Unanticipated regulatory roles for Arabidopsis phytochromes revealed by null mutant analysis

Wei Hu[a], Keara A. Franklin[b1], Robert A. Sharrock[c], Matthew A. Jones[d], Stacey L. Harmer[d] and J. Clark Lagarias[a1]

[a]Department of Molecular and Cellular Biology, University of California, Davis, CA 95616; [b]School of Biological Sciences, University of Bristol, Bristol, BS8 1UG, United Kingdom; [c]Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717; and [d]Department of Plant Biology, University of California, Davis, CA 95616

Submitted to Proceedings of the National Academy of Sciences of the United States of America

In view of the extensive literature on phytochrome mutants in the Ler accession of Arabidopsis, we sought to secure a phytochrome null line in the same genetic background for comparative studies. Here we report the isolation and phenotypic characterization of phyABCDE quintuple mutants and a new phyABDE quadruple mutant in the Ler background. Unlike earlier studies, these lines possess a functional allele of FT permitting measurements of photoperiod-dependent flowering behavior. Comparative studies of both classes of mutants establish that phytochromes are dispensable for completion of Arabidopsis life cycle under red light, despite the lack of a transcriptomic response, and also indicate that phyC is non-functional in the absence of other phytochromes. Phytochrome-less plants can produce chlorophyll for photosynthesis under continuous red light, yet require elevated fluence rates for survival. Unexpectedly, our analyses reveal both light-dependent and -independent roles for phytochromes to regulate the Arabidopsis circadian clock. The rapid transition of these mutants from vegetative to reproductive growth, as well as their insensitivity to photoperiod, establish a dual role for phytochromes to arrest and to promote progression of plant development in response to the prevailing light environment.

circadian clock | flowering | photomorphogenesis | photoperiodism | plant development

Plants rely on light as an energy source for photosynthesis and thus possess photosensor proteins to mediate responses to changes in light quantity, spectral quality, direction and duration for optimal growth and development. Notable among these are the phytochromes, linear tetrapyrrole (bilin) containing light sensors, which primarily detect the level of red (R) and far-red (FR) light in the environment (1). The long wavelength region of the visible light spectrum is critical for plant development, since both the production of chlorophyll and optimal function of the photosynthetic apparatus heavily rely on the absolute and relative flux of R and FR. It is for this reason that the phytochrome (phy) family has expanded and diversified amongst the extant seed plants (2). Molecular phylogenetic reconstructions provide evidence for three primary phy lineages, encoded by the *PHYA*, *PHYB* and *PHYC* gene families, reflecting two rounds of duplications of an ancestral phy gene concomitant with the emergence of seed plants on land (3). While nearly all angiosperms possess representatives of these three lineages, additional rounds of duplication of the *PHYB* locus have yielded new members, e.g. *PHYD* and *PHYE*, in some eudicot plant lineages such as *Arabidopsis thaliana* (4, 5).

Our present understanding of the regulatory roles of individual phys is best known for the model eudicot *Arabidopsis thaliana* and the model monocot *Oryza sativa* (rice) owing to the extensive genetic and molecular resources for these species. The picture drawn from physiological analysis of *phy* mutants in these species indicates that these three classes of phys possess overlapping and distinct roles to entrain plant development with the prevailing light environment (6, 7). Moreover, such studies indicate that phyA performs a dominant role during seedling establishment in low light environments, while phyB is the major regulator of shade avoidance behavior in adult plants. The function of phyC has been more difficult to establish, although its role in photoperiod detection and modulation of phyB responses has been observed in both plant species. Based on these and other studies, it is also clear that the regulatory roles of these three phy classes have continued to diverge within various plant lineages (5).

From studies on Arabidopsis, phyA appears to be the exclusive FR sensor while phyB is the predominant R sensor, with phyC-E playing a less prominent role in R sensing (8-12). In rice by contrast, phyB and phyA function as redundant R sensors, while phyA and phyC both perceive FR (13). This reflects a profound photosensory divergence of phyA and phyC lineages in eudicots and monocots. All rice and Arabidopsis phys are dimeric proteins, some of which, e.g. phyB-E in Arabidopsis and phyB-C in rice, can form heterodimers with each other (13, 14). The functional significance of heterodimer formation is unclear, although previous studies indicate that phyCs fail to homodimerize (15) and require other phys (i.e. phyB or phyD) to accumulate in both Arabidopsis and rice (9, 13, 15). The ability to homodimerize might have been lost multiple times in evolution since Arabidopsis phyE, like phyC, is also an obligate heterodimer (15).

Owing to the regulatory complexity introduced by phy heterodimerization, understanding the specific role of individual phys requires removal of all other phy species. As a baseline for such analyses, it is important to establish the phenotype of a given plant species that lacks all of its phys. A rice *phyABC* triple null mutant in the Nipponbare cultivar was the first reported phyless plant species (16). Blind to both R and FR as evaluated by seedling photomorphogenesis, rice *phyABC* seedlings failed to accumulate detectable chlorophyll under continuous R (Rc) and lacked a transcriptomic response to a R pulse. This mutant was able to complete its life cycle under white light however, albeit with greatly altered morphology (e.g. increased elongation of internodes even during vegetative stages) and reduced fertility due to an anther dehiscence defect (16). By contrast, an Arabidopsis *phyABCDE* quintuple null mutant in the Col accession necessitated the presence of a *flowering locus T* (*ft*-1) mutation to ensure germination (17). Unlike rice null mutants, the Arabidopsis *phyABCDE* mutants retained the ability to synthesize some chlorophyll under R yet failed to develop beyond the cotyledon stage. The retention of rhythmic leaf movement in this mutant

Reserved for Publication Footnotes

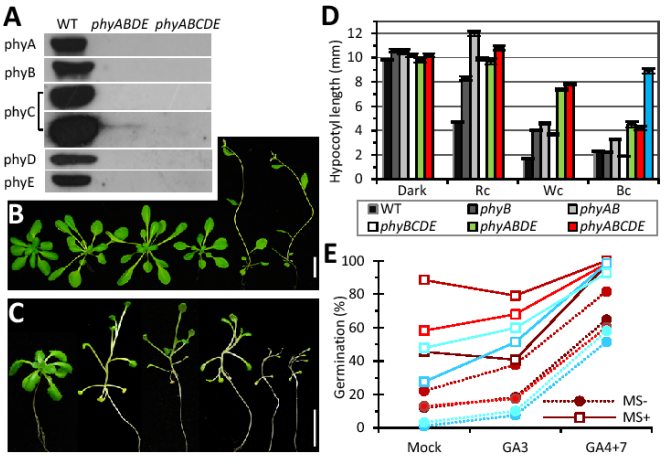

Fig. 1. *phyABCDE* and *phyABDE* mutants are photomorphogenically similar. (A) Immunoblot analysis confirms the identities of *phyAB(C)DE* mutants; the weak phyC band of *phyABDE* is detected after long exposure (bottom blot). (B) White light-grown adult plants on soil under short-day conditions for 6 weeks, from left to right: WT (L*er*), *phyB*, *phyAB*, *phyBCDE*, *phyABDE* and *phyABCDE*, bar = 2 cm. (C) Rc50-grown, 5-week-old adult plants on soil, the plant order is same as (B), bar = 1 cm. (D) Hypocotyl lengths of 4-d-old seedlings grown in darkness or under 50 μmol m^{-2} s^{-1} fluence rate of continuous red (Rc), white (Wc) or blue (Bc) light (mean ± SEM, n= 30∼50). (E) Germination of *phyAB(C)DE* mutants vary and are promoted more effectively by GA_{4+7} than by GA_3. Seeds were sown on phytagar plates with (MS+) or without MS salts (MS-) and supplied with or without 100 μM GA, stratified for 4 days and then grown under Rc50 for 4 days before germination scoring. All mutant lines tested were independently grown and harvested; the two *phyABDE* lines are plotted in blue and the three *phyABCDE* lines in red.

