

# Mutant Analyses Define Multiple Roles for Phytochrome C in Arabidopsis Photomorphogenesis

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The analysis of Arabidopsis mutants deficient in the A, B, D, and E phytochromes has revealed that each of these phytochrome isoforms has both distinct and overlapping roles throughout plant photomorphogenesis. Although overexpression studies of phytochrome C (*phyC*) have suggested photomorphogenic roles for this receptor, conclusive evidence of function has been lacking as a result of the absence of mutants in the *PHYC* locus. Here, we describe the isolation of a T-DNA insertion mutant of *phyC* (*phyC-1*), the subsequent creation of mutant lines deficient in multiple phytochrome combinations, and the physiological characterization of these lines. In addition to operating as a weak red light sensor, *phyC* may perform a significant role in the modulation of other photoreceptors. *phyA* and *phyC* appear to act redundantly to modulate the *phyB*-mediated inhibition of hypocotyl elongation in red light and to function together to regulate rosette leaf morphology. In addition, *phyC* performs a significant role in the modulation of blue light sensing. Several of these phenotypes are supported by the parallel analysis of a quadruple mutant deficient in phytochromes A, B, D, and E, which thus contains only active *phyC*. Together, these data suggest that *phyC* has multiple functions throughout plant development that may include working as a coactivator with other phytochromes and the cryptochrome blue light receptors.

## INTRODUCTION

Light signals regulate plant growth and development through the action of specialized photoreceptors, which include the phytochromes, cryptochromes, and phototropins, working alone and in combination (reviewed by Quail, 2002). The cryptochrome and phototropin families use a flavin as the chromophore and are activated by UVA/blue light. These receptors direct a number of light-dependent responses, including hypocotyl/cotyledon growth and phototropism, pigmentation, and stomatal aperture (Briggs and Huala, 1999; Cashmore et al., 1999). Phytochromes constitute a family of bilin-containing photoreceptors that sense red light (R) and far-red light (FR) through photointerconversion between a Pr form and a Pfr form. Because Pr is inactive and Pfr is active, phytochromes can act as reversible “light switches” to control numerous aspects of plant physiology and development. Responses under phytochrome control span the life cycle of plants, including seed germination, chloroplast development, stem and leaf growth, shade avoidance, flowering, and senescence (Kendrick and Kronenberg, 1994).

In Arabidopsis, five discrete phytochrome-encoding genes, *PHYA* to *PHYE*, are present. They can be clustered by amino acid sequence similarity of the encoded proteins into three subfamilies, *phyA* and *phyC*, *phyB* and *phyD*, and *phyE* (Clack et al., 1994; Mathews and Sharrock, 1997). Studies with *phy* mu-

tants and transgenic lines that ectopically overexpress individual phytochromes indicate that each photoreceptor adopts both distinct and overlapping roles throughout photomorphogenesis (Whitelam and Harberd, 1994). The best understood receptors are the *phyA*, *phyB*, *phyD*, and *phyE* isoforms, for which Arabidopsis mutants deficient in each have been studied alone and in various mutant combinations. For example, despite showing no obvious control under white light, *phyA* has a dominant role in mediating hypocotyl inhibition under FR and FR-enriched environments (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), blue light sensing (Neff and Chory, 1998), and floral promotion (Johnson et al., 1994). By contrast, *phyB* functions in the inhibition of hypocotyl elongation under R, a response enhanced by synergistic coactions with *phyA* (Koornneef et al., 1980; Somers et al., 1991; Reed et al., 1994). Deficiency of *phyB* also results in pronounced petiole elongation, retarded leaf development, and early flowering, phenotypes characteristic of the shade-avoidance syndrome (Somers et al., 1991; Smith and Whitelam, 1997). Consistent with its phylogenetic relationship to *phyB*, *phyD* functions in shade-avoidance responses (Aukerman et al., 1997; Devlin et al., 1999) and also interacts genetically with *cry1*, suggesting that *phyD* has a role in modulating blue light perception (Hennig et al., 1999). *phyE* participates in light-regulated germination, maintenance of rosette habit, and shade avoidance (Devlin et al., 1998).

*phyC* is the only phytochrome in Arabidopsis in which mutant alleles have not been isolated to date. The lack of a *phyC* mutant has severely limited a functional analysis of this phytochrome and of the interactions among members of the phytochrome family. A preliminary understanding of *phyC* in leaf development and leaf expansion was proposed based on the effects of the ectopic expression of the *PHYC* gene. Overexpression of *PHYC* yielded

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Arabidopsis plants with larger primary leaves (Qin et al., 1997), whereas heterologous overexpression of the Arabidopsis *PHYC* gene in tobacco produced plants with significantly larger mature leaves and an enhanced night-break response (Halliday et al., 1994). Here, we describe the isolation of a *phyC* mutant allele of Arabidopsis that appears deficient in *phyC* photoreceptor (*phyC-1*). Analyses of this mutant and mutant combinations deficient in multiple phytochromes revealed functional roles for *phyC* throughout Arabidopsis development and provide a unique insight into coactions between *phyC* and other photoreceptors.

