

# Blue Light–Dependent *In Vivo* and *In Vitro* Phosphorylation of Arabidopsis Cryptochrome 1

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Cryptochromes are photolyase-like blue/UV-A light receptors that regulate various light responses in animals and plants. Arabidopsis cryptochrome 1 (*cry1*) is the major photoreceptor mediating blue light inhibition of hypocotyl elongation. The initial photochemistry underlying cryptochrome function and regulation remain poorly understood. We report here a study of the blue light–dependent phosphorylation of Arabidopsis *cry1*. *Cry1* is detected primarily as unphosphorylated protein in etiolated seedlings, but it is phosphorylated in plants exposed to blue light. *Cry1* phosphorylation increases in response to increased fluence of blue light, whereas the phosphorylated *cry1* disappears rapidly when plants are transferred from light to dark. Light-dependent *cry1* phosphorylation appears specific to blue light, because little *cry1* phosphorylation is detected in seedlings treated with red light or far-red light, and it is largely independent from phytochrome actions, because no phytochrome mutants tested significantly affect *cry1* phosphorylation. The Arabidopsis *cry1* protein expressed and purified from insect cells is phosphorylated *in vitro* in a blue light–dependent manner, consistent with *cry1* undergoing auto-phosphorylation. To determine whether *cry1* phosphorylation is associated with its function or regulation, we isolated and characterized missense *cry1* mutants that express full-length CRY1 apoprotein. Mutant residues are found throughout the CRY1 coding sequence, but none of these inactive *cry1* mutant proteins shows blue light–induced phosphorylation. These results demonstrate that blue light–dependent *cry1* phosphorylation is closely associated with the function or regulation of the photoreceptor and that the overall structure of *cry1* is critical to its phosphorylation.

## INTRODUCTION

Plants rely on at least three types of photosensory receptors to regulate growth and development in response to the changing light environment. These photoreceptors include red/far-red light receptor phytochromes (Quail et al., 1995; Nagy and Schafer, 2002), blue/UV-A light receptor phototropins (Briggs and Huala, 1999; Briggs and Christie, 2002), and cryptochromes (Cashmore et al., 1999; Lin, 2002; Lin and Shalitin, 2003). Arabidopsis has at least two cryptochromes, *cry1* and *cry2*, which mediate, among other responses, deetiolation and photoperiodic responses, respectively (Koornneef et al., 1980, 1991; Ahmad and Cashmore, 1993; Guo et al., 1998; Mockler et al., 1999, 2003; El-Din El-Assal et al., 2001). It has been found that cryptochromes interact with phyB and COP1 (Mas et al., 2000; Yang et al., 2000, 2001; Wang et al., 2001) and that cryptochromes mediate the light regulation of gene expression (Somers et al., 1998; Ma et al., 2001; Yanovsky and Kay, 2002).

Light-dependent protein phosphorylation plays important roles in the function of photoreceptors. For example, it has been shown that phytochromes and phototropins are light-regulated protein kinases that catalyze the phosphorylation of their respective photoreceptors and possibly other proteins (Huala et al., 1997; Christie et al., 1998; Yeh and Lagarias, 1998;

Fankhauser et al., 1999). It also has been shown that cryptochromes are phosphoproteins in Arabidopsis and mammalian cells (Eide et al., 2002; Shalitin et al., 2002). Arabidopsis *cry2* is phosphorylated in seedlings exposed to blue light but not in seedlings exposed to a similar range of light fluence of red or far-red light. Mutations in multiple phytochrome genes showed little effect on the blue light–dependent *cry2* phosphorylation (Shalitin et al., 2002). On the other hand, it has been reported that Arabidopsis *cry1* was phosphorylated by phyA *in vitro* and that *cry1* phosphorylation *in vivo* could be induced under red light but suppressed by far-red light (Ahmad et al., 1998). Therefore, it remains unclear whether blue light–dependent phosphorylation is a common light response associated with plant cryptochromes and whether protein phosphorylation is associated with cryptochrome-mediated blue light responses in general. To address these questions, we investigated the light-dependent protein phosphorylation of Arabidopsis *cry1*.