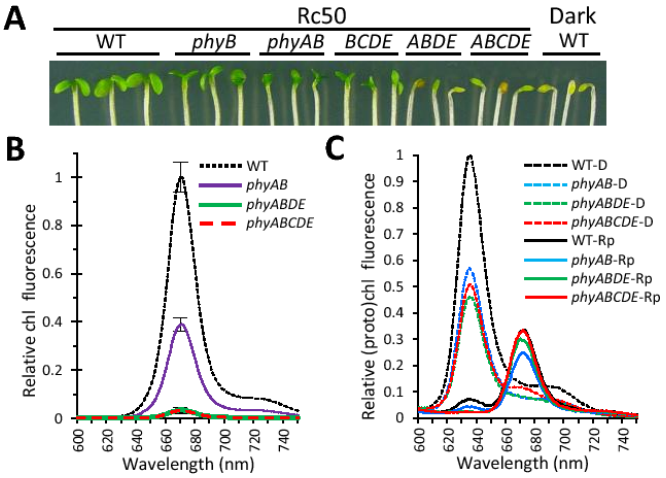

Fig. 2. *phyAB(C)DE* mutants can synthesize a low level of chlorophyll under red light. (A) Rc50-grown, 5-d-old *phyAB(C)DE* seedlings have a nearly etiolated phenotype with marginal greening; some seedlings have cotyledons fully enclosed by a seed coat. (B) Five-day-old *phyAB(C)DE* seedlings accumulate very low levels of chlorophyll (n = 3, SD is given for the peak value). (C) Dark-grown *phyAB(C)DE* seedlings can efficiently photoconvert dark-accumulated protochlorophyllide into chlorophyll(ide) after exposure to Rc50 for 15 min, similar to WT and *phyAB* (n = 3).

also indicated that phys are dispensable for clock maintenance (17).

In view of the extensive literature on *phy* mutants in the L*er* accession of Arabidopsis, we sought to secure a *phy* null line in the same genetic background. The present work describes the isolation and phenotypic characterization of a *phyABCDE* quintuple mutant and a new *phyABDE* quadruple mutant in the L*er* background. Since both possess a functional allele of *FT*, these

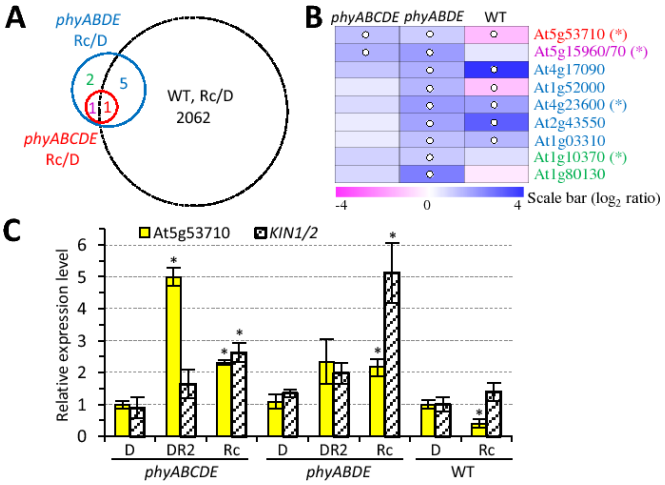

Fig. 3. Transcriptomic analysis of *phyAB(C)DE* response to red light. (A) Venn diagram of red light responsive genes in 4-d-old WT, *phyABDE* and *phyABCDE* (D=dark, Rc = 50 μmol m^{-2} s^{-1} red light). (B) Expression patterns (Rc vs D) of the 9 Rc responsive genes in *phyABDE*; white dots denote significantly differential expression; (*) indicates stress-responsive genes. (C) Expression levels of the two Rc-inducible genes in *phyABCDE*. Expression levels are normalized to WT-D of each gene; DR2 = 4 d darkness followed by 2 hours of Rc50 exposure; * denotes statistical significance (adjusted *p* value < 0.05) from the same genotype grown in the dark.

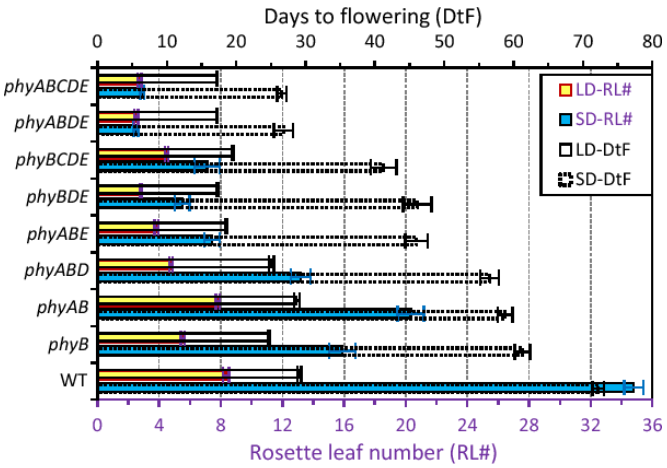

Fig. 4. Flowering of *phyAB(C)DE* mutants is insensitive to photoperiod. The data are presented as mean with SEM (n = 20). LD: long-day conditions (16h L/8h D), SD: short-day conditions (8h L/16h D).

new mutants permit measurements of photoperiod-dependent flowering behavior in the absence of phys and in the presence of stand-alone phyC. Our studies show that phys are not required for the completion of the Arabidopsis life cycle under high fluence rate R despite an almost complete lack of transcriptomic response to R in *phyAB(C)DE lines*, establish that Arabidopsis phyC is non-functional in the absence of other phys, and provide unanticipated insight into the regulatory role of phys in the circadian clock function.

Results

Isolation of *phyABCDE* null mutants in the L*er* accession. A *phyA-201,B-1,C-1,D-1,E-1* null mutant (abbreviated as *phyABCDE* hereafter) was obtained from a cross between the transgenic line YHB^{g}/*phyAB* #5 that expresses a constitutively active allele of *PHYB* (18) and *phyBCDE* (19) both in the L*er* background. After confirming the viability of *phyABCDE*,

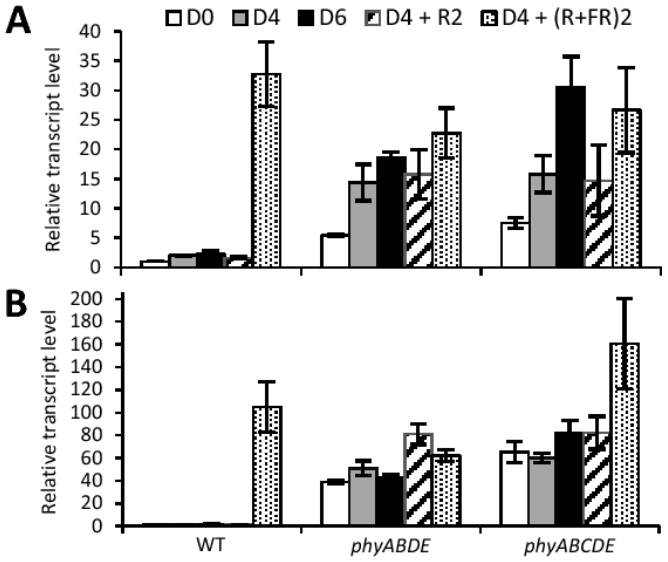

Fig. 5. Expression response of *ATHB2* (A) and *PIL1* (B) to various light treatments. Three-week-old plants grown on soil under SD conditions (8h L/16h D) at 16°C were transferred to darkness for 4 or 6 hours, or 4 hours followed by 2 hours of red light (30 μmol m^{-2} s^{-1}), or by 2 hours of red plus far-red light (R:FR = 0.2) treatments. Expression levels are the means from 3 biological replicates ± SD.