## RESULTS

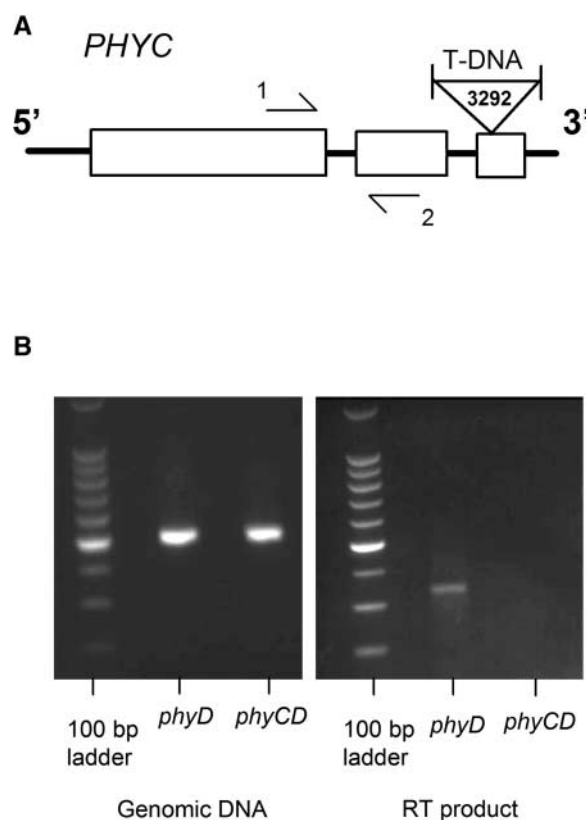
### Identification and Characterization of the *phyC-1* Mutation

The *phyC-1* mutation was identified in a PCR-based screen of the various T-DNA-transformed populations of Arabidopsis for those bearing insertions in the *PHYC* locus (Krysan et al., 1999). Fine mapping of the *phyC-1* mutation, present in the *phyD*-deficient Wassilewskija-2 (*Ws*) background (*phyD-1*), detected a T-DNA insertion in the third exon, with an insertion point at 3292 bp (Figure 1A) (Cowl et al., 1994). The T-DNA insertion contained a functional phosphinothricin acetyltransferase gene (*BAR*) whose product confers resistance to the herbicide phosphinothricin (BASTA). This resistance marker segregated during backcrossing to the wild type in a dominant 3:1 ratio, which is expected of a line with an insertion of T-DNA at a single locus.

Based on its position within the coding region, we predicted that the *phyC-1* mutant would disrupt the synthesis of a full-length apoprotein and thus potentially represent a null *phyC* allele. In support of this notion, accumulation of the *PHYC* mRNA was not detected using reverse transcription (RT)-PCR analysis. As can be seen in Figure 1B, primers that would amplify a region between the first and second exons generated the expected 510-bp product from genomic DNA from both *Ws* (*phyD*) and homozygous *phyC-1* (*phyCD*) plants. However, when cDNA derived from seedling mRNA was used as the PCR template, we were able to amplify a *PHYC* cDNA product of the expected size (374 bp) only from *phyD* plants and not from the *phyCD* mutant (Figure 1B).

### Effect of the *phyC-1* Mutation on Leaf Development

Owing to the fact that the *phyC-1* mutation was isolated in the *phyD*-deficient *Ws* background (Aukerman et al., 1997), all mutant combinations tested are in the *Ws* ecotype and therefore are deficient in *phyD*. In addition, *Ws* plants containing a functional *phyD* (Aukerman et al., 1997) have been analyzed alongside the mutant combinations to assess the possible contribution of *phyD* to each response. Like the other *phy* mutants, analyses of the *phyC-1* mutant indicate that *PHYC* is not an essential gene in Arabidopsis. Homozygous *phyCD* plants were viable and displayed a phenotype indistinguishable from that of wild-type *Ws* (*phyD*) plants when grown in 8-h-light/16-h-dark cycles (Figure 2A). In addition, germination rate, development in the dark, root growth, pigmentation, flowering time, detection of day extensions, and the shade-avoidance response were nor-