## RESULTS

### Light-Dependent Phosphorylation of *cry1*

To determine whether Arabidopsis *cry1*, like *cry2*, is phosphorylated in response to blue light, we first tested whether *cry1* might be metabolically labeled by <sup>32</sup>P in dark-grown or light-treated plants. Etiolated seedlings were excised above the roots, placed in test tubes containing [<sup>32</sup>P]orthophosphate, and incubated in the dark for 3 h. The tissue aliquots then were exposed to blue light; the *cry1* protein was immunoprecipitated before or after light treatment and examined using immunoblot

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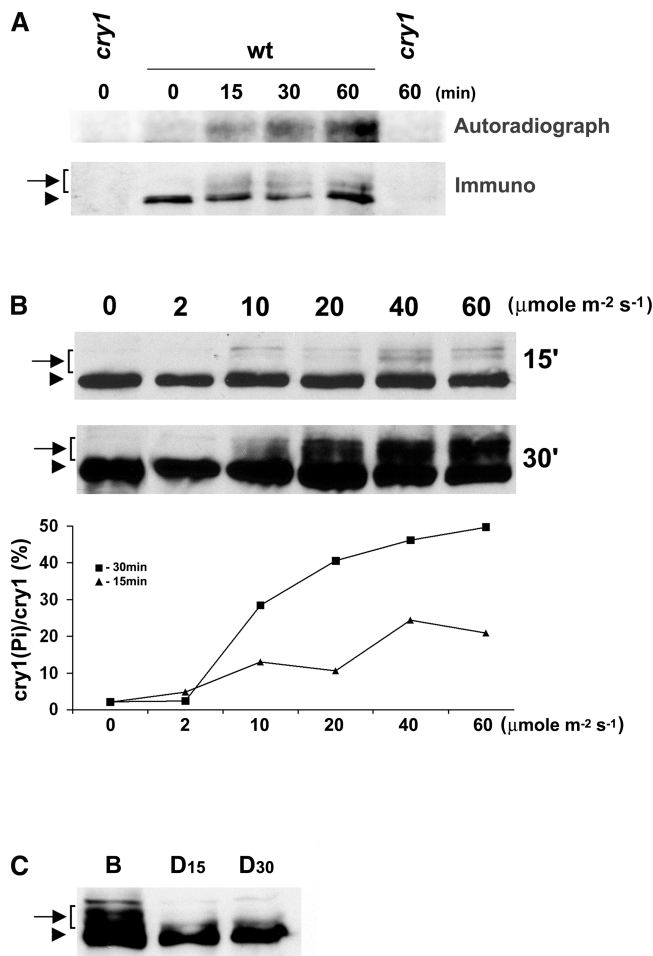
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analysis and autoradiography. As shown in Figure 1, cry1 was radioactively labeled by  $^{32}\text{P}$  in seedlings exposed to blue light for 15 min. By contrast, little  $^{32}\text{P}$  labeling of cry1 was detected in etiolated seedlings that were not treated with blue light (Figure 1A). Relatively more radioactively labeled cry1 was immunoprecipitated from seedlings exposed to blue light for an extended time (Figure 1A). These results clearly demonstrate that, like *Arabidopsis* cry2, cry1 also undergoes blue light-induced protein phosphorylation. As with many phosphoproteins, cry1 phosphorylation results in slow migration on a SDS-PAGE gel (Figure 1B). The immunoblot shows that in addition to a fast-migrating cry1 band detected in both etiolated and light-treated seedlings, at least two or three slow-migrating bands recognized by the anti-CRY1 antibodies were detected in plants treated with blue light (Figure 1B). The slow-migrating bands were sensitive to the phosphatase treatment (see below), indicating that they are phosphorylated forms of cry1. The multiple slow-migrating cry1 bands apparently represent differentially phosphorylated cry1 isoforms, suggesting that the phosphorylation of cry1 occurs at more than one residue.

