 2007).

Not only are the contributions of the different phytochromes to germination environment-dependent, but also two phytochromes, PHYA and PHYD, exhibited evidence for an entirely different role: suppression of germination. This is a major differentiation of behavior among the phytochromes because the two processes—germination and dormancy—are in direct opposition to each other. This differentiation of function has the potential to lead to a complex regulation of germination in response to different seasonal environmental factors.

Thus, the members of this duplicated-gene family each appear to contribute to the regulation of the single process of germination but under different environmental conditions. This diversified but related function of the phytochromes suggests an interesting role of gene duplication in the evolution of environmental sensitivity. The duplication of a gene enables the independent regulation of loci that may encode slightly different gene products (here, phytochromes) that may have different environmental stabilities or activities. For example, a phytochrome that promotes germination in low temperature (e.g., PHYE; Heschel et al. 2007) may be expressed only when other favorable environmental cues are also present, whereas a phytochrome that promotes germination after high temperatures

(e.g., PHYD) may be expressed only when different environmental cues are present. This enables a mechanism whereby germination can occur only under specific combinations or sequences of environmental conditions. Gene duplication may thereby enable the independent evolution of a process as it occurs in different environmental conditions and may consequently contribute to the fine-tuned responses of processes such as germination to complex environmental conditions.

Germination is a critical stage during the life cycle of plants that is under intense natural selection. The diverse activity of multiple phytochromes during germination suggests that they have a combined function of great ecological importance that has not been fully recognized. Complex patterns of functional redundancy and diversity among the different phytochromes provide a potential mechanism to regulate germination responses to multiple environmental factors in a manner that strongly affects the fitness of plants. The precise nature and mechanism of the differentiation of function under different environmental conditions needs to be more fully elucidated. The functional diversification in the phytochrome gene family with regard to germination indicates that gene duplication can contribute to the evolution of a precise environmental signaling and response system.

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