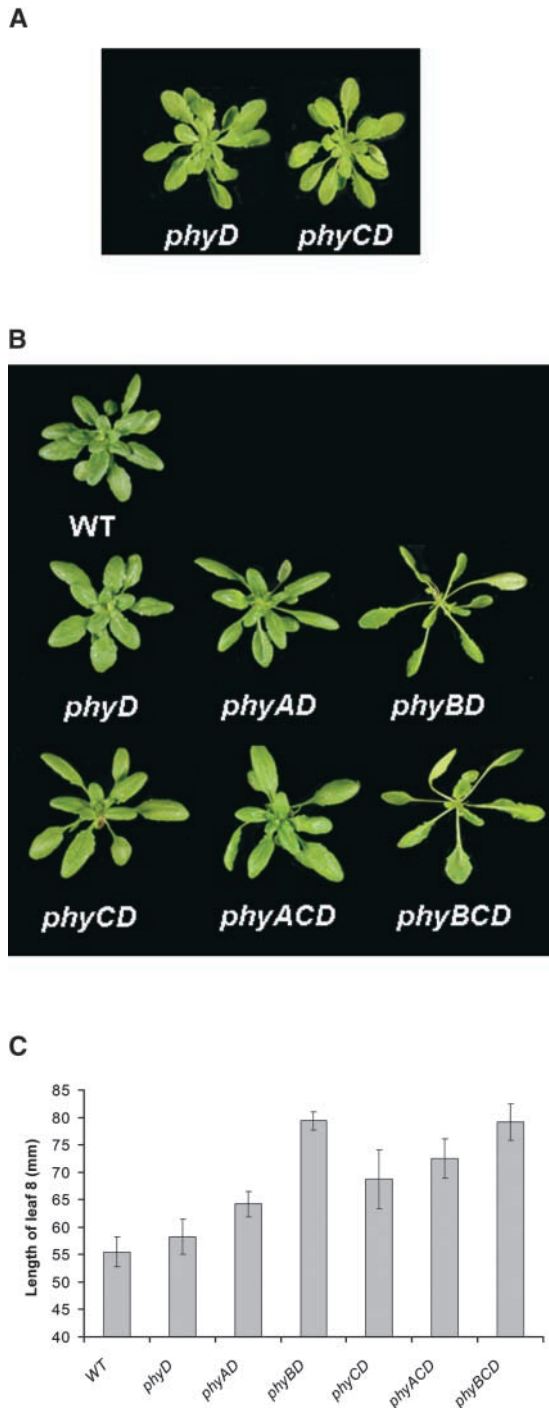
**Figure 1.** Mapping and Analysis of *phyC-1* Mutation.

**(A)** The *phyC-1* mutation results from a T-DNA insertion at the 5' end of the third exon of the *PHYC* gene. The position of the insertion point was mapped to 3292 bp. Boxes represent exons, and lines represent introns. Arrows 1 and 2 represent the positions of primers used for RT-PCR analysis.

**(B)** PCR analyses of *PHYC* using genomic DNA and cDNA from light-grown seedlings of *phyD* and *phyCD* plants. Intron-spanning primers produced a 510-bp product from all genomic samples and a 374-bp product from *phyD* RT samples only.

mal (data not shown). When plants were grown under 16-h-light/8-h-dark cycles, the loss of *phyC* resulted in elongated petioles and an increase in lamina length, phenotypes recorded as an increase in rosette leaf length (Figures 2B and 2C). *phyCD* mutants displayed an increase in leaf length of >10 mm compared with *phyD* plants, suggesting a significant role for *phyC* in regulating rosette leaf morphology. Similar increases were recorded in *phyAD* double and *phyACD* triple mutants but were not observed in monogenic *phyD* plants. The most pronounced leaf elongation was recorded in *phyBD* double mutants, an effect not enhanced significantly by the subsequent loss of *phyC*.

Overexpression of *PHYC* in Arabidopsis resulted in increased expansion of the first true (primary) leaves (Qin et al., 1997). Therefore, the role of *phyC* in primary leaf expansion was investigated in mutant plants. Sequential removal of phytochromes resulted in decreased primary leaf expansion, an effect most marked in *phyB*-deficient backgrounds (Figure 3). Contrary to published overexpression studies, however (Qin et al., 1997), the loss of



**Figure 2.** Rosette Leaf Phenotypes of *phy* Mutant Combinations at 28 Days.

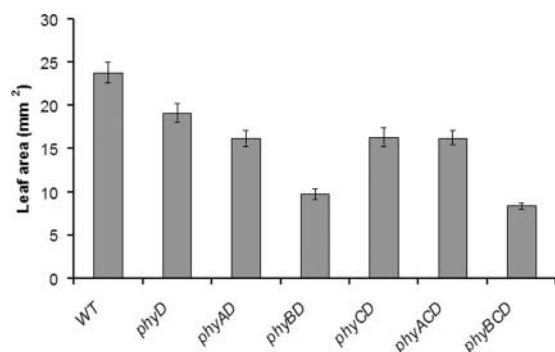
(A) Mature plant phenotypes at 28 days of *phyD* and *phyCD* growth under 8-h-light/16-h-dark cycles of white light ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 22°C. (B) Mature plant phenotypes of *phy* mutant combinations grown under similar conditions with 16-h-light/8-h-dark cycles. WT, wild type. (C) Length of leaf 8 in plants grown under 16-h-light/8-h-dark cycles (mean  $\pm$  SE;  $n = 20$ ).

*phyC* appeared to have no obvious effect on the total area of primary leaves.

