The *out of phase 1* Mutant Defines a Role for PHYB in Circadian Phase Control in Arabidopsis[1]

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Arabidopsis displays circadian rhythms in stomatal aperture, stomatal conductance, and CO_2 assimilation, each of which peaks around the middle of the day. The rhythmic opening and closing of stomata confers a rhythm in sensitivity and resistance, respectively, to the toxic gas sulfur dioxide. Using this physiological assay as a basis for a mutant screen, we isolated mutants with defects in circadian timing. Here, we characterize one mutant, *out of phase 1* (*oop1*), with the circadian phenotype of altered phase. That is, the timing of the peak (acrophase) of multiple circadian rhythms (leaf movement, CO_2 assimilation, and *LIGHT-HARVESTING CHLOROPHYLL a/b-BINDING PROTEIN* transcription) is early with respect to wild type, although all circadian rhythms retain normal period length. This is the first such mutant to be characterized in Arabidopsis. *oop1* also displays a strong photoperception defect in red light characteristic of *phytochrome B* (*phyB*) mutants. The *oop1* mutation is a nonsense mutation of *PHYB* that results in a truncated protein of 904 amino acids. The defect in circadian phasing is seen in seedlings entrained by a light-dark cycle but not in seedlings entrained by a temperature cycle. Thus, PHYB contributes light information critical for proper determination of circadian phase.

Circadian rhythms are endogenous rhythms with periods of approximately 24 h. Circadian systems have been extensively described in cyanobacteria, *Neurospora crassa*, fruitfly (*Drosophila melanogaster*), mice, and humans; genetic and molecular biological studies have identified a number of the components of circadian systems in these model organisms (Johnson, 2001; Loros and Dunlap, 2001; Reppert and Weaver, 2001; Williams and Sehgal, 2001). One common theme emerging from these studies is that circadian oscillators are composed of two interconnected feedback loops (Glossop et al., 1999; Lee et al., 2000; Shearman et al., 2000; Alabadí et al., 2001; Denault et al., 2001). Although plants have provided many examples of circadian rhythmic outputs, including photoreceptor gene expression, and the study of the photoreceptors of plant circadian input pathways is well advanced (Casal, 2000; Devlin and Kay, 2001), our understanding of the plant circadian system remains incomplete (Harmer et al., 2001; McClung, 2001; McClung et al., 2002). Moreover, with the determination of the complete sequence of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000), it is evident that no obvious Arabidopsis orthologs to most known clock proteins can be found, demonstrating that at least part of the Arabidopsis clock mechanism is novel.

A number of loci have been implicated in the Arabidopsis circadian clock mechanism. At present, the best characterized are *TIMING OF CAB 1 EXPRESSION* (*TOC1*), *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), and *LATE ELONGATED HYPOCOTYL* (*LHY*; Alabadí et al., 2001; Makino et al., 2002; Matsushika et al., 2002). *toc1* loss-of-function mutations shorten the period of multiple rhythms, including leaf movement, stomatal conductance (Somers et al., 1998b), and transcription and mRNA accumulation of all clock-regulated genes examined (Millar et al., 1995a; Kreps and Simon, 1997; Strayer et al., 2000). In plants carrying a strong loss-of-function allele of *TOC1* (*toc1-2*), oscillations of *LHY* and *CCA1* mRNA exhibit greatly reduced mRNA abundance, consistent with a role of TOC1 as a positive regulator of *CCA1/LHY* (Alabadí et al., 2001). *CCA1* and *LHY* encode single Myb domain transcription factors, which, when overexpressed, result in arrhythmicity of multiple clock outputs, including leaf movement and mRNA abundance of all clock-regulated genes tested to date (Schaffer et al., 1998; Wang and Tobin, 1998; Fowler et al., 1999; Matsushika et al., 2002). Critically, overexpression of either *LHY* or *CCA1* results in nonoscillating low-level accumulation of *TOC1* mRNA, indicating that CCA1/LHY act as negative regulators of *TOC1* expression. The roles of CCA1 and LHY in

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oscillator function are thought to be at least partially redundant, because loss of CCA1 function shortens the period of mRNA oscillation in at least three clock-controlled genes, but the plants retain rhythmicity (Green and Tobin, 1999).

In all circadian systems, light acts as a powerful resetting signal to induce or repress the expression of clock genes, and photoreceptor mutants show defects in rhythmicity in constant conditions (Devlin and Kay, 2001). In plants, it has recently become clear that photoperception is itself a circadian output because transcription of both *CRYPTOCHROME* (*CRY*) genes and four of five *PHYTOCHROME* (*PHY*) genes (*PHYC* is the sole exception) is clock-regulated (Bognár et al., 1999; Tóth et al., 2001). A number of other components of light input pathways have been identified. For example, *early flowering 3* (*elf3*) mutants are conditionally arrhythmic in continuous light and *ELF3*, which encodes a novel protein (Hicks et al., 2001), plays a role in gating light signals to the clock (Hicks et al., 1996; McWatters et al., 2000; Covington et al., 2001; Liu et al., 2001). Mutational analysis has identified several other components that may participate in light input to the Arabidopsis circadian clock. Loss of function of *ZEITLUPE* (*ZTL*, also called *ADAGIO1*, *LOV KELCH PROTEIN 1* [*LKP1*], and *TOC7*) lengthens the period of all the rhythms affected by *toc1* (Somers et al., 2000). In addition, leaf movement becomes arrhythmic in red light in the *adagio1* mutant (Jarillo et al., 2001). Overexpression of the related *LKP2* results in arrhythmicity (Schultz et al., 2001). ZTL family members possess known functional domains arranged in a novel fashion: a single PAS/LOV-domain, an F-box, and six Kelch repeats, suggesting a role for light-regulated protein degradation in clock function (Kiyosue and Wada, 2000; Somers et al., 2000; Jarillo et al., 2001). ZTL (ADO1) physically interacts with both PHYB and CRY1 (Jarillo et al., 2001), although a role in photoreceptor degradation has not been established.

We wished to develop an independent and complementary approach to the identification of components of the Arabidopsis circadian system. Circadian rhythms in stomatal aperture and in the responsiveness of guard cells to environmental stimuli have been described in a number of plants (Webb, 1998). In beans, there is circadian control of the underlying biochemical reactions of the Calvin cycle in addition to control of stomatal aperture and gas exchange (Hennessey and Field, 1991). In accordance, we first established that Arabidopsis exhibits circadian rhythms in stomatal opening and CO_2 assimilation. We then demonstrated that these rhythms were correlated with a circadian rhythm in resistance and sensitivity to the air pollutant, sulfur dioxide (SO_2). We took advantage of this rhythm to screen a population of mutagenized M2 plants for individuals that exhibited damaged leaves in response to SO_2 exposure at a time when wild-type plants were resistant. Included among the mutants identified in this screen are several in which the circadian period is altered (either lengthened or shortened from that seen in wild-type plants) or in which circadian phase is disrupted. We show here that one of the mutants isolated from the screen, *out of phase 1* (*oop1*) exhibits a defect in circadian phase but retains wild-type period length. That is, the peak in several rhythms, including leaf movement, CO_2 assimilation, and *LIGHT-HARVESTING CHLOROPHYLL a/b BINDING PROTEIN* (*LHCB*) transcription, occurs earlier (phase leads) than is seen in wild type. This phenotype has not been previously described in Arabidopsis. *oop1* is a new allele of the red-light photoreceptor *PHYB* gene; *oop1* plants accumulate a truncated PHYB lacking most of the C-terminal kinase domain and behave as strong *phyB* mutants in red light. Surprisingly, the phase phenotype observed in *oop1* mutants is found in *phyB-9* mutants as well, indicating that it is the loss of PHYB function that confers the phase alteration. A blue-light-dependent enhancement of the hypocotyl phenotype seen in *oop1* mutants indicates an alteration of CRY1 signaling, although this is not seen in *phyB-9* seedlings and so does not seem to contribute to the phase alteration.