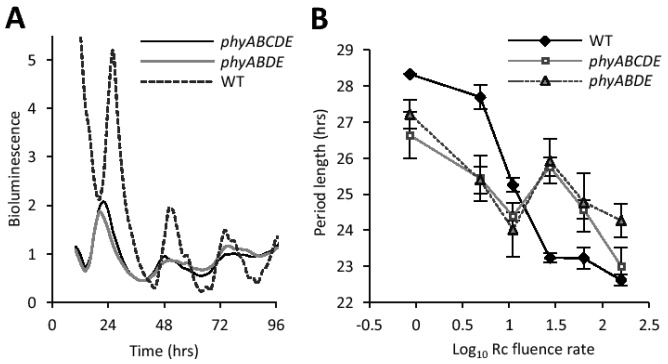

Fig. 6. Circadian rhythms in the *phyAB(C)DE* mutants. (A) Normalized bioluminescence of seedlings containing a *pCCA1:LUC2* reporter construct. Plants were entrained to 12L:12D cycles for 6 d before being moved to 27 μmol m^{-2} s^{-1} Rc. Data presented for each line was normalized to the average bioluminescence over 72 h following background subtraction. (B) *phyAB(C)DE* mutants have a shorter period in comparison to WT at low fluence rates, but a longer period at higher red light fluence rates. Seedlings were entrained as in (A) before being moved to Rc at the indicated fluence rate. Error bars indicate SEM (n≥6).

additional mutant lines were obtained from a direct cross between *phyABDE* (20) and *phyBCDE*. Besides genotyping at the DNA level, immunoblot analyses were performed to validate the identities of newly isolated *phyABDE* and *phyABCDE* mutants (Fig. 1*A*). As expected, the protein levels of phyA, phyB, phyD and phyE were undetectable in *both* mutant lines, while phyC was not present in *phyABCDE* and detectable in *phyABDE* only after long exposure of the film. The phyC level in *phyABDE* was less than that in other *phy* mutants examined (15).

Seedling photobiology and seed germination of *phyAB(C)DE* mutants. We next sought to compare the *phyABDE* and *phyABCDE* mutants to define any possible physiological activities regulated by the low level of phyC in *phyABDE*. As shown in Fig. 1*B*, white light-grown *phyABDE* and *phyABCDE* (collectively called *phyAB(C)DE* for simplicity as needed hereafter) adult plants were both similarly slender and were capable of reproductive development. Grown under Rc at a moderate fluence rate (50 μmol m^{-2} s^{-1}) on soil, most *phyAB(C)DE* plants could not survive, but some were able to produce 3 to 4 tiny rudimentary leaves (Fig. 1*C*). Under a higher fluence rate of Rc (150 μmol m^{-2} s^{-1}), *phyAB(C)DE* mutants produced flowers and set seeds (Fig. S1*A*), suggesting that phy-less Arabidopsis plants can fulfill their life cycle when provided sufficient R illumination. When grown on MS salt medium, however, the mutants performed considerably worse than on soil, exhibiting similar phenotypes to the *phy* null mutant in the Col background (Fig. S1*B*, S1*C*) (17).

Examined at the seedling stage, *phyABDE* and *phyABCDE* mutants were indistinguishable under all light conditions (Fig. 1*D*, S1*D*). Both mutants were etiolated under Rc, and had longer hypocotyls than *phyAB* and *phyBCDE* under Wc. Under Bc, *phyB* and *phyBCDE* were similar to WT, while *phyAB* was longer than WT, indicating that phyA modulates blue light-induced photomorphogenesis, consistent with a previous finding (21). *phyAB(C)DE* seedlings were longer than *phyAB*, but still much shorter than *cry1cry2*, showing that blue light signaling is moderately impaired in *phyAB(C)DE* mutants. Photomorphogenesis under FRc was as deficient in *phyAB(C)DE* as in *phyA* (Fig. S1*D*), consistent with previous conclusions that phyA is the sole FR photoreceptor in Arabidopsis.

The *phyABCDE* mutant in the Col accession was reported to require the *ft* mutation and GA_4 treatment for efficient seed germination (17). This was not the case for the L*er phyAB(C)DE* mutants. Independently grown and harvested *phyAB(C)DE* seeds exhibited variable germination capacity, with some lines exhibiting > 80% germination rate on the MS salt plates (Fig. 1*E*). Comparing the germination of the same mutant line on phytagar plates with and without the MS salts, it is evident that some nutrient elements of the MS salts greatly promote *phyAB(C)DE* germination. Most of time, *phyAB(C)DE* seeds exhibited >40% germination, which is sufficient for analysis work using seedlings as the materials. Consistent with the previous report (17), we demonstrated that GA_4 promotes more effectively than GA_3 of germination of the mutant lines with low germination capacity (Fig. S2*A*). We also found that 25μM of GA_4 was as effective as 100 μM for promoting good germination (Fig. S2*B*).

***phyAB(C)DE* mutants can synthesize chlorophyll under red light.** Rc-grown *phyAB(C)DE* seedlings were etiolated, and some had cotyledons that were completely enclosed by testa and never expanded (Fig. 2*A*). Occasionally, a few seedlings seemed pale green. Chlorophyll fluorescence assay showed that the Rc-grown mutants indeed can synthesize chlorophyll (indicated by their peak fluorescence at 670 nm) at a level approximately 30∼50 fold lower than WT (Fig. 2*B*). The newly isolated *phyABDE* lines from this study had the same chlorophyll level as *phyABCDE*. The original/parental *phyABDE* line (20) repeatedly had a chlorophyll level two-fold higher than the newly isolated *phyAB(C)DE* mutants under various Rc irradiation levels and seedling ages tested (Fig. S3). In addition, the original *phyABDE* line exhibited unusually long hypocotyls even in darkness, and narrower and longer leaves under Wc - phenotypes not observed in the new *phyABDE* lines. When dark-grown seedlings were exposed to R for 15 min, *phyAB(C)DE* converted protochlorophyllide into chlorophyll(ide) to a similar extent as WT and *phyAB* (Fig. 2*C*). When 4 d-old, dark-grown seedlings were exposed to Rc over a 24 h period, *phyAB(C)DE* mutants accumulated chlorophyll 10- and 3-fold lower than that of WT and *phyAB*, respectively (Fig. S4). During the first 3 h Rc, there was no difference in chlorophyll accumulation between *phyAB* and *phyAB(C)DE*, implying that phyC-E contribute to prolonged light-dependent chlorophyll accumulation in Arabidopsis. When exposed to Wc, *phyAB(C)DE* accumulated chlorophyll at a much higher level than under Rc, confirming that these mutants are more robust under wide spectrum light. Collectively, the *phy* null mutants retained a basal

capability of chlorophyll synthesis under R, and there was no significant difference between *phyABDE* and *phyABCDE*.