**phyA and phyC May Act Redundantly to Modulate the phyB-Mediated Inhibition of Hypocotyl Growth under Red Light**

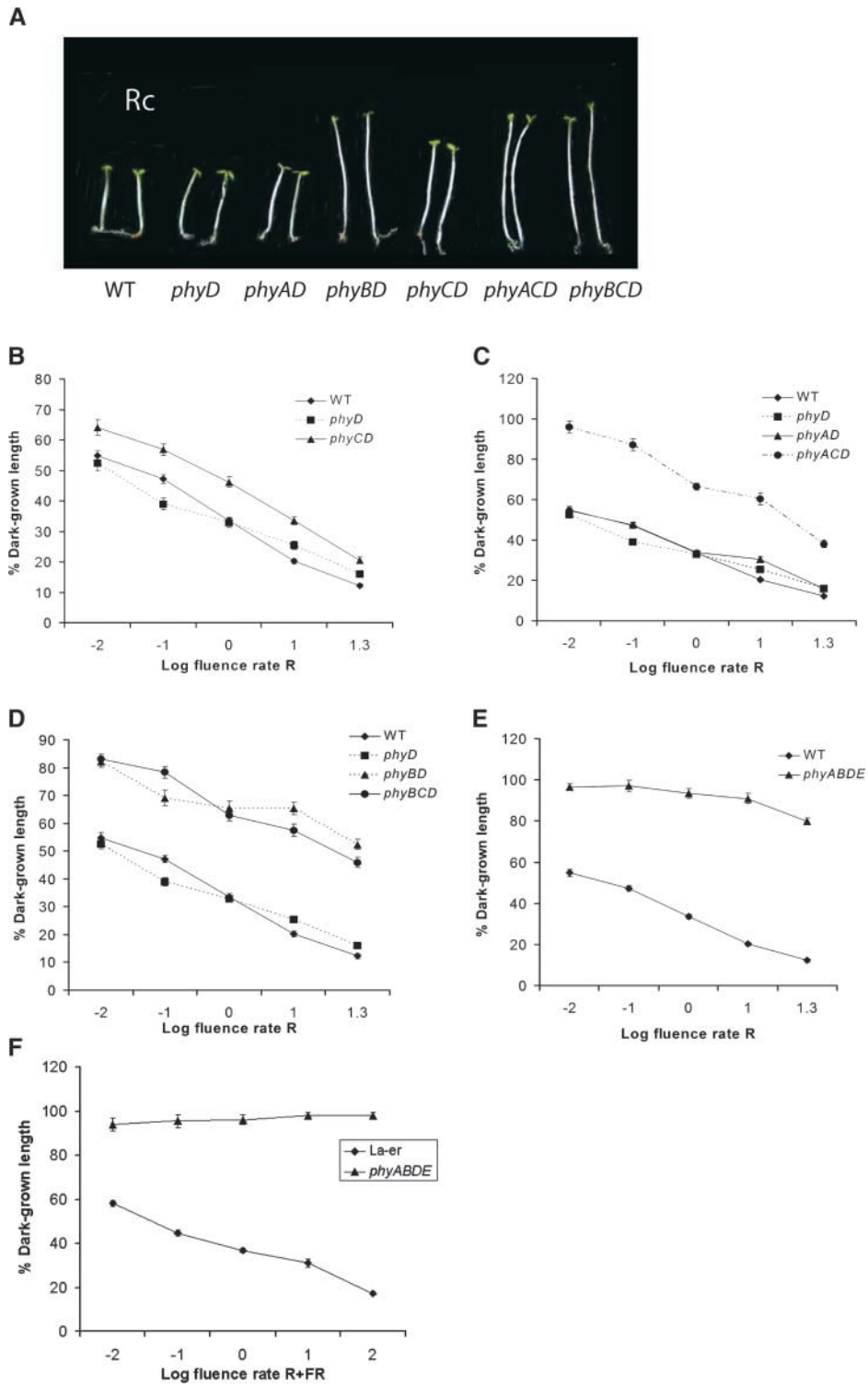
Mutants deficient in *phyC* displayed elongated hypocotyls when grown under continuous red light (Rc) (Figure 4A). The inhibition of hypocotyl elongation under Rc is a well-documented phyB-mediated phenomenon (Koomneef et al., 1980; Somers et al., 1991). In plants containing all five phytochromes (wild type), this response is amplified with increasing fluence rate (Figure 4B). Plants deficient in *phyD* (*phyD*) were phenotypically comparable to wild-type plants, whereas the additional loss of *phyC* (*phyCD*) resulted in significantly longer hypocotyls at all fluence rates used (Figure 4B). Such data indicate that *phyC* can function as a R sensor. Consistent with previous observations, the loss of *phyA* did not significantly impair hypocotyl inhibition by R at any fluence rate (Whitelam et al., 1993) (Figure 4C). However, the combined loss of *phyA* and *phyC* (*phyACD*) resulted in a marked loss of sensitivity to R with respect to the inhibition of hypocotyl elongation at all fluence rates. This effect was most pronounced at low fluence rates, where the inhibition of hypocotyl elongation in the *phyACD* triple mutant was negligible, but was still marked at higher fluence rates. Interestingly, the extent of R hyposensitivity in the *phyACD* triple mutant was comparable to that observed in the *phyBD* double mutant (Figure 4D). In addition, the *phyBCD* triple mutant behaved similarly to the *phyBD* double mutant at all fluence rates of R, suggesting that the presence of *phyB* is required to observe a role for *phyC* in this response (Figure 4D).

The *phyABDE* quadruple mutant displayed inhibition of hypocotyl growth only at higher fluence rates of R (Figure 4E). This response was fully reversible by supplementation with FR (Figure 4F). Thus, we conclude that, in isolation from other phytochromes, *phyC* inhibits hypocotyl elongation under high fluence rates of R in a FR-reversible manner.