RESULTS

Arabidopsis Displays Circadian Rhythms in Stomatal Aperture and CO_2 Assimilation

Direct microscopic examination of epidermal peels from rosette leaves of Columbia (Col) plants grown under a 14-h:10-h light-dark cycle for 4 weeks revealed a diurnal oscillation in stomatal opening (Fig. 1A), as has been described in many species (Webb, 1998). The proportion of open stomata is greatest during the middle of the light period and is least late in the dark period. This rhythm persists in continuous conditions (extended dark) and, therefore, is under circadian control (Fig. 1B). This rhythm in stomatal aperture confers a rhythm in stomatal conductance in continuous light (data not shown), as has been described elsewhere (Somers et al., 1998b).

These oscillations in stomatal aperture and stomatal conductance are correlated with a rhythm in CO_2 assimilation (Fig. 2). Under entraining light-dark conditions the rate of CO_2 assimilation is high throughout the light period and becomes negative as a result of respiration in the dark (Fig. 2A). CO_2 assimilation responds immediately to the onset and offset of illumination. We attribute the apparent increase in CO_2 fixation occasionally observed before light onset to an artifact of our experimental system in which the lights went on during the sampling interval, yielding an intermediate rate of CO_2 assimilation, rather than interpreting this as evidence of dawn anticipation. Upon release into continuous light (Fig. 2B), the rate of CO_2 assimilation continues to oscillate, exhibiting a circadian rhythm with a period of 23.59 $\pm$ 0.23 h (mean $\pm$ SE, $n = 20$).

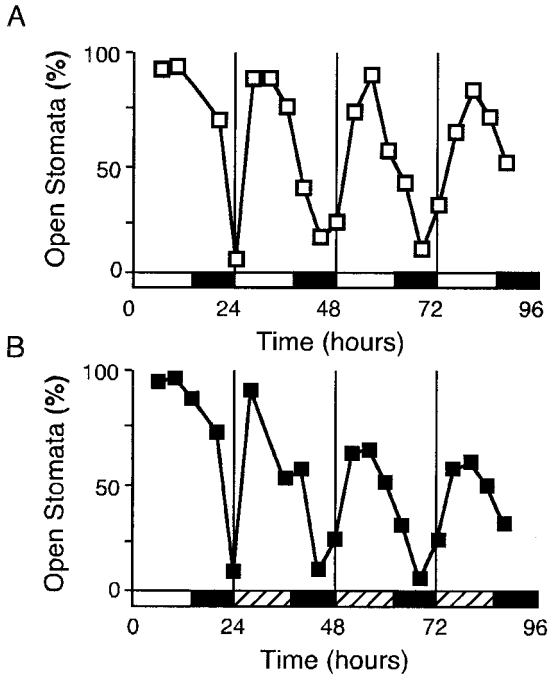

Figure 1. Circadian rhythm in stomatal aperture in Arabidopsis. A, Epidermal peels were prepared from Col plants grown in a 14-h:10-h light-dark cycle. B, As in A, but plants were grown in a 14-h:10-h light-dark cycle and transferred into extended darkness. Hatched bars indicate subjective day. Each peel was scored by microscopy for the percentage of open stomates. Each point represents the mean of duplicate samples, each of at least 100 stomates.

Circadian Rhythm in Resistance to SO_2

Most gas exchange occurs through stomata, and closed stomata render plants more tolerant to toxic gases (Mansfield and Freer-Smith, 1984). We asked whether the circadian rhythms in stomatal opening and conductance could generate a circadian rhythm in the tolerance of Arabidopsis to SO_2. Plants were grown in soil in a 14-h:10-h light-dark cycle for 3 to 4 weeks and transferred into continuous light. Replicate pots of plants were exposed to SO_2 at 2-h intervals over 48 h in continuous light. Plants exposed to SO_2 when the stomata were open developed pronounced necrotic leaf lesions (Fig. 3, A and C), whereas plants exposed when the stomata were maximally closed exhibited no visible necrosis (Fig. 3B). Plants were resistant only when treated in a narrow temporal window of approximately 1-h duration, 2 h before subjective dawn. Plants treated 2 h earlier, 2 h later, and at all other times were sensitive and developed necrotic lesions. The circadian oscillation in resistance persisted for at least 2 d in continuous light (data not shown). Because the rhythm in CO_2 assimilation persists for as long as 6 d in continuous light (Fig. 2B), we expect the rhythm in SO_2 resistance to persist after 48 h; however, this has not been experimentally determined.

Such a narrow window of resistance to SO_2 suggested an easy and sensitive physiological screen to identify mutants with altered circadian properties. Col plants grown in a light-dark cycle and transferred into continuous light would be resistant to a SO_2 treatment 2 h before subjective dawn (Fig. 3B). Any mutant unable to control properly its circadian rhythm in stomatal opening would, thus, develop necrotic lesions similar to those shown in Figure 3, A and C. This approach should allow the identification of both short- and long-period mutants in a single screening protocol (Fig. 3D). A short-period mutant would already have advanced through the period of tolerance and become sensitive because of its open stomata. A long-period mutant conversely would not yet have reached the tolerance period (closed stomata) and would also be sensitive to SO_2. Moreover, mutants in which the time of day of maximal stomatal closure and, hence, maximal SO_2 resistance has been shifted (i.e. circadian phase is altered) would also be expected to develop necrotic lesions.

M2 populations of ethyl methanesulfonate-mutagenized Col plants grown in a 14-h:10-h light-dark cycle and transferred into continuous light were treated with SO_2 2 h before subjective dawn on the 2nd d after transfer, when wild-type plants are resistant to SO_2. Plants that developed necrotic lesions were allowed to regrow from undamaged young leaves and to set seeds. The screen involved 6,500

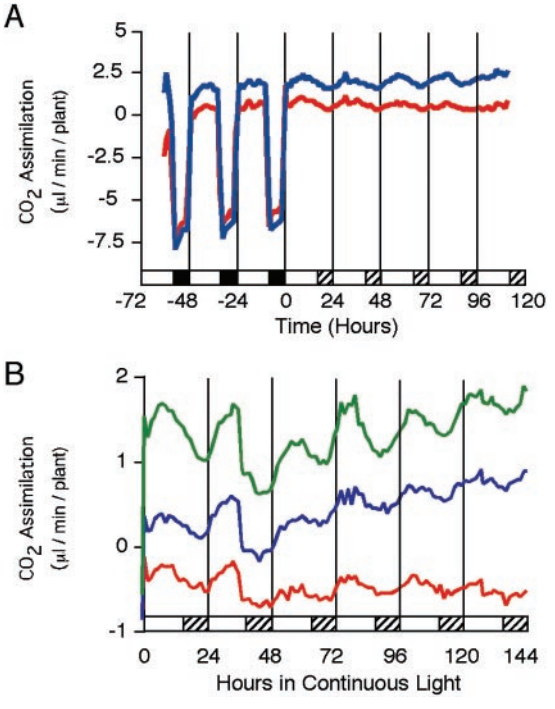

Figure 2. The rhythm in stomatal aperture correlates with a rhythm in CO_2 fixation. A, Rates of CO_2 exchange of representative individual 5- to 6-week-old Col plants grown in a 16-h:8-h light-dark cycle and transferred into continuous light. Hatched bars indicate subjective night. For the first 3 d, plants were maintained under entraining conditions and, therefore, show respiration during the night. B, Rates of CO_2 exchange of several representative individual Col plants after transfer into continuous light. Hatched bars indicate subjective night.