***phyAB(C)DE* mutants are nearly transcriptionally blind to red light.** To determine global gene expression changes in *phyAB(C)DE* mutants in response to R, we performed transcriptomic analysis using Affymetrix ATH1 microarray chips. Our previous work indicated that 2112 genes had statistically significant, more than two-fold (SSTF) expression change in WT grown for 4 days under Rc50 compared to WT grown in the dark (18). In the present studies, WT control microarray measurements revealed a similar number of Rc-regulated SSTF genes (i.e. 2068 genes) after normalization of the WT dataset with the *phyAB(C)DE* mutant datasets. By contrast, only 2 and 9 genes exhibited SSTF expression changes in Rc50-grown *phyABCDE* and *phyABDE*, respectively (Fig. 3*A*). The 2 genes from *phyABCDE* were among the 9 genes from *phyABDE*. Four of the 9 genes are stress responsive loci, suggesting that plants lacking phys are more sensitive to light stress (Fig. 3*B*). In addition, 2 genes showed an opposite expression pattern in the *phyAB(C)DE* mutants and WT, so the light regulation of these genes was masked by the presence of phys. We also measured transcriptomic changes in mutant seedlings in response to 2 h of R following 4 days of dark growth. Once again, only 4 and 1 genes in *phyABCDE* and *phyABDE*, respectively, exhibited SSTF expression changes to the short-time R treatment (SI Dataset 1). Notably, At5g53710 encoding an unknown stress-responsive protein was consistently induced in *phyABCDE* by 2 h- or 4 d-R exposure, reinforcing the interpretation that *phyABCDE* perceives R as a stress (Fig. 3*C*). Overall, we conclude that *phyAB(C)DE* mutants are nearly blind to R at the transcriptomic level.

Flowering behavior of *phyAB(C)DE* mutants is insensitive to photoperiod. The initial *phyABCDE* lines isolated from crosses of *YHB*^{s}*/phyAB* x *phyBCDE* and of *phyABDE* x *phyBCDE* flowered consistently later than *phyABDE* (Fig. S5*A*). This observation led to a speculation that phyC may promote early flowering. When overexpression of Col or L*er* alleles of *PHYC* in *phyABCDE* (independent line n=16 and 40, respectively) failed to confer the early flowering phenotype of *phyABDE*, we transformed *phyABDE* mutants with a *PHYC* RNAi construct to knock down the already very low level of phyC. A delayed flowering phenotype was not observed in 84 independent transformants. To test whether the later flowering trait was due to a mutation linked to any of the *phy* alleles, *phyABCDE* was backcrossed to L*er*. While most of newly resultant *phyABCDE* lines were late flowering, a small number of *phyABCDE* lines flowered as early as *phyABDE*. We also isolated early- (predominant) and late-flowering (rare) *phyABDE* lines from the backcrossed F2 population. Thus, the later flowering behavior of the parental *phyABCDE* line was not due to the *phyC* mutation, but reflected an unknown *phyC*-linked locus in the Ws background from which the *phyC-1* allele was originally isolated (19). Alternatively, this result could be due to hybrid vigor between L*er* and Ws on Chromosome V. Fig. S5*B* shows morphological differences between early- and late-flowering *phyABCDE* lines. The two types of *phyABCDE* mutants were indistinguishable under Rc.

Based on genotyping (see below), we determined that the early flowering behavior is the authentic phenotype of the *phyABCDE* mutant. Evaluated by rosette leaf number, authentic *phyABDE* and *phyABCDE* lines flowered very early under both LD and SD conditions (Fig. 4). Both exhibited a delay in days to flowering under SD, however, probably due to insufficient photosynthesis that limited growth and development (Fig. 4 and Fig. S6). The flowering behavior of *phyAB(C)DE* illustrates their insensitivity to photoperiod, as neither mutant displayed flowering delay under SD. By comparison, *phyBDE* mutants flowered as early as *phyAB(C)DE* under LD, but later under SD, indicating that phyA can delay flowering under SD in the absence of type-II phys (Fig. 4). Indeed, *phyBCDE* mutants flower later than *phyABCDE* lines under SD (Fig. 4). The flowering phenotypes of *phyAB(C)DE* lines support the conclusion that phyC does not regulate flowering in the absence of other phys.

Genotyping distinguishes between early- and late-flowering *phyAB(C)DE* lines. Seedling microarray data revealed that the parental late-flowering *phyABCDE* line had unusually high expression of *FLC*, a flowering repressive gene that integrates signals from both vernalization and autonomous pathways (22). Indeed, the *FLC* expression in the early-flowering *phyABCDE* line was reduced to a level similar to WT (Fig. S5*C*). Although both *FLC* and *PHYC* are located on Chromosome V (ChrV), the long distance between *FLC* (at 3.2 Mb) and *PHYC* (at 14.0 Mb) is inconsistent with the close linkage between *phyC* and the late-flowering locus inferred by genetic analyses. Association mapping excluded linkage of loci on the bottom arm of ChrV with the flowering behavior. The parental *phyBCDE* line used for constructing *phyABCDE* was found to contain Ws alleles in the entire top arm of ChrV, presumably from the original Ws *phyC-1* mutant (Fig. S5*D*). The Ws NGA76 marker allele at 10.4Mb always co-segregated with *phyC-1*, and was not linked with flowering phenotype. That the pericentric Ws NGA76 marker co-segregated with the pericentric *phyC-1* allele is consistent with the rare recombination frequency of loci near the centromere (23). By contrast, 3 markers at the top arm of ChrV were linked with the flowering phenotype to varying degrees. The early-flowering *phyABCDE* lines all had L*er* alleles for these markers, whereas the late-flowering lines contained Ws alleles. We thus conclude that a variant Ws locus in the top arm of ChrV that activates *FLC* expression is responsible for the delayed flowering of the parental *phyABCDE* lines (Fig. S5*D*). Fine mapping of this locus is beyond the scope of this work. An early-flowering *phyABCDE* line with all L*er* alleles in this region was further backcrossed with L*er* WT. All progeny *phyABCDE* and *phyABDE* mutant lines from this second backcross flowered early. These data support that the early-flowering phenotype of L*er phyAB(C)DE* mutants is authentic.

***ATHB2* retains response to changes in R/FR ratio in *phyAB(C)DE* mutants.** A previous study of the *phyABDE* mutant showed that the shade-inducible gene *ATHB2* was still responsive to the change in R/FR ratio - a result attributed to the residual phyC function (20). We therefore re-examined this response in newly isolated *phyAB(C)DE* mutants under the same growth conditions and treatment (20). As expected from previous studies (24), transfer of WT plants to simulated shade (R:FR = 0.2) resulted in dramatic increase in *ATHB2* transcript abundance when compared with R treatment alone (Fig. 5*A*). While the *ATHB2* transcript levels in light-grown *phyAB(C)DE* were already elevated compared with WT, they further increased in response to transfer to darkness (Fig. 5*A*). A similar but weaker increase was also seen in WT, implying that other processes can suppress *ATHB2* expression in the light, e.g. photosynthesis. The transcript increase was more pronounced in *phyAB(C)DE* when the dark period was extended from 4 to 6 h, while 2h R treatment following 4 h dark prevented this enhancement. By contrast, when the 2 h R treatment was replaced with simulated shade (R:FR = 0.2) with the same R fluence rate, *ATHB2* expression increased (the *p* values of statistical significance were slightly higher than 0.05 due to great variation among biological replicate sets). As both mutants behaved similarly to simulated shade, the residual phyC does not contribute to the expression alternation of *ATHB2*. *PIL1*, another shade-inducible gene, maintained a very high expression level in light-grown *phyAB(C)DE* and did not respond significantly to the dark treatment (Fig. 5*B*). Intriguingly, *phyABCDE*, but not *phyABDE*, displayed a marked, but variable, increase in *PIL1* transcript abundance following simulated shade treatment. This

cannot be attributed to phy function but may represent a stress response in these plants.