**Figure 3.** Primary Leaf Areas of *phy* Mutant Combinations at 18 Days.

Seedlings were grown under continuous white light ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at 22°C (mean  $\pm$  SE;  $n = 20$ ). WT, wild type.



**Figure 4.** Hypocotyl Lengths of *phy* Mutant Combinations Grown under Different Fluence Rates of R.

**(A)** Seedling phenotypes of *phy* mutant combinations grown for 3 days under Rc at  $0.1 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at  $22^\circ\text{C}$ . WT, wild type.

**(B) to (F)** Hypocotyl lengths of *phy* mutant combinations grown under different fluence rates of R. Seedlings were grown under continuous irradiation at  $22^\circ\text{C}$  for 3 days (mean  $\pm$  SE;  $n = 40$ ).

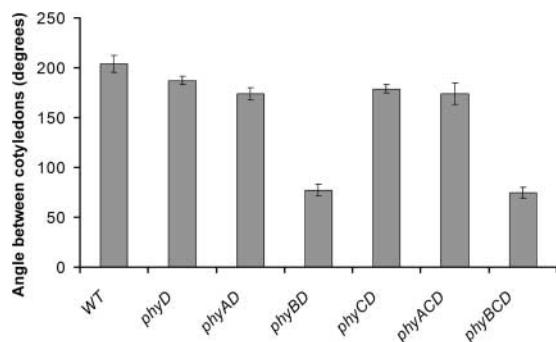


### Loss of phyC Does Not Impair Cotyledon Opening under R

The question of whether phyC deficiency confers a general hyposensitivity to R was addressed by investigating cotyledon opening in *phy* mutant combinations grown under Rc. The regulation of this opening response has been attributed primarily to phyB (Neff and Chory, 1998). The results presented here support this hypothesis, with phyA and phyD performing minor additional roles (Figure 5). The loss of phyC, however, appears to confer no obvious impairment of cotyledon opening under these conditions. Such data confirm that phyC responses observed in the hypocotyl are specific and not the result of a general insensitivity to R.

### phyC Modulates Blue Light-Sensing Mechanisms to Inhibit Hypocotyl Elongation

The inhibition of hypocotyl elongation under blue light (B) has been shown to involve the interaction of phytochrome and cryptochrome signaling (Neff and Chory, 1998). At low fluence rates of B, *phyABDE* quadruple mutants displayed a marked increase in hypocotyl length compared with wild-type Landsberg *erecta* (*Ler*) plants (Figure 6A). However, inhibition was increased significantly with increasing fluence rate. To assess the possible contribution of phyC in this response, hypocotyl lengths were recorded in *phyC* mutant combinations grown under different fluence rates of B. Mutants deficient in phyD consistently lacked a phenotype and were comparable to wild-type plants (Figure 6B). The additional loss of phyC (*phyCD*) conferred hyposensitivity to B at low fluence rates, implicating a role for phyC in B sensing (Figure 6B). Increased hypocotyl lengths were recorded in *phyAD* double mutants at all fluence rates of B (Figure 6C). The combined loss of phyA and phyC (*phyACD*), however, resulted in a marked increase in hypocotyl length at low fluence rates only (Figure 6C). Conversely, the loss of phyB conferred a mild B hyposensitivity only at higher fluence rates, which potentially represents the B-mediated conversion of phyB to its Pfr form (Figure 6D). Unlike the R-mediated response, however, the *phyBCD* triple mutant displayed greater hypocotyl length than the *phyBD* double mutant at all fluence rates of B (Figure 6D).



**Figure 5.** Cotyledon Angles in *phy* Mutant Combinations at 3 Days.

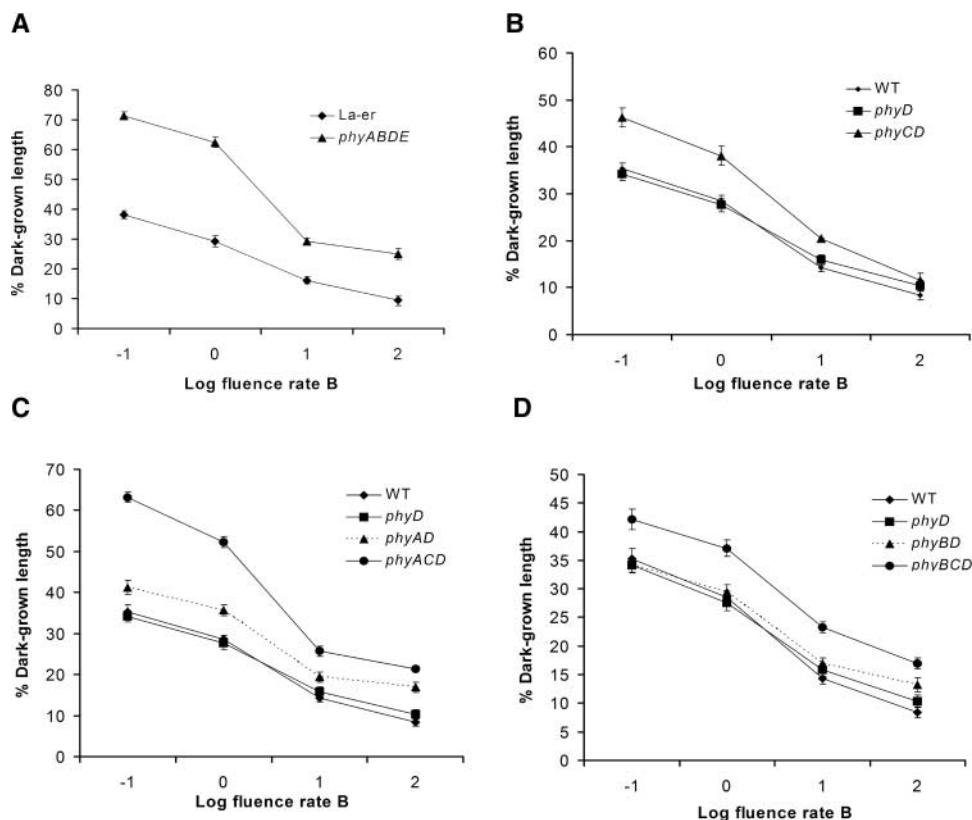
Seedlings were grown at 22°C under continuous R at 3  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (mean  $\pm$  SE;  $n = 20$ ). WT, wild type.