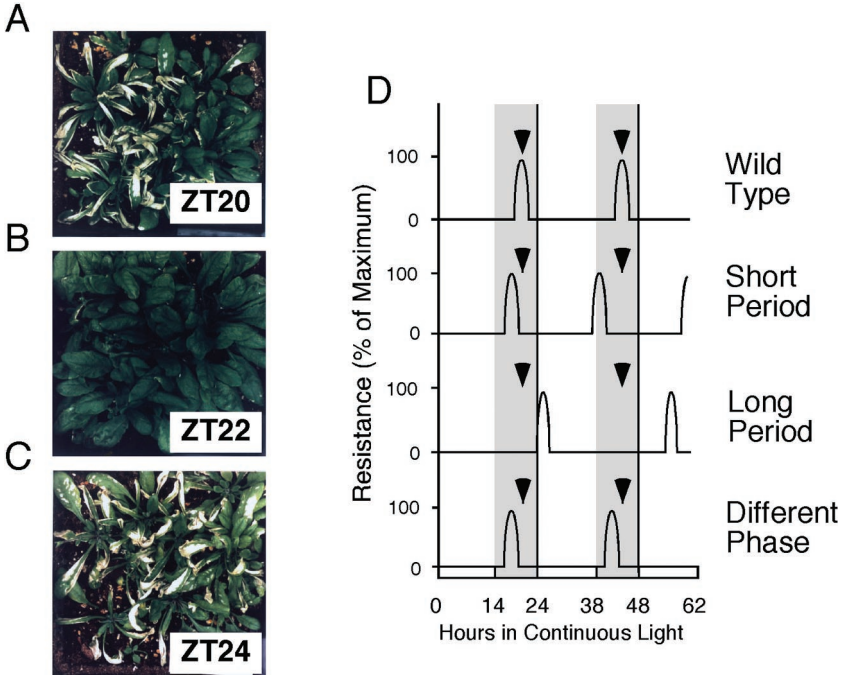

Figure 3. Arabidopsis exhibits a rhythm in sensitivity and resistance to SO_2. Col plants were grown in a 14-h:10-h light-dark cycle, transferred into continuous light, and then treated with SO_2 for 30 min each time, at 2-h intervals. A, Plants gassed 4 h before subjective dawn (ZT20, where ZT refers to Zeitgeber Time, defined as the number of h after the onset of illumination). B, Plants gassed 2 h before subjective dawn (ZT22). C, Plants gassed at subjective dawn (ZT24). D, Diagrammatic representation of the screening strategy for putative clock mutants based on resistance to SO_2. Wild-type (WT) plants are resistant to the treatment only approximately 2 h before subjective dawn (ZT22 and ZT46) and, thus, do not develop lesions when treated with SO_2 at ZT46. A short-period mutant would already have advanced through the period of resistance and become sensitive because of its open stomata. A long-period mutant would not yet have reached the tolerance period (closed stomata) and would also be sensitive to SO_2. Phase mutants would display resistance to SO_2 with a WT period, but the timing of the window of resistance is shifted earlier (as shown) or later than that of Col plants. Plants were grown in a 12-h:12-h light-dark cycle and transferred into continuous light at T = 0; the gray area indicates subjective night.

ethyl methanesulfonate-mutagenized plants from 13 independent M2 pools. Approximately 100 putative mutants were isolated that developed necrotic lesions when wild type did not. Of these, approximately one-third developed necrotic lesions without exposure to SO_2 and so were not studied further.

The CO_2 assimilation patterns of the remaining 65 mutants were analyzed to identify those with altered circadian rhythm properties. Twelve mutants with altered period lengths and/or altered phase relationships were found and fell into the three expected classes (long period, short period, and altered phase) of mutants. The period mutants have been designated *circadian timing defective*. The remaining mutants that exhibited wild-type period lengths in CO_2 assimilation rhythm but in which the phase of the peak of CO_2 assimilation was either earlier (phase leading) or later (phase lagging) than in wild type have been designated *out of phase* (*oop*). Because no mutants with this phenotype (leading circadian phase but wild-type period) have yet been described in Arabidopsis, one of these, *oop1*, was chosen for further characterization.

Characterization of *oop1*, a Circadian Phase Mutant of Arabidopsis

We compared rhythmicity of *oop1* with wild-type Col plants in terms of both period and phase of the peak (acrophase; Fig. 4; Table I). To facilitate comparison of phase among rhythms with slightly different periods, phase values were normalized to the period length of the rhythmic trace and are recorded in circadian time (CT = phase/period × 24 h). The period of the CO_2 assimilation rhythm in *oop1* was similar to that of the wild-type Col; in contrast, the acrophase (peak) of the rhythm occurred 3.4 h earlier than in Col (Fig. 4A; Table I). This difference in phase of CO_2 assimilation was significant (Student's two-tailed heteroscedastic *t* test, $P = 0.0036$).

Leaf movement allows us to look at an unrelated clock-controlled output. Although the periods of the rhythms in Col and *oop1* were not significantly different, the acrophase of the cotyledon movement in *oop1* occurred 3.6 h earlier than in Col (Fig. 4B; Table I), consistent with the results seen for CO_2 assimilation. Again, the difference in circadian phase between Col