***phyAB(C)DE* mutants maintain circadian rhythms under Rc, with reduced responsiveness of period to fluence rate.** Both temperature and light cues ensure correct synchronization between the endogenous clock and the environment (25, 26), with phys affecting circadian phase, period and output amplitude of gene expression (27, 28). To test whether circadian rhythms of gene expression are maintained in *phyAB(C)DE* seedlings under Rc, we introduced the clock-regulated, enhanced luciferase reporter *pCCA1::LUC2* into both mutants. Both *phyABDE* and *phyABCDE* seedlings retained robust rhythms of bioluminescence following transfer from 12L:12D light cycles to Rc although the initial phase of peak bioluminescence for the two mutants were earlier than that of the WT (Fig. 6*A*). The periods were similar for both mutants, however the amplitude of rhythmic bioluminescence in both mutants was greatly reduced in comparison to WT. Since circadian periods of many diurnal species, including plants, are shortened in response to higher fluence rates of constant light, a phenomenon formalized by Aschoff (29), we undertook comparative period measurements under a range of Rc fluence rates. For the WT as expected, we observed a fluence rate-dependent shortening of circadian period with the seedlings most responsive between ~5 to ~30 μmol m^{-2} s^{-1} Rc (Fig. 6*B*). Intriguingly, *phyAB(C)DE* seedlings did not simply display longer period phenotypes as might be expected from data reported for single *phyA* and *phyB* mutants (27). Instead, the period of *phyAB(C)*DE mutants was much less dependent on the fluence rate of Rc, exhibiting a shorter period than WT under lower fluence rates and a longer period under higher fluence rates (Fig. 6*B*). No measurable difference was observed between *phyABDE* and *phyABCDE* mutants. These data show that phys are not required for clock maintenance under Rc, and implicate that phys can both increase and decrease the rate of the clock.

Discussion

Although Arabidopsis *phy* null mutants in the Col accession have been described previously (17), null mutants have not been secured in the L*er* accession for which an extensive literature on phy function is available. In contrast to the earlier report, we show that the L*er* phy-less mutant is robust, and as such, represents a valuable tool for studying the photoregulatory functions of individual phys and their interaction with other family members in an otherwise isogenic background. The L*er* phy-less mutant phenotype is quite stable, and re-segregated mutants from two backcrosses with the L*er* WT continue to produce viable seeds for propagation for multiple generations. This indicates that residual phy transmitted to the progeny is dispensable for continued viability. Our studies also reinforce that phyC requires other phys for activity, because all phenotypes examined for *phyABDE* are indistinguishable from those of *phyABCDE*. While this loss of function is in part owed to greatly reduced phyC protein accumulation, the residual phyC in the *phyABDE* mutant lacks any photo-regulated activity. These findings are consistent with the observation that, in Arabidopsis, phyC is an obligate heterodimer with either phyB or phyD (9, 14, 15), implicating monomeric phyC to be non-functional and/or degraded. This agrees with the observation that the rice *phyAB* mutant is essentially the same as the rice *phyABC* mutant phenotypically (16), yet contrasts with earlier observations that implicate regulatory function of phyC in the absence of other phys (8, 17). The reason for this difference is unclear, but may reflect cryptic mutations at other loci that were not removed in the genotypes previously examined.

Phy-less plants are viable, but developmentally challenged. Our studies show that *phyABCDE* plants are viable, although their survival is conditional on the growth environment as reported previously (16, 17). We believe that survival reflects retention of minimal photosynthetic development, as *phyABCDE* null plants can synthesize sufficient chlorophyll and develop functional chloroplasts even under Rc. Only under elevated fluence rates of Rc can the quintuple mutant complete the life cycle however, arguably due to enhanced chlorophyll synthesis and light harvesting. The poor cotyledon expansion of *phyABCDE* seedlings frequently prevented shedding of their seed coats, which may contribute to arrested seedling development and death. To a lesser extent, this also occurred in *phyABCDE* seedlings grown in white light (Fig. S7). The proportion of arrested development in the mutant population was much higher under SD than under LD conditions, suggesting that phy-less plants rely on high irradiation levels for survival.

Only 2 genes were SSTF induced in *phyABCDE* seedlings under Rc, both of which are stress-related implying that the mutants perceive R as a stress. The *phyABDE* employed in the microarray studies was the original parental line that had more chlorophyll than *phyABCDE*. It is not surprising that this mutant had seven more SSTF-regulated genes, two of which are involved in starch metabolism. Compared with the >2000 SSTF Rc-regulated genes in WT, the few SSTF genes in the two mutants reinforce the conclusion that the *phyAB(C)DE* mutants are nearly blind to R. Growth on MS agar plates was also stressful for *phyAB(C)DE* mutants. Even with sucrose supplementation, most quintuple plants failed to develop beyond the seedling stage - a problem observed in the previous study (17). In contrast to the Arabidopsis *phyAB(C)DE* mutants, rice *phyABC* mutants do not synthesize sufficient chlorophyll under Rc for development beyond the seedling stage (16). Under broad-spectrum white light, *phyABCDE* null mutants fared much better, presumably due to the activities of the cryptochrome, phototropin or other blue/UVA light sensors or due to enhanced photosynthetic light conversion. The *ft* mutation was previously found necessary for germination of Col *phyABCDE* seeds (17). We too found that germination was reduced in some L*er phyAB(C)DE* mutants, which could be mostly rescued by GA_4 treatment. However, some seed lots of the *phyAB(C)DE* mutants showed robust germination suggesting that the physiological state of adult plants at the time of seed set plays a significant role in seed germination.

Flowering is insensitive to photoperiod in the absence of phys. The *ft-1* mutation present in the Col *phyABCDE* mutant makes flowering measurements problematic, thus the photoperiod response of flowering was not addressed previously (17). Moreover, the *ft-1* allele was originally derived from the L*er* background, so genetic background effects could also complicate the interpretation of the flowering phenotypes of the mutant. Indeed, we encountered a similar problem when we examined the flowering of the originally isolated *phyABCDE* mutant that flowered later than the *phyABDE* mutant. After monitored genetic background cleanup by backcrossing, *phyABCDE* flowered as early as *phyABDE* under both LD and SD conditions, possessing only 2 or 3 rosette leaves at bolting (Fig. 4). Thus, the phy-less mutants appeared to be insensitive to photoperiod. In this regard, the rice chromophore-deficient *se* (30) and *phyABC* mutants (16) are both insensitive to photoperiod. Measured as days to flowering, both rice *se* and *phyABC* mutants flowered slightly later under SD than under LD conditions, similar to Arabidopsis *phyAB(C)DE* mutants. This has been rationalized by the slower growth of the rice *phyABC* mutants under SD conditions (30), thus the same appears true for the Arabidopsis *phyAB(C)DE* mutants. These data indicate that the elimination of phys confers photoperiod insensitivity. Given that phys alter CO stability in the photoperiodic pathway (31), we hypothesize that phy-less mutants have increased CO stability and therefore high FT expression, rendering them insensitive to photoperiod and early flowering. Alternatively, the photosynthetic deficiency of these mutants may contribute to a general stress that induces early

flowering regardless of the light environment. Finally, previous genetic studies implicate phyC in the delay of flowering under SD photoperiods while also supporting the conclusion that the L*er PHYC* allele is poorly active (9, 32). It is thus conceivable that the reduced regulatory activity of L*er* phyC is responsible for the observed photoperiod insensitivity of our *phyABDE* mutants. While experiments to assess this possibility are beyond the scope of this investigation, a potential polymorphism in a flowering locus linked to the pericentric *PHYC* allele on ChrV (as was observed here) cannot be dismissed as an explanation for the previous observations.