### DISCUSSION

Here, we have described the isolation and initial characterization of an Arabidopsis mutant deficient in phyC. The *phyC-1* mutation is the product of a T-DNA insertion at the 5' end of the third exon of *PHYC* and results in the loss of normal *PHYC* transcript. Thus, *phyC-1* is likely a null allele of the *PHYC* locus. Isolated in the *Ws* ecotype, this mutant also lacks functional phyD (Aukerman et al., 1997). The subsequent creation and analysis of multiple *phy* mutant combinations revealed roles for phyC throughout Arabidopsis photomorphogenesis. Observations that the homologous and heterologous overexpression of Arabidopsis *PHYC* resulted in plants with larger primary and mature leaves, respectively, presupposed a role for this phytochrome in leaf development (Qin et al., 1997; Halliday et al., 1997). Phenotypic analyses of *phy* mutant combinations have revealed a role for phyC in the regulation of rosette leaf morphology. Additionally, we provide further evidence for roles of phyA and phyB in these responses (Halliday et al., 1994; Franklin et al., 2003). When grown under 16-h-light/8-h-dark cycles, loss of phyC resulted in an elongated leaf phenotype similar to that of phyA-deficient mutants. In *Ler*, a role for phyA in the regulation of rosette leaf morphology was observed only in a *phyBDE* background, suggesting possible ecotypic differences between *Ws* and *Ler* (Franklin et al., 2003) (data not shown).

In contrast to studies of Arabidopsis plants that overexpress *PHYC* (Qin et al., 1997), no significant role for phyC in primary leaf expansion was identified using the *phyC-1* mutant under identical conditions. It is possible that the increased primary leaf expansion observed in overexpressing lines represents an ecotypic difference between *Ws* and *Nossen*. Alternatively, such a phenotype may represent an artifact resulting from ecotypic constitutive expression of a phytochrome rather than an endogenous phyC-mediated phenomenon. In support of the latter possibility, the constitutive expression of other phytochromes in Arabidopsis were shown previously to elicit aberrant phenotypes inconsistent with mutant analyses. For example, constitutive expression of phyB was shown to result in aberrant phyA responses under FR (Short, 1999), whereas constitutive expression of phyA was shown to partially complement phyB deficiency (Halliday et al., 1999). These observations suggest that overexpressed phytochromes can display atypical functions and adopt roles normally performed by other photoreceptors, thus strengthening the need for mutational analysis of photoreceptor-deficient lines.

The role of phyB in the R-mediated inhibition of hypocotyl elongation is well documented and has been exploited as a screen for the deficiency of this phytochrome in mutagenized populations (Koorneef et al., 1980; Somers et al., 1991). Additional minor roles in this response also have been recorded for phyA and phyD (Reed et al., 1994; Aukerman et al., 1997). The results presented here reveal that a deficiency of phyC also impairs the R-mediated inhibition of hypocotyl growth. Significantly, the hyposensitivity to R conferred by the loss of phyC is amplified by the additional loss of phyA. Because the loss of phyA alone has no effect on sensitivity to R, this finding suggests that phyA and phyC act redundantly to regulate sensitivity to R. Furthermore, given that phyB is the major photoreceptor that mediates



**Figure 6.** Hypocotyl Lengths of *phy* Mutant Combinations Grown under Different Fluence Rates of B. Seedlings were grown under continuous irradiation at 22°C for 3 days (mean  $\pm$  SE;  $n = 40$ ). WT, wild type.

the inhibition of hypocotyl elongation by R, the pronounced hypersensitivity to R observed in *phyACD* triple mutants at low fluence rates of R raises the possibility that in the absence of *phyA* and *phyC*, there is an impairment of *phyB* function.