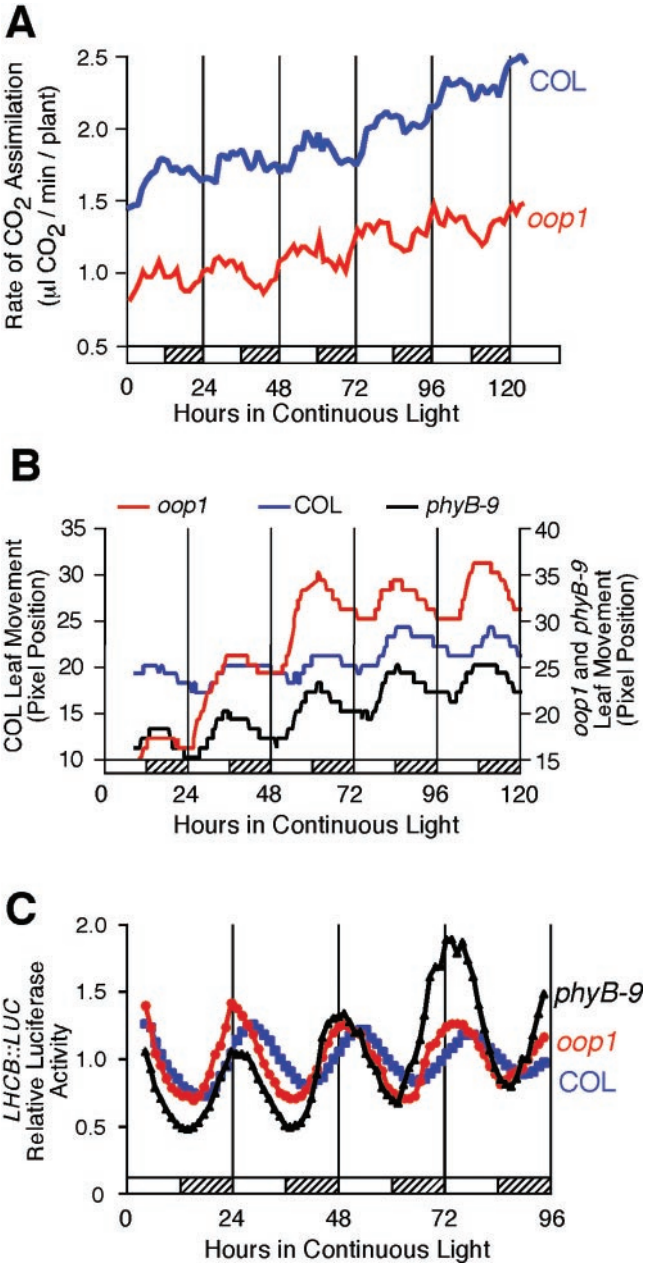

Figure 4. The *oop1* mutation causes an altered phase in circadian rhythms. A, Net CO_2 fixation levels in *oop1* and Col. *oop1* and Col plants were grown for 4 weeks in a 12-h:12-h light-dark cycle and transferred into continuous light. CO_2 assimilation levels were recorded for 6 d. B, Cotyledon movement in Col and *oop1* seedlings. *oop1* and Col seedlings were grown for 4 to 5 d in a 12-h:12-h light-dark cycle and transferred to 24-well cloning plates, one seedling per well, in continuous light. Cotyledon movement was recorded for 7 d. C, *LHCB::LUC* transcription. Col, *oop1*, and *phyB-9* seedlings homozygous for the *LHCB::LUC* transgene were grown in a 12-h:12-h light-dark cycle and transferred into continuous light at T = 0. Luciferase activity was recorded from each group of seedlings after transfer into continuous light and temperature conditions and is presented as the average of multiple seedlings (see Table I) for four complete circadian cycles. Hatched bars indicate subjective night. Col, Blue squares; *oop1*, red circles; and *phyB-9*, black triangles.

and *oop1* was statistically significant (Student's two-tailed heteroscedastic *t* test, $P < 0.0001$).

We also determined the effect of the *oop1* mutation on transcription of a luciferase transgene driven by the *LHCB 1*1* (*CAB2*) promoter (*LHCB::LUC*). Luciferase activity was recorded over 4 d in continuous light after 7 d of entrainment in a 12-h:12-h light-dark (Fig. 4C; Table I). Again, the period of the rhythm in *LHCB* transcription was not statistically different in *oop1* versus Col seedlings. However, the phase of the rhythm in *LHCB* transcription was 1.9 h earlier, and this difference was statistically significant (Student's two-tailed heteroscedastic *t* test, $P = 0.0459$).

These rhythms are measured at different stages of development: Leaf movement and *LHCB* transcription were recorded on 5- to 12-d-old seedlings, whereas CO_2 assimilation was measured on 4- to 5-week-old plants. Thus, the *oop1* mutation affects multiple rhythmic outputs at different developmental stages. Moreover, the *oop1* mutation alters the phase of rhythms that display distinct circadian phases. The period of the rhythm in CO_2 assimilation was shorter than that for leaf movement or *LHCB* transcription. We suspect that differences in the growth conditions, possibly including the light conditions, as well as the age of the plants for each assay, may account for the variation in period lengths for the measured rhythms (Table I; for details, see "Materials and Methods").

oop1 Is Primarily Impaired in Red-Light Photoperception

Initial observations of the *oop1* mutant suggested that its hypocotyl was longer than that of wild-type seedlings when grown under continuous white light. Mutant seedlings were, thus, grown under different light qualities (100 μmol m^{-2} s^{-1} white, 20 μmol m^{-2} s^{-1} red, 25 μmol m^{-2} s^{-1} blue, and 10 μmol m^{-2} s^{-1} far-red) to better define the response of *oop1* seedlings to light. Hypocotyls of *oop1* seedlings are much longer than Col under white and red light but not under blue or far red light (Fig. 5, A and B), indicating that *oop1* is primarily impaired in red-light photoperception through PHYB. The extent of the hypocotyl elongation phenotype seen in *oop1* under red light is similar to that of *phyB-9*, a null allele of *PHYB* in the Col background (Reed et al., 1993), suggesting that the *oop1* mutation eliminates PHYB-dependent signaling. In white light, the *oop1* hypocotyl phenotype was inherited as a single recessive Mendelian locus. As with *phyB* loss of function alleles, in red light, the hypocotyl length of seedlings heterozygous for *oop1* is intermediate between wild-type and *oop1* homozygotes (data not shown), suggesting semidominant or incompletely recessive inheritance of the *oop1* mutation.

Table I. *Alterations in circadian phase in oop1 and phyB-9 mutants*

Values are mean ± SD.

Rhythm	Genotype	Period	Phase[a]
		h	*CT h*
CO_2 Assimilation	Col	22.20 ± 0.52	13.58 ± 1.50 ($n = 5$)
	oop1	22.80 ± 0.36 ($P = 0.0667$)[b]	10.15 ± 1.15 ($n = 5$; $P = 0.0036$)
Leaf Movement	Col	24.24 ± 0.90	17.32 ± 1.54 ($n = 13$)
	oop1	24.30 ± 0.54 ($P = 0.8007$)	13.69 ± 1.62 ($n = 24$; $P < 0.0001$)
	phyB-9	23.72 ± 1.02 ($P = 0.2213$)	15.15 ± 3.39 ($n = 9$; $P = 0.0548$)
LHCB::LUC	Col	24.53 ± 0.88	3.81 ± 2.15 ($n = 7$)
	oop1	24.22 ± 0.80 ($P = 0.4104$)	1.93 ± 1.91 ($n = 17$; $P = 0.0459$)
	phyB-9	24.23 ± 0.74 ($P = 0.3830$)	1.32 ± 2.14 ($n = 21$; $P = 0.0131$)

[a] Phase values are normalized to the period length and are given in circadian time (CT = phase/period × 24 h). [b] *P* value from Student's two-tailed heteroscedastic *t* test comparing the mutant value with Col is given in parentheses. The *oop1* and *phyB-9* values were not significantly different within any treatment.

The *oop1* Locus Is *PHYB*

Cloning the mutated gene responsible for the phase defect seen in several rhythms was the next step in trying to understand the molecular determinants of phase regulation in Arabidopsis. For low-resolution mapping, 41 lines homozygous for the *oop1* mutation were selected from the F_2 progeny of a cross of *oop1* to Landsberg *erecta*, based on their long hypocotyl in red light. Using CAPS, RFLP, and simple sequence length polymorphism markers, the *oop1* mutation was located centromere proximal, on the bottom arm of chromosome 2 (Fig. 6A). A large region, from 29 to 36 cM, was identified in which no recombinant chromosomes could be detected. Of particular interest to us was the fact that *PHYB* lies at 34.46 cM within this region. We determined the genomic sequence of *PHYB* in the *oop1* background. As shown in Figure 6B, two mutations were found on two overlapping PCR products, a missense mutation (P872L), and a nonsense mutation, introducing a stop codon after amino acid 904 (Q905X). These mutations were confirmed by sequencing independent PCR amplification products from independent DNA preparations and with the creation of mutation-specific dCAPS markers (Fig. 6C).

$PHYB^{oop1}$ is predicted to encode a truncated protein of an apparent molecular mass of approximately 95 kD. This truncation would eliminate the C terminus of phytochrome, which is the region with most divergence among PHYs. The PHYB-specific monoclonal antibody B6B3 (Hirschfeld et al., 1998) fails to detect any PHYB protein in *oop1* (Fig. 6D), presumably because the epitope to this antibody is C-terminal to the *oop1* truncation. However, a second monoclonal antibody (3B5), which recognizes all five Arabidopsis PHYs (Hirschfeld et al., 1998), detects in *oop1* homozygotes both a reduced quantity of full-length PHYs and a peptide of smaller M_r than a full-length PHY protein (Fig. 6D). This protein has the predicted approximately 95-kD size for a truncated $PHYB^{oop1}$ protein lacking the last 268 amino acids. The large C-terminal truncation may reduce protein stability and contribute to the low levels of the PHYB photoreceptor in *oop1*. We, therefore, conclude that *oop1* is a new allele of the *PHYB* gene encoding the red-light photoreceptor PHYB and that the *oop1* mutant accumulates low levels of a truncated PHYB lacking most of the kinase domain (Fig. 6E).