We next analyzed the fluence response of cry1 phosphorylation. As shown in Figure 1B, cry1 phosphorylation increased in plants exposed to a higher fluence rate of blue light or exposed to blue light for a longer time. Cry1 phosphorylation was barely detectable in seedlings treated with the lowest fluence of blue light tested ( $2 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 30 min) (Figure 1B). At the highest fluence rate of blue light tested ( $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), the level of cry1 phosphorylation was approximately four times greater in seedlings exposed to light for 30 min than that in seedlings exposed to light for 15 min (Figure 1B). In seedlings treated with blue light for 30 min, the level of overall cry1 phosphorylation increased in response to the increased fluence rate of blue light (Figure 1B). These kinetics features of cry1 phosphorylation are in contrast to the bell-shaped fluence response curve of cry2 phosphorylation, for which the relative phosphorylation increases in seedlings exposed to relatively low fluence but decreases at higher fluence (Shalitin et al., 2002). The different fluence responses of cry1 and cry2 phosphorylation are consistent with the fact that cry1 and cry2 are light-stable and light-labile proteins, respectively, and that the phosphorylation of cry2 triggers its degradation (Shalitin et al., 2002).

We found that the total fluence used in the cry1 phosphorylation assay was significantly lower than the total fluence needed to show the gross morphological change resulting from the blue light inhibition of hypocotyl growth. Such a morphological change usually requires exposure of the seedlings to comparable fluence rates of blue light for days instead of minutes. This finding is consistent with cryptochrome phosphorylation being required for its function. We also noted that cry1 was not phosphorylated to completion under the conditions tested. For example, at the highest fluence tested ( $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  blue light for 30 min), the slow-migrating isoforms that represent phosphorylated cry1 accounted for  $\sim 50\%$  of the total cry1 pool (Figure 1B). It is less likely that the nonphosphorylated cry1 represents an artifact of nonspecific phosphatase activity during homogenization, because a phosphatase inhibitor (NaF) was included in the homogenization buffer (see Methods). The response of cry1 phosphorylation to changing light conditions



**Figure 1.** Blue Light-Dependent Phosphorylation of cry1.

**(A)** Five-day-old etiolated seedlings were cut above the roots and incubated with  $300 \mu\text{Ci}$  of  $^{32}\text{P}\text{-H}_3\text{PO}_4$  in water for 3 h in the dark. The tissue aliquots were exposed to blue light ( $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for the time indicated or kept in darkness. The cry1 protein was immunoprecipitated with anti-CRY1 antibodies, separated on a 10% SDS-PAGE gel, blotted to a membrane, and analyzed by autoradiography (Autoradiograph) and then by probing an immunoblot with anti-cry1 antibodies (Immuno).

**(B)** Immunoblots of samples prepared from wild-type seedlings exposed to blue light for 15 min (15') or 30 min (30') at the fluence rate indicated were probed with the anti-CRY1 antibody. The signals of the slow-migrating bands (indicated by the arrow-bracket) are normalized to the fast-migrating band in the same lane (arrowhead), represented as [cry1(Pi)/cry1 (%)], and plotted against the fluence rates (graph at bottom).

**(C)** Immunoblot showing dephosphorylation of cry1 in the dark. Etiolated seedlings were exposed to blue light (B;  $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 1 h and then transferred to dark. Samples were prepared before or after seedlings were transferred to darkness for 15 or 30 min (D<sub>15</sub> and D<sub>30</sub>). Arrows with brackets indicate phosphorylated cry1; arrowheads represent nonphosphorylated cry1. wt, wild type.