Circadian clock period length is nearly insensitive to Rc fluence rate in phy-less plants. Col *phyABCDE* plants were previously shown to maintain circadian rhythms of leaf movement under Wc, but not under Rc (17). Using the *pCCA1::LUC2* reporter, we show that L*er phyAB(C)DE* mutants can maintain rhythmicity of *CCA1* expression under Rc. However, a dramatic reduction in the amplitude of the bioluminescence signals in *phyAB(C)DE* seedlings was observed compared to WT controls. It was previously shown that Rc induces *CCA1* expression (33) and that the amplitude of *CCA1* rhythmic expression is reduced in the *phyB-9* mutant (34). Similarly, the amplitude of *CCA1* promoter-driven luciferase expression is dampened in darkness (35). The reduced amplitude we observed is thus likely a consequence of impaired Rc perception in *phyAB(C)DE*. Since *phyAB(C)DE* seedlings are smaller with delayed cotyledon expansion and true leaf emergence when grown under 12 L/12 D cycles, however, we cannot fully distinguish between this hypothesis and the possibility that the reduced bioluminescence is a consequence of delayed development. These data indicate that the previously reported arrhythmicity in leaf movement under Rc (17) is not caused by complete loss of oscillator function, but instead might reflect an overall low amplitude of clock-regulated processes and/or the extremely small leaves of the *phyAB(C)DE* seedlings.

In addition to reduced bioluminescence, we observed an early phase of *pCCA1::LUC2* peak activity in the *phyAB(C)DE* mutants immediately following transfer to Rc. An early phase phenotype has previously been reported for *phyB* mutants harboring a *pLHCB::LUC* transgene in the Col accession under Wc, i.e. *phyB-9* and *oop1* (28). The early phase phenotypes of *phyB-9* and *oop1* were not evident in seedlings entrained to temperature cycles, suggesting that light signaling defects contribute to this phenotype (28). Intriguingly, *phyB-9* has also been reported to differentially affect the phase of several clock components under Wc (34). In this latter study, the phase of *pCCA1::LUC+* and *pTOC1::LUC+* was comparatively unaffected whereas *GI* and *PRR9* promoters had early phases compared to WT (34). Impaired phy signaling to multiple points of the circadian system likely underlies the early phase phenotype of *phyAB(C)DE* seedlings.

Increased R fluence rates lead to a shortening of the circadian clock in Arabidopsis (27, 29). This response was impaired in *phyAB(C)DE* seedlings, consistent with the expectation that phys contribute to this fluence rate-dependent period shortening. However, we observed a modest shortening of circadian period in *phyAB(C)DE* as fluence rate increased, suggesting that phyless seedlings maintained some sensitivity to R. Such sensitivity may derive from a metabolic signal induced by enhanced photosynthesis under increasing fluence rates or by increased oxidative stress. Interestingly, at fluence rates less than 10 μmol m^{-2} s^{-1} Rc, we observed an increased pace in the circadian oscillator in *phyAB(C)DE* compared to WT. This non-intuitive shortening of circadian period in *phyB-9* and higher order *phy* mutants under Wc has previously been reported (17, 34). Such data suggest that phys do not simply act as a light-induced accelerant of the clock mechanism. Instead, we hypothesize that P_r forms of phys act to delay the circadian system under low fluence rates whereas light-activated P_{fr} forms act to increase the pace of the oscillator under higher light intensities.

Materials and Methods

Plant materials, immunoblot analyses, phenotypic analyses, (proto)chlorophyll(ide) measurements, microarray analysis, real-time RT-PCR, and **luciferase imaging assays** are described in *SI Materials and Methods*.

Acknowledgements. We thank Matt Rolston for performing Affymetrix chip hybridization and scanning work, and Koby Schwartz for providing the *pCCA1::LUC2* construct. We also thank the late Professor Garry Whitelam, under whose supervision the initial *phyABDE* and *phyBCDE* mutants were constructed. The work is supported by grants from the National Institutes of Health (Grant GM068552 to J.C.L and GM069418 to S.L.H.), the National Science Foundation (Grant IOS-0920766 to R.A.S.), and by a Royal Society University Research Fellowship to K.A.F..

1. Rockwell NC, Su YS, & Lagarias JC (2006) Phytochrome structure and signaling mechanisms. *Ann Rev Plant Biol* 57:837-858.
2. Sharrock RA (2008) The phytochrome red/far-red photoreceptor superfamily. *Genome Biol* 9:230.
3. Mathews S (2006) Phytochrome-mediated development in land plants: red light sensing evolves to meet the challenges of changing light environments. *Mol Ecol* 15:3483-3503.
4. Sharrock RA & Quail PH (1989) Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution, and differential expression of a plant regulatory photoreceptor family. *Genes Dev* 3:1745-1757.
5. Mathews S (2010) Evolutionary studies illuminate the structural-functional model of plant phytochromes. *Plant Cell* 22:4-16.
6. Franklin KA & Quail PH (2010) Phytochrome functions in Arabidopsis development. *J Exp Bot* 61:11-24.
7. Takano M, Xianzhi X, & Inagaki N (2011) Mutational dissection of the phytochrome genetic systems in rice. *Plant Mut Breed Biotech*, (FAO/IAEA).
8. Franklin KA, Davis SJ, Stoddart WM, Vierstra RD, & Whitelam GC (2003) Mutant analyses define multiple roles for phytochrome C in Arabidopsis photomorphogenesis. *Plant Cell* 15:1981-1989.
9. Monte E, *et al.* (2003) Isolation and characterization of phyC mutants in Arabidopsis reveals complex crosstalk between phytochrome signaling pathways. *Plant Cell* 15:1962-1980.
10. Whitelam GC, *et al.* (1993) Phytochrome A null mutants of Arabidopsis display a wild-type phenotype in white light. *Plant Cell* 5:757-768.
11. Reed JW, Nagpal P, Poole DS, Furuya M, & Chory J (1993) Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. *Plant Cell* 5:147-157.
12. Parks BM & Quail PH (1993) hy8, a new class of arabidopsis long hypocotyl mutants deficient in functional phytochrome A. *Plant Cell* 5:39-48.
13. Takano M, *et al.* (2005) Distinct and cooperative functions of phytochromes A, B, and C in the control of deetiolation and flowering in rice. *Plant Cell* 17:3311-3325.
14. Sharrock RA & Clack T (2004) Heterodimerization of type II phytochromes in Arabidopsis. *Proc Natl Acad Sci U S A* 101:11500-11505.
15. Clack T, *et al.* (2009) Obligate heterodimerization of Arabidopsis phytochromes C and E and interaction with the PIF3 basic helix-loop-helix transcription factor. *Plant Cell* 21:786-799.
16. Takano M, *et al.* (2009) Phytochromes are the sole photoreceptors for perceiving red/far-red light in rice. *Proc Natl Acad Sci U S A* 106:14705-14710.
17. Strasser B, Sanchez-Lamas M, Yanovsky MJ, Casal JJ, & Cerdán PD (2010) Arabidopsis thaliana life without phytochromes. *Proc Natl Acad Sci U S A* 107:4776-4781.
18. Hu W, Su YS, & Lagarias JC (2009) A light-independent allele of phytochrome B faithfully recapitulates photomorphogenic transcriptional networks. *Mol Plant* 2:166-182.
19. Franklin KA, Allen T, & Whitelam GC (2007) Phytochrome A is an irradiance-dependent red light sensor. *Plant J* 50:108-117.
20. Franklin KA, *et al.* (2003) Phytochromes B, D, and E act redundantly to control multiple physiological responses in Arabidopsis. *Plant Physiol* 131:1340-1346.
21. Neff MM & Chory J (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. *Plant Physiol* 118:27-36.
22. Parcy F (2005) Flowering: a time for integration. *Int J Dev Biol* 49:585-593.
23. Singer T, *et al.* (2006) A high-resolution map of Arabidopsis recombinant inbred lines by whole-genome exon array hybridization. *PLoS Genet* 2:e144.
24. Carabelli M, Morelli G, Whitelam G, & Ruberti I (1996) Twilight-zone and canopy shade induction of the Athb-2 homeobox gene in green plants. *Proc Natl Acad Sci U S A* 93:3530-3535.
25. Fankhauser C & Staiger D (2002) Photoreceptors in Arabidopsis thaliana: light perception, signal transduction and entrainment of the endogenous clock. *Planta* 216:1-16.
26. Jones MA (2009) Entrainment of the Arabidopsis circadian clock. *Journal of Plant Biology* 52:202-209.
27. Somers DE, Devlin PF, & Kay SA (1998) Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *Science* 282:1488-1490.
28. Salome PA, *et al.* (2002) The out of phase 1 mutant defines a role for PHYB in circadian phase control in Arabidopsis. *Plant Physiol* 129:1674-1685.
29. Aschoff J (1960) Exogenous and endogenous components in circadian rhythms. *Cold Spring Harb Symp Quant Biol* 25:11-28.
30. Izawa T, Oikawa T, Tokutomi S, Okuno K, & Shimamoto K (2000) Phytochromes confer the photoperiodic control of flowering in rice (a short-day plant). *Plant J* 22:391-399.
31. Jackson SD (2009) Plant responses to photoperiod. *New Phytol* 181:517-531.