Altered Phase in *LHCB::LUC* Transcription Is a General Property of *phyB* Mutants

The isolation of a new *phyB* allele that confers altered phasing of several circadian rhythms was unexpected. The role of PHYB has been well documented for the establishment of the proper period length in red light and in entrainment of the circadian clock. In intermediate and high-fluence red light, loss of PHYB function lengthens the period, whereas PHYB overexpression shortens the period (Somers et al., 1998a; Devlin and Kay, 2000, 2001). Little has been reported on the effect of *phyB* mutations on circadian rhythms under white light. In white light, overexpression of PHYB does not shorten nor does loss of PHYB function lengthen the period for *LHCB* transcription (D.E. Somers, personal communication), consistent with our observations that neither *oop1* nor *phyB-9* confer lengthened period in white light. Blue-light signaling through PHYA, CRY1, and CRY2 also is important in the establishment of period length (Somers et al., 1998a; Devlin and Kay, 2000, 2001), and we suspect that blue-light signaling is sufficient to establish wild-type period length in *oop1* under white light. We have considered two explanations of the leading (early) phase observed in *oop1*. The truncated PHYB protein accumulating in *oop1* could display dominant negative interference with another signal transduction pathway and, together with the loss of PHYB activity, cause the leading phase. As an alternative, the phase alteration could be a general feature of *phyB* mutants. In accordance, we crossed a *LHCB::LUC* transcriptional fusion into the *oop1* and *phyB-9* mutant backgrounds. Luciferase activity was recorded over 4 d in contin-

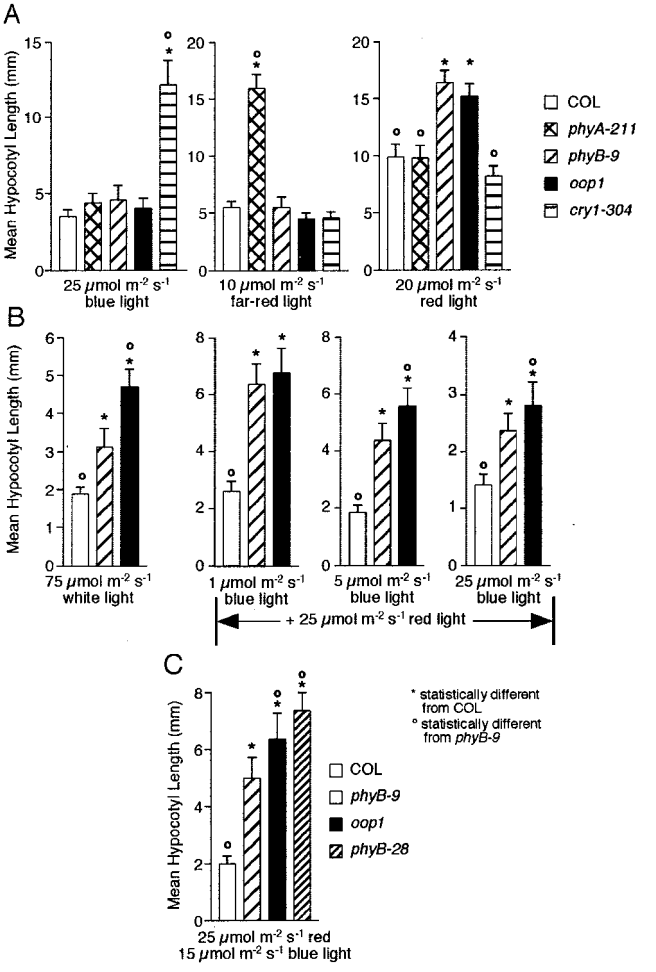

Figure 5. The *oop1* mutant is primarily impaired in red-light perception. A, *oop1* was grown in continuous blue, far-red, or red light for 5 d before hypocotyl length was measured with NIH Image v1.62. The known photoreceptor mutants *phyA-211*, *phyB-9*, and *cry1–304* were also used as controls for loss of PHYA-, PHYB-, or CRY1-mediated light perception, respectively. Hypocotyl length (mean ± SD) is given for each genotype and treatment. B, Hypocotyl phenotype of *oop1* and *phyB-9* in response to combinations of red and blue lights. Col, *oop1*, and *phyB-9* seedlings were grown as described in A, under 25 μmol m^{-2} s^{-1} red light combined with 1, 5, or 25 μmol m^{-2} s^{-1} blue light. C, Enhancement of hypocotyl phenotype in *oop1* and *phyB-28*. Col, *oop1*, *phyB-9*, and *phyB-28* seedlings were grown as described in A, under a combination of 25 μmol m^{-2} s^{-1} red light and 15 μmol m^{-2} s^{-1} blue light. *, Hypocotyl length is significantly different (Student's two-tailed heteroscedastic *t* test, $P < 0.001$) from Col; °, hypocotyl length is significantly different ($P < 0.001$) from *phyB-9*.

uous light after 7 d of entrainment in a 12-h:12-h light-dark cycle. F3 seedlings homozygous for *oop1 LHCB::LUC* and *phyB-9 LHCB::LUC* show the same leading phase when compared with seedlings of their respective wild types (Fig. 4C; Table I). This is consistent with the *oop1* mutant phenotype resulting from the mutation of the *PHYB* locus as opposed to resulting from a mutation in a second locus tightly linked to PHYB. Moreover, because *phyB-9* is a complete loss-of-function allele (Reed et al., 1993), it is the loss of PHYB function in white light that is responsible for the leading phase of *LHCB::LUC* transcription and of leaf movement (Table I). Importantly, this phase alteration is not seen in *oop1* or *phyB-9* seedlings entrained by a 12-h:12-h temperature cycle of 12°C: 22°C (Fig. 7; Col phase = 3.30 ± 3.27 CT h [n = 9], *oop1* phase = 3.14 ± 2.75 CT h [n = 19], and *phyB-9* phase = 2.83 ± 2.46 CT h [n = 24]). This shows that the phase alteration is solely due to the loss of photoperception through PHYB and does not indicate a defect in the clock oscillator, which retains a wild-type period.

PHYB^{oop1} Affects Blue-Light Photoperception

When grown in white light, *oop1* seedlings displayed a longer hypocotyl than *phyB-9* seedlings (Fig. 5B), raising the possibility that *oop1* may affect more than PHYB signaling in white light. We grew the seedlings under a combination of red or far-red light (25 μmol m^{-2} s^{-1}) plus blue light at 1, 5, or 15 μmol m^{-2} s^{-1}. Although no differences between *oop1* and *phyB-9* could be seen in combined far-red plus blue lights (data not shown), hypocotyls of *oop1* are significantly longer than those of Col, *cry1-304*, and *phyB-9* when seedlings are grown in mixtures of red and blue lights. This effect was observed at 5 and 25 μmol m^{-2} s^{-1} blue light, but not at 1 μmol m^{-2} s^{-1} blue light (Fig. 5B).