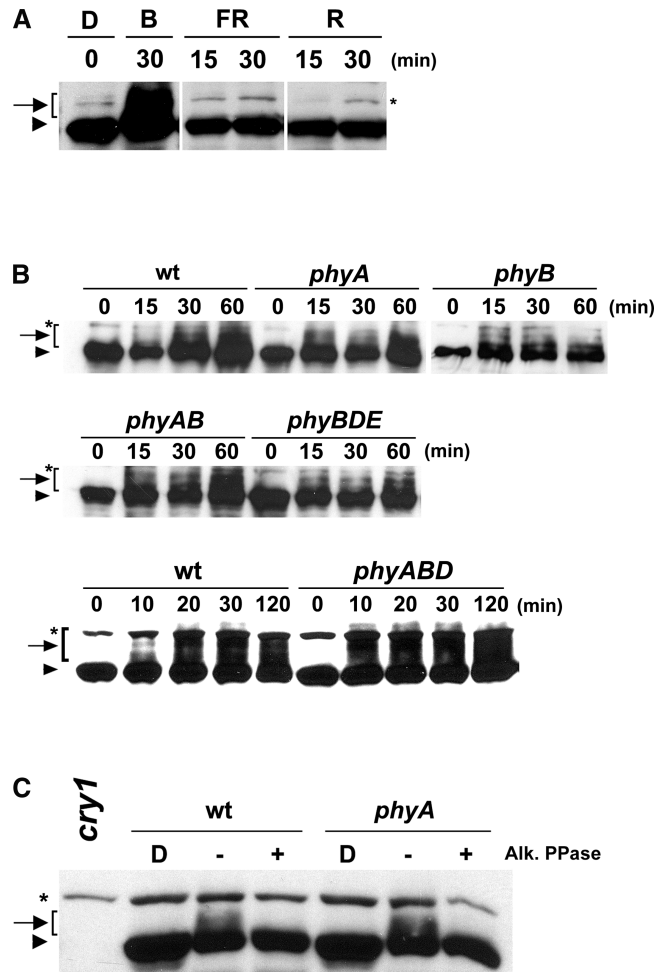
was examined further in seedlings transferred from blue light to dark (Figure 1C). In this experiment, the phosphorylated cry1 disappeared almost completely within 15 min after plants were transferred to dark. Because cry1 protein is relatively stable in both dark and light conditions, this result indicates that cry1 is likely dephosphorylated by a protein phosphatase.

A recent study showed the involvement of a protein phosphatase, PP7, in the blue light inhibition of hypocotyl elongation (Moller et al., 2003). It would be interesting to determine whether PP7 is involved in cryptochrome dephosphorylation. Because cry1 function depends specifically on blue light, the observation that cry1 phosphorylation is blue light dependent would be consistent with the hypothesis that the unphosphorylated cry1 is inactive but the phosphorylated cry1 is the active photoreceptor. However, alternative interpretations cannot be excluded at present. For example, blue light might activate cry1 via a change of conformation rather than by phosphorylation of the protein, and the active cry1 might trigger phosphorylation and inactivation of the photoreceptor as a negative feedback regulation. Provided that phosphorylated and unphosphorylated cryptochrome molecules have different physiological activities, the presence of both isoforms in plants grown in light would allow a rapid adjustment of photoreceptor activity in response to the changing light environment.

#### Mutations of Phytochromes Have Little Effect on cry1 Phosphorylation

To further investigate whether cry1 phosphorylation is specific to blue light, we analyzed cry1 protein in seedlings exposed to different wavelengths of light. Figure 2 shows that cry1 phosphorylation was detected in seedlings exposed to blue light for 30 min, but little phosphorylation of cry1 was detected in seedlings exposed to a similar fluence range of red light, far-red light, or dark (Figure 2A). To determine whether phytochromes affect blue light-dependent cry1 phosphorylation, we analyzed cry1 phosphorylation in phytochrome mutants. As shown in Figure 2B, cry1 phosphorylation clearly was detected in the *phyA* and *phyB* monogenic mutants as well as in those mutant lines that are impaired in multiple phytochrome genes, including *phyAB*, *phyBDE*, and *phyABD* (Franklin et al., 2003). The slow-migrating cry1 isoforms in wild-type and *phyA* mutant seedlings disappeared completely after phosphatase treatment (Figure 2C). This result confirmed that the slow-migrating bands recognized by the anti-CRY1 antibody were phosphorylated cry1 and that the *phyA* mutation did not abolish the blue light-induced cry1 phosphorylation.

The findings that cryptochromes are phosphorylated in blue light but not in red or far-red light (Figure 2A) and that none of the phytochrome mutants tested showed an easily discernible effect in the phosphorylation of cry1 (Figures 2B and 2C) suggest that no single phytochrome tested is solely responsible for the blue light-induced phosphorylation of cry1. However, because it is difficult to precisely quantify each of the multiple bands of phosphorylated cry1, a minor or quantitative effect of phytochrome mutations on the blue light-dependent phosphorylation of cry1 cannot be excluded. For example, it appears that cry1 phosphorylation may occur slightly faster in the phy-



**Figure 2.** Blue Light Specificity and the Effect of Phytochrome Mutations on cry1 Phosphorylation.

(A) Immunoblot showing cry1 phosphorylation in seedlings exposed to blue light (B;  $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), far-red light (FR;  $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), or red light (R;  $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for the time indicated or kept in the dark (D).