32. Balasubramanian S, *et al.* (2006) The PHYTOCHROME C photoreceptor gene mediates natural variation in flowering and growth responses of Arabidopsis thaliana. *Nat Genet* 38:711-715.
33. Wang ZY & Tobin EM (1998) Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93:1207-1217.
34. Palagyi A, *et al.* (2010) Functional analysis of amino-terminal domains of the photoreceptor phytochrome B. *Plant Physiol* 153:1834-1845.
35. Kim JY, Song HR, Taylor BL, & Carre IA (2003) Light-regulated translation mediates gated induction of the Arabidopsis clock protein LHY. *EMBO J* 22:935-944.

Supporting Information

Hu *et al* Unanticipated regulatory roles for Arabidopsis phytochromes revealed by null mutant analysis

SI Materials and Methods

Plant Materials. To obtain the *phyA-201,B-1,C-1,D-1,E-1* (*phyABCDE*) quintuple mutant, *YHB*^{g}/*phyA-201,B-5* transgenic line #5 (1) was crossed with the *phyB-1,C-1,D-1,E-1* (*phyBCDE*) mutant (2); one of the subsequent F_2 lines was determined as *(YHB*^{g}*+/-)/phyA-201,B-1,C-1,D-1,E-1*, and homozygous *phyABCDE* was isolated from the F_3 progeny. Similar *phyABCDE* mutant lines were also obtained from the cross between *phyA-201,B-1,D-1,E-1* (*phyABDE*) (3) and *phyBCDE*. A backcross of the *phyABCDE* line obtained from the first endeavor to L*er* wild type was performed, and new early-flowering *phyABCDE* and *phyABDE* lines were isolated among the F_2 progeny. An early-flowering *phyABCDE* line in the F_5 generation was backcrossed a second time to L*er* to purify the genetic background and to confirm the inheritance of early-flowering phenotype. *PhyABD* (4), *phyABE* (5) and *phyBDE* (6) were employed as controls for flowering tests. Plasmid *pEarleyGate301-pCCA1::LUC2* that contains an 800 bp region of the *CCA1* promoter was used to transform L*er* and a series of *YHB*^{g}#5-containing transgenic plants. A resultant *pCCA1::LUC2*/*YHB*^{g}/*phyABDE* line was crossed with an early-flowering *phyABCDE* line. Homozygous *pCCA1::LUC2*/*phyABDE* and *pCCA1::LUC2*/*phyABCDE* lines were isolated from the segregating F2 population. Primers used for genotyping are shown in Table S1.

Immunoblot Analysis. Protein extraction and immunoblot analyses were performed as previously described (7). In this study, we have validated that the anti-phyC MnAb C11 detects the N-terminus of phyC, whereas C13 detects the C-terminal region. Since the T-DNA in the *phyC-1* mutant was inserted close to the stop codon (8, 9), a mixture of C11 and C13 was used to ensure that any truncated phyC protein derived from the *phyC-1* mutant would be detected.

Phenotypic Analyses. Seeds were sown on 1x basal MS salts solidified with 0.8% phytoblend agar (Caisson Laboratories), followed by 4 day stratification in darkness before germination induction. The red and white light sources were described previously (10). Blue light was provided by SANYO LEDs (peaked at 472 nm). The light fluence rates were adjusted to 50 μmol m^{-2} s^{-1} for seedling measurements, if not otherwise specified. For germination tests, 100 μM of GA_3 (Sigma) or GA_{4+7} (PhytoTechnology; GA_4/GA_7=6/3) was directly mixed into the growth medium. Temperature was adjusted to 20ºC for all experiments if not otherwise specified. For flowering tests, seedlings were grown on MS plates for 5 days before transferring onto soil; the tests was also repeated with seeds directly sown on soil with similar results.

(Proto)Chlorophyll(ide) Measurements. Ten seedlings were homogenized in 1.0 ml of ice-cold 80% $NaHCO_3$-saturated acetone, extracted in darkness at 4 °C for 1 hr, and centrifuged for 10 min at 13000 rpm at 4 °C. The pigment supernatant was used to measure autofluorescence levels of protochlorophyllide (peak at 634 nm) and chlorophyll(ide) (peak at 670 nm) using a PTI QM-6/2005SE fluorometer equipped with a red-enhanced photomultiplier tube (Photon Technology International). The excitation wavelength was 440 nm and the emission scan spectrum was 600-750 nm with a bandwidth of 1 nm.

Microarray Analysis. The *phyABDE* and *phyABCDE* mutants used for microarray analyses were the parental lines, rather than those lines obtained later from the backcross with L*er* WT. Seedling growth, RNA extraction, cRNA synthesis and data analysis were similar to previous work (1) except that no temperature cycle was employed in the first two days of growth, and that the GeneChip 3' IVT express kit rather than the One-Cycle Target Labeling kits (both from Affymetrix) was used to synthesize cRNA. Three biological replicate samples were assayed for dark- and Rc50-grown mutants, and two for mutants that were dark grown for 4 days followed by 2 h Rc50 exposure. One dark-grown WT sample was also assayed as a control for comparison with the previous ATH1 microarray dataset (1). Microarray data are deposited in the NCBI Gene Expression Omnibus with an accession number GSE31587.