The observation that *phyC* deficiency does not result in further hypocotyl elongation in the *phyB* mutant background supports the proposal that *phyC* acts to modulate the *phyB*-mediated inhibition of hypocotyl elongation under low fluence rates of R. However, because it is known that mutants deficient in *phyB* contain reduced levels of *phyC* (Hirschfeld et al., 1998), it is likely that this will contribute to the similarity of the *phyBD* and *phyBCD* phenotypes. Nevertheless, it is clear that there are several situations in which the residual *phyC* present in a *phyB* mutant background is sufficient to display detectable biological activity (Franklin et al., 2003). The very striking insensitivity of *phyACD* triple mutants to R demonstrates a redundant interaction between *phyA* and *phyC*. Such redundancy is consistent with the relatively close phylogenetic relationship between these two phytochromes, based on amino acid sequence relationships (50% identity) (Mathews and Sharrock, 1997). Despite this functional similarity, the loss of *phyC* conferred no impairment of hypocotyl inhibition under continuous FR, the spectral region effective in promoting *phyA*-mediated responses (data not shown).

Cotyledon opening in R was not affected by *phyC* deficiency. This finding indicates that although *phyC* functions as a R sensor for the inhibition of hypocotyl elongation, it is not a general R sensor affecting many aspects of seedling deetiolation. The modest inhibition of hypocotyl elongation observed in *phyABDE* quadruple mutants grown under high-fluence-rate R and the FR reversibility of this response confirm previous observations that, in isolation from other phytochromes, *phyC* is a weak R/FR-reversible photoreceptor (Franklin et al., 2003).

Sensitivity to blue wavelengths is conferred primarily by the B photoreceptors (cryptochromes and phototropins). *cry1* and *cry2* are flavoproteins that show structural similarity to DNA photolyases and regulate an array of developmental responses throughout Arabidopsis photomorphogenesis (Briggs and Huala, 1999; Cashmore et al., 1999). Despite uncertainty regarding the exact nature of the coaction, it is accepted that B-mediated deetiolation involves the genetic interactions of both phytochrome and cryptochrome signaling (Yanovsky et al., 1995; Ahmad and Cashmore, 1997; Casal and Mazzella, 1998). The proposal that the inhibition of hypocotyl elongation in B is dependent on the presence of either *phyA* or *phyB* (Ahmad and Cashmore, 1997) was contradicted by evidence showing a nearly wild-type response in *phyAB* double mutants at high fluence rates of B (Poppe et al., 1998). The significant inhibition of hypocotyl elon-

gation observed in *phyABDE* quadruple mutants grown under similar conditions suggested that phyD and phyE also are not necessary for this response.

This fact raises the question of the possible role of phyC in responses to B. Hypocotyl measurements in *phyC* mutant combinations subsequently revealed that phyC-deficient plants display B hyposensitivity, an effect most evident at low fluence rates of B. Under these conditions, it has been shown that cry2 function is predominant in the regulation of hypocotyl elongation (Lin et al., 1998). Thus, the hyposensitivity of phyC mutants to low fluence rates of B indicates a possible functional interaction between phyC and cry2. Consistent with published studies (Ahmad and Cashmore, 1997), we observed elongated hypocotyls in phyA-deficient mutants exposed to all fluence rates of B. This hyposensitivity observed in *phyAD* plants is consistent with the proposal that phyA acts as a blue light photoreceptor. The combined deficiency of phyA and phyC (*phyACD*) resulted in an additive response, with significant B hyposensitivity observed only at low fluence rates. Therefore, it is possible that phyA and phyC operate via independent mechanisms to inhibit hypocotyl extension in B.

In contrast to studies in R, phyC deficiency generated hyposensitivity to B in a *phyB* background. This hyposensitivity indicates that the residual reduced phyC present in a *phyB* background (Hirschfeld et al., 1998) is nevertheless sufficient to mediate a significant biological response. This finding is in accord with reports that phyC in the *phyABDE* quadruple mutant background is capable of mediating a number of R/FR reversible responses (Franklin et al., 2003) (Figures 4E and 4F).

We conclude that phyC is a weak R sensor that may perform a significant role in the modulation of other photoreceptors. We did not identify a role for phyC in the regulation of root development, flowering time, chlorophyll synthesis, or shade-avoidance responses. The analysis of multiple *phy* mutant combinations suggests functional redundancy between phyA and phyC in the modulation of phyB function, in addition to revealing a possible role for phyC in B sensing. Similarities between phyA and phyC function also were observed in the regulation of rosette leaf morphology, although phyC was unable to mediate FR high-irradiance responses, which are characteristic of phyA. Given the relatively close phylogenetic relationship between these two phytochromes and their overlapping physiological roles, we speculate that the functional divergence of phyA and phyC is partially caused by the high levels of phyA in etiolated tissue and its rapid degradation upon photoconversion to Pfr, neither of which is apparent in phyC (Sharrock and Quail, 1989). The data presented here have revealed roles for the least well characterized of the phytochromes and provide more complete insight into the functional divisions between members of this family of plant photoreceptors. Because there are now mutations in all five phytochromes, the continuing analysis of all possible mutant combinations should conclusively define the roles of each receptor as they work alone or in combination.