Because the enhancement of the hypocotyl phenotype is seen only when red light and fluence rates of blue light 5 μmol m^{-2} s^{-1} and higher are combined, we conclude that *oop1* causes a red-light-dependent alteration of CRY1 signaling. This does not reflect changes in CRY1 protein levels, which are unaltered in *oop1* plants (data not shown). Although CRY2 has been shown to interact with PHYB in vivo (Más et al., 2000), CRY1 is the primary blue-light photoreceptor at high-fluence rates, whereas CRY2 and PHYA are both important in the perception of blue light at low-fluence rates (Lin, 2000). A similar enhancement of hypocotyl elongation was seen in the *phyB-28* mutant (Fig. 5C), which lacks most of the kinase domain due to a frame shift at amino acid 991 that results in the premature termination of the protein at amino acid 995 (Krall and Reed, 2000). The phenotype in combined red plus blue lights was even more pronounced than in *oop1*, presumably because *phyB-28* accumulates more truncated PHYB than *oop1* (Krall and Reed, 2000). Because *oop1* and *phyB-28* were isolated from two independent screens and show the same phenotype with respect to hypocotyl elongation in combined lights, we believe this phenotype to be due to an interference with another signaling pathway, possibly originating from CRY1, rather than to the presence of a second, linked mutation in the *oop1* background. Consistent with the *oop1* mutation interfering with blue-light signaling through

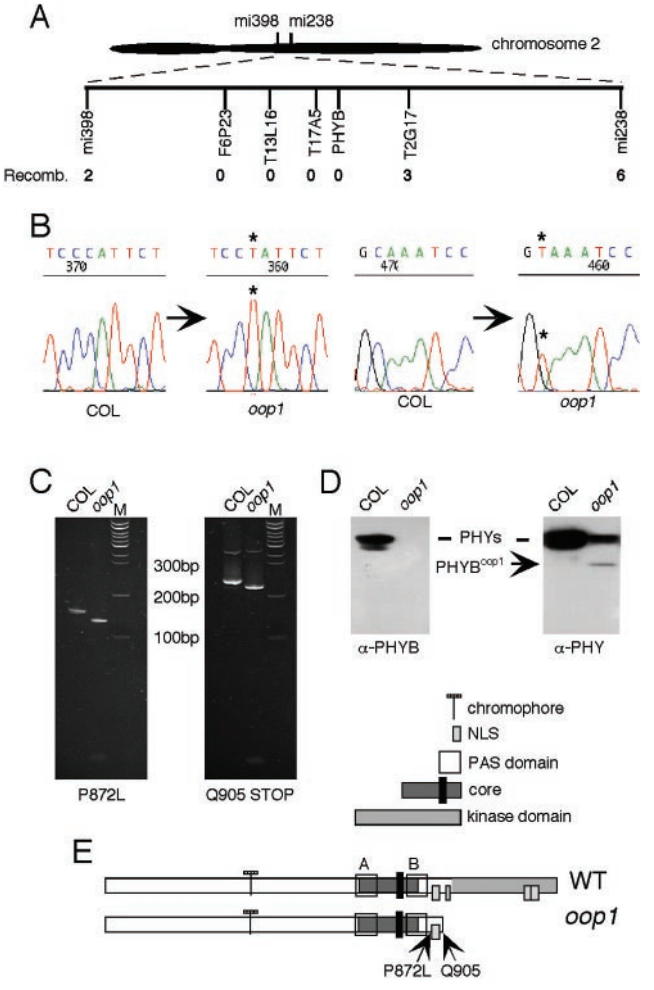

Figure 6. *oop1* is a new allele of *PHYB*. A, Map position of the *oop1* locus. The number of recombinant chromosomes among a population of 41 plants homozygous for the *oop1* mutation is indicated for each marker. mi398 and mi238 are RFLP markers at 29.27 and 39.02 cM, respectively. B, Mutations in the $PHYB^{oop1}$ gene. Sequencing results from Col and *oop1* are shown. The positions of the mutations are highlighted by asterisks. C, dCAPS analysis of PHYB in *oop1*. Two dCAPS markers were developed, one specific to each mutation. The PCR products were amplified from Col and *oop1* DNA and digested with *Sty*I for the P872L mutation and *Bsr*GI for the Q905X mutation. The restriction digests were run on a 10% (w/v) acrylamide gel and stained with ethidium bromide. Left, dCAPS results obtained for the P872L mutation. Right, Q905X mutation. M indicates the 100-bp Plus ladder (MBI Fermentas, Hanover, MD). D, Western-blot analysis of *oop1* plants. Left, The membrane was probed with a PHYB-specific monoclonal antibody that recognizes an epitope in the carboxy terminus, which is not retained in *oop1*. This antibody fails to detect full-length PHYB protein in *oop1*. Right, The membrane was probed with a monoclonal antibody raised against a conserved epitope in the central region of the molecule, which recognizes all phytochromes; the *oop1* sample shows a smaller protein species of the M_r predicted for a PHYB protein comprising the first 904 amino acids only. E, Maps of PHYB and $PHYB^{oop1}$. The functional domains of PHYB are indicated, as well as the position of the two mutations in *oop1*. The Q905X mutation introduces a premature stop codon at amino acid 904 and causes the loss of the His-kinase related domain and three of the four putative nuclear localization sequences.

CRY1, *oop1* plants grown in short day conditions display elongated internodes before the transition to reproductive meristem has occurred (data not shown). This phenotype is similar to, although not as extreme as, that seen in *phyB cry1* double mutants grown in the same conditions (Casal and Mazzella, 1998).

DISCUSSION

The *oop1* phenotype in which the phases but not the periods of multiple rhythms are affected in white light is unusual and may allow insight into the mechanisms of phase determination. *oop1* is a new allele of the red-light photoreceptor *PHYB* gene. This represents the first report of PHYB as a regulator of circadian phase, which adds to its known involvement in controlling the period of the clock in red light (Somers et al., 1998a; Devlin and Kay, 2000, 2001). *oop1* plants accumulate a truncated form of PHYB that lacks the C-terminal Ser/Thr kinase domain (Yeh and Lagarias, 1998), but that retains the two PAS domains, which mediate protein-protein interactions (Lindebro et al., 1995; Ni et al., 1998). Elongation of hypocotyls in red light shows that *oop1* is a putative null allele of *PHYB* in red light, which suggests that this truncated PHYB protein is not active. The characterization of *phyB-9*, a known null *PHYB* allele (Reed et al., 1993), shows that loss of PHYB function affects the phase of *LHCB::LUC* transcription. The similar phase alteration caused by these two *phyB* mutations suggests that the truncated photoreceptor accumulating in *oop1* is not responsible for the circadian phenotype but rather, it is the loss of PHYB signaling that is primarily responsible for changing the phase.

At least three possible mechanisms may explain the phase alteration seen in *oop1* and *phyB-9*. PHYB signaling could affect light input to the clock. As an alternative, PHYB signaling could directly affect levels of a critical clock component. Finally, PHYB signaling could affect a component of an output pathway acting downstream of the oscillator. Because the period length is unaffected by the *oop1* or *phyB-9* mutations, we conclude that oscillator function is not affected, and it is unlikely that an oscillator component is the direct target of PHYB signaling. However, our data are inadequate to distinguish between a defect in light input to the clock and light modulation of an output component. It is often difficult to distinguish between input and output pathways based solely on an altered clock parameter like period or phase (Roenneberg and Merrow, 1998; Foster and Lucas, 1999; Merrow et al., 1999). As noted above, the expression of Arabidopsis *PHY* and *CRY* photoreceptor genes oscillates, indicating that the ability to perceive light (a clock input) is modulated by the clock and, hence, is a clock output (Bognár et al., 1999; Tóth et al., 2001). However, we do note that because the