(B) Immunoblot showing cry1 phosphorylation in samples prepared from the wild type or from *phyA*, *phyB*, *phyA phyB*, *phyBDE*, and *phyABD* mutants. Seedlings were grown in the dark and then exposed to blue light ( $20 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for the time indicated. wt, wild type.

(C) Immunoblot showing cry1 phosphorylation in samples prepared from wild-type or *phyA* seedlings grown in the dark and then exposed to blue light ( $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 1 h. Protein extracts from light-treated seedlings were incubated with alkaline phosphatase (Alk. PPase) at  $30^\circ\text{C}$  for 30 min (+) or analyzed without phosphatase treatment (-). Asterisks indicated a band nonspecifically recognized by the antibody.

tochrome mutants tested than in the wild-type controls (Figure 2B), although this remains to be examined more carefully.

#### Cry1 Phosphorylation Is Closely Associated with Its Function or Regulation

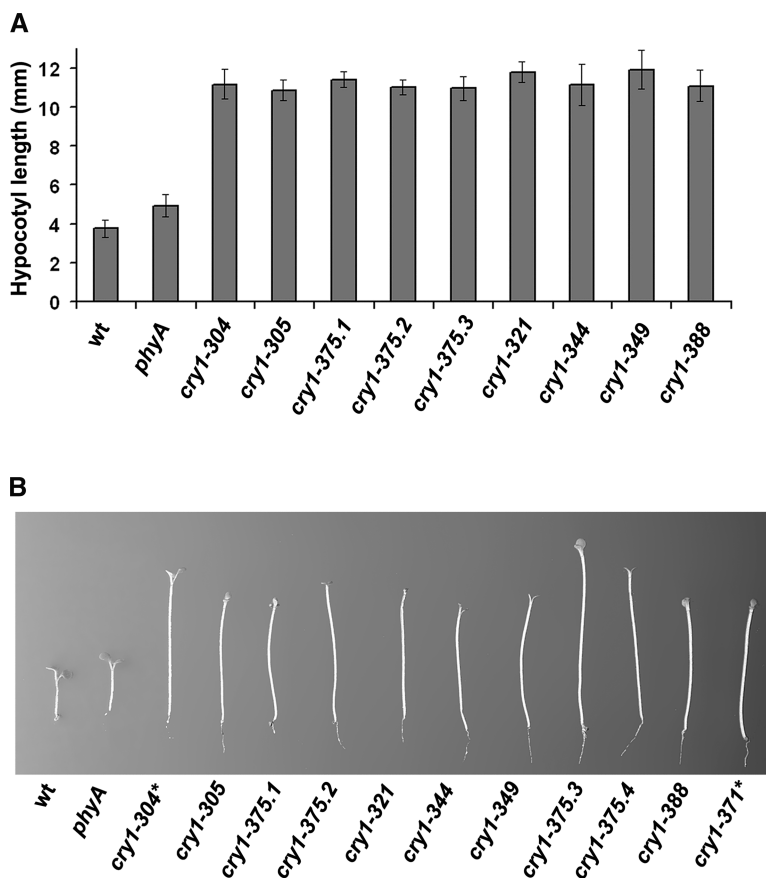
The structure-function relationship is critical to our understanding of the molecular mechanisms underlying cryptochrome

phosphorylation and function. Although we previously reported *cry2* phosphorylation in *Arabidopsis* (Shalitin et al., 2002), a direct test of the structure-function relationship of *cry2* phosphorylation has been difficult, because few missense *cry2* mutants are available (Koornneef et al., 1991; Guo et al., 1998). Isolation of a large number of *cry2* mutations has been difficult (H. Yang and C. Lin, unpublished data), because the late-flowering phenotype is associated not only with *cry2* but also with mutations in other flowering-time genes and the long-hypocotyl phenotype of *cry2* is not robust enough for efficient genetic screening (Guo et al., 1998; Lin et al., 1998). By contrast, a large number of *cry1* mutants in *Arabidopsis* were isolated that showed long hypocotyls when grown in continuous blue light (Koornneef et al., 1980; Liscum and Hangarter, 1991; Ahmad et al., 1995; Bagnall et al., 1996; Bruggemann et al., 1996).