Real-time RT-PCR of *ATHB2* and *PIL1* Expression. Plant growth, light treatment and RNA extraction were essentially same as before (3). Plants were grown on soil under SD conditions (8h L/16h D) at 16°C for 3 weeks; an hour after dawn on the 22nd day, plants were transferred to darkness for 0, 4 or 6h, or dark for 4 hours and then followed by 2 h of Rc (30 μmol m^{-2} s^{-1}), or by 2 h of Rc plus FRc (R:FR=0.2) with the same photon irradiance of Rc. RNA extraction, cDNA synthesis and qPCR were performed using whole rosettes as described previously (11). Expression values were normalized to *ACTIN2*, using the primers Actin-F (TCAGATGCCCAGAAGTGTTGTTCC) and Actin-R (CCGTACAGATCCTTCCTGATATCC). *ATHB2* and *PIL1* were amplified using the primers ATHB2-F (GAGGTAGACTGCGAGTTCTTACG), ATHB2-R (GCATGTAGAACTGAGGAGAGAGC), PIL1-F (AAATTGCTCTCAGCCATTCGTGG) and PIL1-R (TTCTAAGTTTGAGGCGGACGCAG), respectively. Three biological repeats were performed.

Luciferase Imaging Assays. Plants were entrained for 6 days in 12L:12D cycles under white light on sucrose-free MS media before being sprayed with 3 mM D-luciferin in 0.01% Triton X-100. Plants were then transferred to free-running conditions under red LEDs as previously described (12). Imaging was completed over 5 days and data was processed using Metamorph software (Molecular Devices). Time series data were processed and analyzed using the Fast Fourier transform nonlinear least squares method (13).

SI: References

1. Hu W, Su YS, & Lagarias JC (2009) A light-independent allele of phytochrome B faithfully recapitulates photomorphogenic transcriptional networks. *Mol Plant* 2:166-182.
2. Franklin KA, Allen T, & Whitelam GC (2007) Phytochrome A is an irradiance-dependent red light sensor. *Plant J* 50:108-117.
3. Franklin KA*, et al.* (2003) Phytochromes B, D, and E act redundantly to control multiple physiological responses in Arabidopsis. *Plant Physiol* 131:1340-1346.
4. Devlin PF*, et al.* (1999) Phytochrome D acts in the shade-avoidance syndrome in Arabidopsis by controlling elongation growth and flowering time. *Plant Physiol* 119:909-915.
5. Devlin PF, Patel SR, & Whitelam GC (1998) Phytochrome E influences internode elongation and flowering time in Arabidopsis. *Plant Cell* 10:1479-1487.
6. Shalitin D*, et al.* (2002) Regulation of Arabidopsis cryptochrome 2 by blue-light-dependent phosphorylation. *Nature* 417:763-767.
7. Clack T*, et al.* (2009) Obligate heterodimerization of Arabidopsis phytochromes C and E and interaction with the PIF3 basic helix-loop-helix transcription factor. *Plant Cell* 21:786-799.
8. Franklin KA, Davis SJ, Stoddart WM, Vierstra RD, & Whitelam GC (2003) Mutant analyses define multiple roles for phytochrome C in Arabidopsis photomorphogenesis. *Plant Cell* 15:1981-1989.
9. Monte E*, et al.* (2003) Isolation and characterization of phyC mutants in Arabidopsis reveals complex crosstalk between phytochrome signaling pathways. *Plant Cell* 15:1962-1980.
10. Su YS & Lagarias JC (2007) Light-independent phytochrome signaling mediated by dominant GAF domain tyrosine mutants of Arabidopsis phytochromes in transgenic plants. *Plant Cell* 19:2124-2139.
11. Franklin KA & Whitelam GC (2007) Light-quality regulation of freezing tolerance in Arabidopsis thaliana. *Nat Genet* 39:1410-1413.
12. Jones MA*, et al.* (2010) Jumonji domain protein JMJD5 functions in both the plant and human circadian systems. *Proc Natl Acad Sci U S A* 107:21623-21628.
13. Plautz JD*, et al.* (1997) Quantitative analysis of Drosophila period gene transcription in living animals. *J Biol Rhythms* 12:204-217.

SI: Figure Legends

Fig. S1. Prolonged Rc growth of wild type and *phy* mutants on soil for 6 weeks at the fluence rate of 150 μmol m^{-2} s^{-1} (A), or on MS salt phytoagar plates for 5 weeks at 50 (B) or 150 (C) μmol m^{-2} s^{-1}. The genotypes in (B) and (C) from right to left are WT, *phyB*, *phyAB*, *phyBCDE*, *phyABDE* (two) and *phyABCDE* (two). Bars = 1 cm. (D) Morphological comparison of *phy* mutant seedlings grown for 4 days in the dark or under continuous white light (60 μmol m^{-2} s^{-1}), red light (50 μmol m^{-2} s^{-1}), blue light (50 μmol m^{-2} s^{-1}), and far red light (20 μmol m^{-2} s^{-1}), bars = 5.0 mm.

Fig. S2. GA_4 effectively promotes germination of *phyABCDE* mutant lines that exhibit low germination capacity. (A) Low-germination *phyABCDE* lines (n = 5, independently grown and harvested) were sown on phytoagar plates (Agar) or MS salt phytoagar plates (MS) supplied with or without 100 μM of GA_3 or GA_{4+7} (PhytoTechnology product, GA_4/GA_7 = 6/3); after 4 d stratification, seeds were directly grown under Rc50 for 4 d before germination scoring. (B) Effect of GA_{4+7} concentration on *phyABCDE* germination on MS plates; the 5 independent lines used in (B) are different from those used in (A). Data are presented as mean ± S.D.

Fig. S3. Comparison of parental and newly isolated *phyABDE* lines in this study. (A) 4-d-old seedlings; (B) Relative chlorophyll levels of 5-d-old, Rc50-grown seedlings (data normalized to wild type; n= 4 to 6); (C) the first two rosette leaves of 26-d-old *phyABDE* mutant lines grown under short-day conditions , values are the whole leaf length/width ratio (n=12); (D) Cauline leaves of 36-d-old *phyABDE* mutant lines grown under short-day conditions, values are the leaf length/width ratio (n=10).

Fig. S4. Time course of red (Ri, 50 μmol m^{-2} s^{-1}) and white (Wi, 50 μmol m^{-2} s^{-1}) light induction of chlorophyll synthesis in 4 d-old, dark-grown seedlings (n = 3, mean ± SD), data are normalized to 4-d-old, Rc50-grown *phyAB* seedlings.

Fig. S5. The relatively late flowering phenotype of *phyABCDE* mutant lines is due to genetic impurity. (A) Parental *phyABCDE* lines flower later than *phyABDE*, shown are LD-grown plants (Mean ± S.E.M.). (B) Comparison of early- and late-flowering *phyABCDE* mutant lines. Plants were grown under LD conditions for 23 days. The inset compares the thickness of mature stems below the first internode (mean ± S.D.). (C) Real time PCR confirms higher expression levels of *FLC* in the parental and late-flowering *phyABCDE* lines isolated from

the backcross. (D) Linkage of flowering and molecular markers on Chromosome V; parental *phyBCDE* and *phyABCDE* are relatively late flowering; the *phyC-1* mutation was originated from the Ws accession.

Fig. S6. Morphology of *phy* mutants grown under long-day and short-day conditions for 26 days.
Seedlings were grown on MS medium for 5 days before transferring onto soil for flowering assay. Bars = 2 cm.

Fig. S7. Some *phyABCDE* mutants are developmentally retarded even under white light. The shown *phyABCDE* mutants were directly sown on soil and grown under short-day conditions for 35 days. A portion of mutant seedlings have the cotyledons being completely enclosed by the seed coat and never expand during their life time (right), some can expand their cotyledons but are greatly retarded (middle) compared to the normally developing ones (left). *phyABDE* mutants have similar phenomenon. The proportion of enclosed seedlings is reduced when they are grown under long-day conditions. Bar = 1cm.