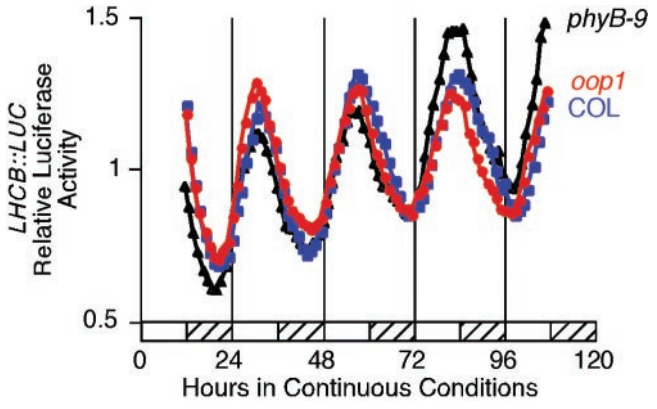

Figure 7. Phase alteration of *LHCB::LUC* transcription in *oop1* is not seen after entrainment by temperature cycles. Col and *oop1* seedlings homozygous for the *LHCB::LUC* transgene were grown for 7 d under entraining conditions consisting of 12 h at 22°C followed by 12 h at 12°C. Luciferase activity was recorded from each group of seedlings after transfer into continuous light and temperature conditions and is presented as the average of multiple seedlings. Col, Blue squares; *oop1*, red circles; and *phyB-9*, black triangles. The hatched bars represent the subjective cold (12°C) period of the day.

phase of multiple rhythms (SO_2 resistance, CO_2 assimilation, leaf movement, and *LHCB::LUC* transcription) is affected in *oop1* and *phyB-9* mutants, if PHYB affects clock output, then the defect must occur at an output component that is common to all of these output pathways, clock-proximal to any branch points that distinguish these output pathways.

Both light and temperature signaling can entrain the circadian clock (Somers et al., 1998a; Devlin and Kay, 2000, 2001). The altered phase in the *oop1* mutant is only evident in response to an entraining light-dark cycle and is not detected after entrainment to temperature cycles. Temperature signaling, which is not affected in *oop1* plants, apparently compensates for the impairment in red-light signaling and is sufficient for the establishment of proper phase in both *oop1* and *phyB-9* plants after entrainment by temperature cycles. However, in the temperature cycles used in Figure 7, the seedlings are grown under continuous light and one might expect that the light signaling defect of *oop1* and *phyB-9* should be evident as a phase-angle alteration. Nonetheless, under these conditions the phase of *LHCB* transcription is identical in all genotypes. This argues that the *oop1* (*phyB*) defect does not affect parametric entrainment under continuous light but, rather, that PHYB is required to establish the wild-type phase relationship (phase angle) during discrete entrainment to light-dark cycles.

Loss of PHYB signaling would be expected to attenuate light input to the clock. This would reduce the acute induction of a light-induced clock component at dawn (lights on) or, conversely, delay the light-regulated degradation of some critical clock component. In either case, the timing of the acrophase after dawn would be affected. One known target of PHYB signaling is PHY INTERACTING FACTOR 3 (PIF3), a bHLH-PAS transcription factor that binds to G-box motifs (Ni et al., 1998). Upon illumination with red light, PHYB relocalizes from the cytoplasm into the nucleus (Kircher et al., 1999; Yamaguchi et al., 1999) where it binds directly to PIF3 and modulates transcriptional activity (Ni et al., 1999; Martínez-García et al., 2000). Transcription of two known clock components, *CCA1* and *LHY*, is activated by PHYB through PIF3 bound to G-boxes in their promoter regions, and in *PIF3* antisense lines, the induction of both *CCA1* and *LHY* is attenuated (Martínez-García et al., 2000). We suggest that the loss of PHYB-mediated induction of *CCA1* and *LHY* at dawn might confer the phase defect seen in *oop1* and *phyB-9* plants. The mutant phase leads (precedes) the phase seen in wild-type plants, which suggests that the PHYB/PIF3 target should function as a negative clock element. This is consistent with the demonstrated role of CCA1 as a negative regulator of *TOC1* transcription (Alabadí et al., 2001). Thus, we suggest that PHYB and PIF3 may constitute an important phase-determination pathway providing input to either the Arabidopsis clock or to a downstream output component. This pathway need not be exclusive to PIF3 signaling and may include one or more of the many PIF3 relatives found in Arabidopsis. PHYA also provides light input through PIF3 to induce *CCA1* expression (Tepperman et al., 2001). *CCA1* and *LHY* expression is still detectable to levels close to wild type in *oop1* and *phyB-9* (data not shown), suggesting that induction of these putative clock components is not exclusively mediated by PHYB. It is interesting to note that both PHYB and PIF3 contain PAS domains, as do the ZTL/LKP2/FKF1 proteins that are implicated in light-regulated protein degradation in the Arabidopsis circadian system (Kiyosue and Wada, 2000; Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001). The PAS domain features prominently in other clock-associated light input pathways including that of *N. crassa*, where the WHITE COLLAR proteins and VIVID (VVD) contain PAS domains (Heintzen et al., 2001; Loros and Dunlap, 2001; Merrow et al., 2001).

Phase mutants have been described in several other clock systems. In fruitfly, two *phase-angle* (*psi-2* and *psi-3*) mutants dramatically alter the phase of the eclosion rhythm, although the role of these genes in the establishment of phase remains unknown (Jackson, 1983). In *N. crassa*, mutation of the *vvd* locus alters the phase of the rhythm in *FREQUENCY* expression (Heintzen et al., 2001). Deletion of a calcium/calmodulin-dependent protein kinase, *camk-1*, confers both a lagging phase and slight period lengthening in *N. crassa* (Yang et al., 2001). In cyanobacteria, inactivation of *cikA*, which encodes a bacteriophytochrome, alters circadian phase but also shortens the period length (Schmitz et al., 2000). Thus, PHYB is analogous to VVD and CikA in play-

ing a role in the determination of both period and phase.

Although the circadian phase defect seen in the *oop1* mutant probably reflects simple loss of PHYB signaling, the observed hypocotyl phenotypes of *oop1* and other *phyB* mutants suggests interactions with other light-signaling pathways. Through its PAS domains, $PHYB^{oop1}$ may be able to interact with at least some PHYB-interacting factors, but would be unable to transduce the light signal to them, because it lacks all of the kinase domain. The enhancement of hypocotyl elongation seen in red plus higher intensities of blue light suggests that $PHYB^{oop1}$ and $PHYB^{phyB\text{-}28}$ interact with a CRY1 signaling pathway, possibly through the titration of some common interacting protein. Because $PHYB^{oop1}$ lacks the three nuclear localization sequences shown to be sufficient for nuclear translocation of a GUS::$PHYB_{898–1172}$ fusion (Sakamoto and Nagatani, 1996), we predict that these potential interactions are cytosolic. PIF3 is constitutively nuclear (Ni et al., 1998) and is, therefore, unlikely to be a partner for this aberrant $PHYB^{oop1}$ interaction. ELF3 affects light input to the circadian clock (McWatters et al., 2000) and light-mediated inhibition of hypocotyl elongation in both red and blue wavelengths (Zagotta et al., 1996). In addition, ELF3 interacts directly with PHYB (Liu et al., 2001). However, ELF3 is a nuclear protein (Liu et al., 2001), so it is unlikely that $PHYB^{oop1}$ titrates ELF3. PHY KINASE SUBSTRATE 1 (PKS1) is cytosolic (Fankhauser et al., 1999), but PKS1 overexpressing or antisense lines show no modified sensitivity to blue light, indicating that PKS1 is unlikely to be the relevant target of $PHYB^{oop1}$. PKS2 (Munich Information Center for Protein Sequences no. At1g14280), a protein 55% identical to PKS1, is predicted to be cytosolic and might represent such in interactor. As an alternative, $PHYB^{oop1}$ could physically interact with CRY1. Labeling studies showed a red-light-dependent, far-red-light reversible phosphorylation of CRY1, which could indicate that PHYB may interact with CRY1 in vivo (Ahmad et al., 1998).