To investigate the role of *cry1* phosphorylation, we isolated and characterized missense *cry1* mutants that showed little blue light inhibition of hypocotyl elongation but still expressed apparently full-length CRY1 apoprotein. It was expected that these missense mutations would allow the identification of the amino acid residues critical for *cry1* function and/or phosphory-

lation. Ethyl methanesulfonate-mutagenized seeds of a *phyA* mutant line were screened to isolate individuals with long hypocotyls when grown in continuous blue light (Figure 3). We then used immunoblot analysis with anti-CRY1 antibodies to select those mutants that expressed apparently full-length CRY1 apoprotein. Most *cry1* mutations isolated expressed little or truncated CRY1 protein (data not shown). However, nine lines were found to express apparently full-length CRY1 apoprotein at a level indistinguishable from that of the wild type, and they were studied further (Figures 3 and 4). As shown in Figure 3, the parental *phyA* mutant line was slightly taller than the wild type when grown under continuous blue light, but the newly isolated *cry1* lines that express full-length CRY1 apoprotein grew significantly taller than the *phyA* parent or the wild type. These newly isolated *cry1* mutants showed very similar phenotypes to the reference *cry1-304* allele or a *cry1 phyA* double mutant that expresses no CRY1 apoprotein (Mockler et al., 1999) (Figure 3B, *cry1-371*), and they all contained mutations in the *CRY1* gene (see below).

We tested whether these *cry1* mutations affected *cry1* phosphorylation. In contrast to the *phyA* mutant that exhibited ap-



**Figure 3.** Long-Hypocotyl Phenotype of the Newly Isolated *cry1* Mutants.

**(A)** Seedlings were grown in continuous blue light ( $25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 5 days, and hypocotyl lengths were measured. wt, wild type.

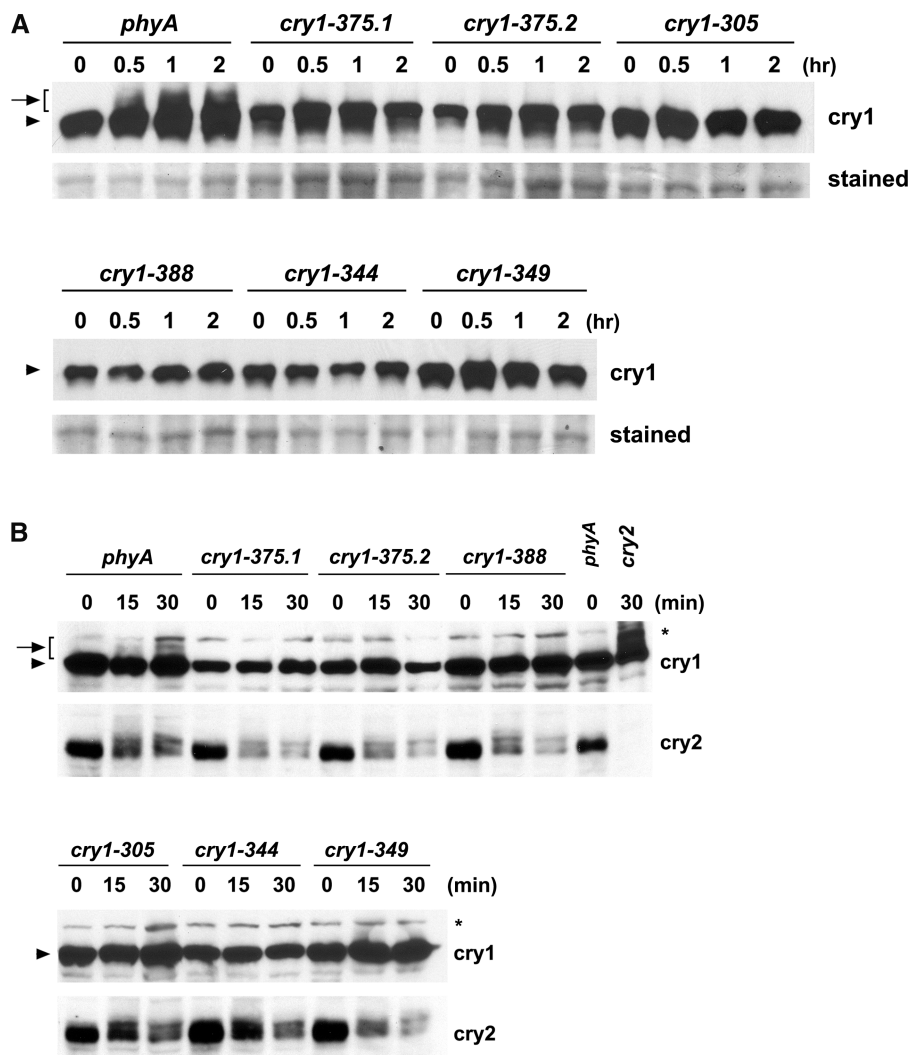
**(B)** Representative seedlings of parental control (*phyA*), a *cry1* reference allele (*cry1-304*), and nine newly isolated *cry1* alleles are shown. *cry1-304* and *cry1-371* express no CRY1 apoprotein, whereas the other alleles express full-length CRY1 apoprotein.