## METHODS

### Isolation of the *phyC* Mutant

A *phyC* allele was isolated using a PCR-based screen of a T-DNA-transformed population of *Arabidopsis thaliana* [Amasino collection, acces-

sion Wassilewskija-2 [Ws]] from the Biotechnology Center at the University of Wisconsin-Madison (Davis et al., 2001). The phyC primers phyC5' 5'-ACTATTAAGCAGCATACGCTTCACTCCTG-3' and phyC3' 5'-GTC-ATTTACTTGGCCAGGGGCTTTCATC-3' were used in conjunction with typical right-border and left-border primers. To define the exact nature of the *phyC-1* mutation, PCR products were purified and sequenced directly. After backcrossing to the wild type, homozygous *phyC-1* lines were isolated by selfing the tagged line and selecting progeny that displayed 100% resistance to phosphinothricin (BASTA).

### Plant Material and Growth Conditions

The Arabidopsis *phyABDE* quadruple mutant (ecotype Landsberg *erecta*) was described previously (Franklin et al., 2003). Ws plants containing a functional *PHYD* gene (designated wild type here) also have been described (Aukerman et al., 1997). All other mutant combinations used are in the naturally phyD-deficient Ws background (designated *phyD*) (Aukerman et al., 1997). Ws mutants deficient in phyA and phyB (*phyA-5* and *phyB-10*) were isolated originally from the Feldman T-DNA-tagged population for aberrant growth responses to far-red and red light, respectively, using screening procedures described elsewhere (Whitelam et al., 1993; Bradley et al., 1996). Triple mutant lines were created by crossing *phyA-5* (*phyAD*) and *phyB-10* (*phyBD*) double mutants with the *phyC-1* (*phyCD*) mutant and selection on BASTA.

Seeds were surface-sterilized in 10% commercial bleach and sown directly onto Lehle medium (Lehle Seeds, Round Rock, TX) supplemented with 0.8% agar. After 4 days of stratification in darkness at 4°C, germination was synchronized by treating seeds with a white light pulse and returning the seeds to the dark for 24 h before transfer to a specialized light regime. For mature leaf analyses, seedlings were germinated under 8-h-light/16-h-dark cycles at 16°C. Uniformly sized seedlings were transplanted to 5-cm × 5-cm × 5-cm pots containing a 3:1 mixture of compost and horticultural silver sand. After another 12 days of growth under the same conditions, plants were transferred to 16-h-light/8-h-dark cycles at 22°C.

### Light Sources

White light was provided by cool-white fluorescent tubes (photon irradiance of 400 to 700 nm) at a fluence rate of  $\sim 100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Red light was provided by light-emitting diodes (LEDs) at  $\lambda_{\text{max}}$  of 665 nm (Farnell, Leeds, UK) filtered through 20 mm of water. Broad-band blue light was provided by blue tubes (FL20S B; Toshiba Lighting and Technology, Tokyo, Japan) wrapped in one layer of deep-blue cinemoid filter (HT120; Lee Filters, Hampshire, UK) (photon irradiance of 400 to 500 nm) supplemented with LEDs ( $\lambda_{\text{max}}$  of 450 nm; Farnell). Far-red light was provided by LEDs at  $\lambda_{\text{max}}$  of 735 nm (Farnell). All light measurements were performed using a EPP2000 fiberoptic spectrometer (Stellarnet, Tampa, FL).

### Physiological Analyses

All physiological analyses were performed at 22°C. Leaf length (including petiole) was measured on leaf 8 after bolting. Leaves were excised and photographed, and their dimensions were measured using Sigma Scan pro5 (SPSS UK, Surrey, UK). Primary leaves were excised after 21 days of growth under white light and photographed, and areas were measured using Sigma Scan pro5 (mean  $\pm$  SE;  $n = 20$ ). For cotyledon-angle measurements, plants were grown under continuous red light ( $3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 3 days. Seedlings were photographed, and angles were measured with a protractor (mean  $\pm$  SE;  $n = 20$ ). For hypocotyl measurements, seedlings were photographed after 3 days of growth,

and hypocotyl lengths were measured using Sigma Scan pro5 (mean  $\pm$  SE;  $n = 40$ ). Data are expressed as percentages of dark-grown length to allow for differences in germination rates between seed batches.

### Reverse Transcription-PCR

Tissue was harvested from 7-day-old white light-grown seedlings. RNA was extracted using an RNeasy mini prep kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and DNA was removed with DNA-free (Ambion, Cambridgeshire, UK). RNA quantification was performed with a TD-360 mini fluorometer (Turner Designs, Sunnyvale, CA) using the ribo-green RNA quantification system (Molecular Probes, Leiden, The Netherlands). Two micrograms of total RNA was placed in a 40- $\mu$ g reverse transcription-PCR system (Qiagen Omniscript) together with a *PHYC*-specific primer situated before the T-DNA insertion (5'-CTCCACATGATTCGGGCATA-3'). A *PHYC* sequence interval was amplified using 30 PCR cycles with the following intron-spanning primers: 5'-TTGCGGTTGATGCTCTGGT-3' and 5'-CTCCACATGATTCGGGCATA-3'. Control PCR was performed with 2  $\mu$ g of genomic DNA extracted from similar seedlings (Qiagen DNeasy).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact G. C. Whitelam, gcw1@le.ac.uk.

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## Mutant Analyses Define Multiple Roles for Phytochrome C in Arabidopsis Photomorphogenesis

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