It has been clear for some time that PHYB plays multiple important roles in light regulation of multiple developmental processes. The characterization of *oop1*, a new allele with a truncation of the carboxy terminus of PHYB, has revealed two previously unknown roles of PHYB: a potential interaction with CRY1-mediated blue-light regulation of hypocotyl elongation, and an important contribution to white-light-mediated phase determination of the circadian clock.

MATERIALS AND METHODS

Plant Growth and Genotypes

Plants were sown on standard soil mixture (Pro-mix "BX," Premier, Rivière-du-Loup, Canada) and watered with Arabidopsis nutrient solution (Somerville and Ogren, 1982). Plants were germinated and grown under fluorescent white light (model TL-741, Philips, Somerset, NJ). The wild-type ecotype was Col-2 (Col, ABRC no. CS907, Ohio State University, Columbus). Several null alleles of photoperception mutants were used in this study. *phyA-211*, *phyB-9*, and *cry1-304* are null alleles of *PHYA*, *PHYB*, and *CRY1*, respectively, and all are in the Col ecotype. The *cry1-304* seeds were a gift of C. Lin (University of California, Los Angeles).

Determination of Stomatal Opening, Stomatal Conductance, and Measurement of CO_2 Assimilation

Epidermal peels were prepared from Col plants grown for 3 weeks under approximately 100 μmol m^{-2} s^{-1} in a 14-h:10-h light-dark cycle or grown in a light-dark cycle and transferred into extended darkness. Stomatal opening was scored by microscopic inspection. For each time point, at least 10 fresh peels were made and at least 10 stomata per peel (at least 100 stomata total) were scored as either open or closed. Each point in Figure 1 represents the mean of duplicate samples. Stomatal conductance was measured with a photosynthesis system (CI-301PS, CID, Vancouver, WA).

For the measurement of CO_2 assimilation, plants were grown under 100 μmol m^{-2} s^{-1} white light in a 12-h:12-h light-dark cycle (Fig. 4A) or in a 16-h:8-h light-dark cycle (Fig. 2) at 20°C to 22°C in soil in 50-mL conical tubes from which the tips had been removed to permit bottom watering. The soil was initially wetted with a nutrient solution (Somerville and Ogren, 1982) and plants were subsequently bottom watered. After approximately 1 week of growth, seedlings were thinned to one per tube. After 3 to 4 weeks, individual plants in their conical tubes were transferred to 100-mL beakers containing approximately 50 mL of deionized water. The upper surface of the beakers was sealed with laboratory film (Parafilm, American National Can, Greenwich, CT) to reduce evaporation. The beakers and the plants were then placed individually into 1-quart Mason jars that were individually attached to Micro Oxymax Respirometers (Columbus Instruments, Columbus, OH). The plants were further entrained in 12-h:12-h light-dark cycle (Fig. 4A) or in 16-h:8-h light-dark cycle (Fig. 2) for another 2 d before being transferred into continuous white light (approximately 100 μmol m^{-2} s^{-1}). CO_2 concentration in the sample chambers was determined by infrared gas analysis at approximately 1-h intervals. Because the system is closed, after each sampling cycle, the air in the sample chamber was replaced with compressed air (Northeast Airgas, White River Junction, VT) of constant composition (350 μL L^{-1} CO_2).

SO_2 Sensitivity/Resistance Assay

For determination of sensitivity to SO_2, 4-week-old Col plants were grown in a 14-h:10-h light-dark cycle, transferred to continuous light for 2 d, and then treated with SO_2 at 2-h intervals over two complete circadian cycles. Time is given in Zeitgeber Time (ZT; zeitgeber is German for time giver), where ZT0 corresponds to lights on; for example, ZT20 corresponds to 20 h from the onset of illumination. The plants were gassed in a black Plexiglas box with an approximate volume of 130 L. SO_2 (445 μL L^{-1}, Merriam-Graves, White River Junction, VT) was added to the box at a flow rate of 5.63 L min^{-1} for 2 min (total of 11.26 L) to yield a final SO_2 concentration of 38.5 μL L^{-1}; plants were left under these conditions for 15 min. The box was then flushed with compressed air at approximately 8 L min^{-1} for 15 min. Plants were removed from the Plexiglas box, returned to the light-dark cycle, and scored for the presence of lesions (water-soaked, wilted areas) 2 h after treatment and again 2 d after treatment, when the lesions dried and turned white.

Leaf Movement Assay

Assessment of rhythmicity in leaf movement was carried out as described (Millar et al., 1995a, 1995b; Hicks et al., 1996). Seeds were surface-sterilized by the vapor-phase method (Clough and Bent, 1998) and then plated on Murashige and Skoog salts + 2% Suc (MS2S). Seedlings were grown in white light for 4 to 5 d in a 12-h:12-h light-dark cycle on MS2S plates and then transferred to 24-well cloning plates (Greiner Labortechnik, Frickenhausen, Germany), one seedling per well. The plates were transferred to continuous white light (30–40 μmol m^{-2} s^{-1}), and leaf movement was recorded every 20 min over 7 d by Panasonic CCTV cameras (model WV-BP120, Matsushita Communications Industrial, Laguna, Philippines). Post-run analysis was performed using the Kujata software program (Millar

et al., 1995a, 1995b), and traces were analyzed by fast Fourier transform-nonlinear least squares (Plautz et al., 1997; Zhong et al., 1997).

Hypocotyl-Length Characterization

Seeds were surface-sterilized by the vapor-phase method (Clough and Bent, 1998) and then plated along the diagonal of MS2S petri plates. The plates were stratified for 3 d at 4°C and released in white light (approximately 100 μmol m^{-2} s^{-1}) for 12 h to induce germination. The plates were then transferred into the dark for 12 h before being released into the appropriate light conditions. For monochromatic light treatments, plates were placed in an E-30LED chamber (Percival, Boone, IA) in combination with red Plexiglas filters (Rohm and Haas no. 2423, Cadillac Plastics, Manchester, NH). For combined light studies, the filters were omitted because both blue and red light were provided. After 5 to 6 d of growth, the plates were scanned in Adobe Photoshop (Adobe Systems, Mountain View, CA), and hypocotyl length was determined by using NIH Image 1.62.

Genetic Mapping of *oop1* and Sequencing of $PHYB^{oop1}$

The map position of *oop1* was determined by the analysis of 41 *oop1* × Landsberg *erecta* F_3 lines homozygous at the *oop1* locus, by a combination of CAPS (Konieczny and Ausubel, 1993), RFLP, and simple sequence length polymorphism (Bell and Ecker, 1994) markers. The $PHYB^{oop1}$ genomic sequence was determined from three overlapping PCR products on both strands.

Luciferase Imaging of Gene Expression

A transcriptional fusion of the *LHCB1*1* promoter and luciferase (*LHCB::LUC*) was introduced into Col, *oop1*, and *phyB-9* plants by crossing and selection on MS2S plates containing 50 μg/mL kanamycin (Sigma Chemicals, St. Louis). Seven-day-old seedlings grown under approximately 100 μmol m^{-2} s^{-1} white light and entrained in a 12-h light:12-h dark photoperiod were transferred into 96-well plates, each well containing 150 μL of MS2S and 30 μL of luciferin (2.5 mg/mL; Biosynth, Staad, Switzerland). The plates were covered with sealing tape and entrained for an additional 2 d before being moved into continuous white light (15–25 μmol m^{-2} s^{-1}) on a Packard TopCount Luminometer. The data were analyzed by fast Fourier transform-nonlinear least squares.

Protein Extraction and Immunoblotting

Proteins were extracted from 8-d-old white light-grown seedlings, ammonium sulfate fractionated, separated on 6% SDS-polyacrylamide gels, and immunoblotted as described by Aukerman et al. (1997). Monoclonal antibodies used were: B6-B3 (PHYB-specific) and 3B5 (universal) as described by Hirschfeld et al. (1998).

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