parently normal cry1 phosphorylation in response to blue light of both high fluence (Figure 4A) and low fluence (Figure 4B), none of the nine *cry1* alleles showed easily detectable cry1 phosphorylation (Figure 4). These results suggest a positive correlation between the light-dependent phosphorylation of cry1 and its activity in mediating the blue light inhibition of hypocotyl elongation. Because both cry1 and cry2 are phosphorylated in response to blue light and their functions are partially redundant (Mockler et al., 1999), we tested if the two cryptochromes would affect the phosphorylation of each other. Figure 4B shows that the *cry2* mutation had no apparent effect on cry1 phosphorylation. Similarly, cry2 phosphorylation clearly was detectable in all of the *cry1* mutant alleles tested at the rel-

atively low fluence range that is optimal to show cry2 phosphorylation (Shalitin et al., 2002) (Figure 4B). It appears that cry1 and cry2 are phosphorylated largely independently in response to blue light.

#### Mutations That Impair cry1 Phosphorylation Are Found throughout the CRY1 Apoprotein

CRY1 is a 681-residue protein comprising an N-terminal photolyase-related domain (~500 amino acids) and a C-terminal domain (~180 amino acids). The CRY1 C-terminal domain is unrelated to the DNA photolyase, but it contains a DAS motif that is rich in Ser and is well conserved in cryptochromes



**Figure 4.** Immunoblots Showing the Lack of cry1 Phosphorylation and Normal cry2 Phosphorylation in Different *cry1* Mutant Alleles.

**(A)** Samples were prepared from etiolated seedlings exposed to  $25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  blue light for the time indicated or from samples kept in the dark (0 h), and immunoblots were probed with anti-CRY1 antibodies (cry1). For a loading control, the membrane was stained with Ponceau red, and a portion of the stained blot showing unspecified proteins is included (stained).

**(B)** Samples were prepared from etiolated seedlings exposed to  $7 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  blue light for the time indicated or from samples kept in the dark (0 min). The immunoblot was probed first with anti-CRY2 antibody (cry2), stripped, and reprobed with anti-CRY1 antibody (cry1).

throughout the plant kingdom (Lin, 2002). To determine which residues of CRY1 might be important for its phosphorylation and function, we sequenced the *CRY1* gene in all nine of the apparently missense *cry1* mutants. As expected, mutations were detected in the *CRY1* coding sequence of every *cry1* allele tested (Figure 5). The mutated residues are distributed throughout the sequence of CRY1 apoprotein (Figure 5). Among the nine independent *cry1* lines sequenced, seven contain mutations in the photolyase-related domain and two have mutations in the C-terminal domain (*cry1-321* and *cry1-349*); four contain the same mutation, G347R (*cry1-375.1*, *-375.2*, *-375.3*, and *-375.4*). The sequence corresponding to Gly-347 apparently is prone to mutagenesis, because G347R also was reported in two previously isolated *cry1* alleles, *hy4-15* and *hy4-16* (Ahmad et al., 1995). One mutation results in the conservative change R536K in the C-terminal domain (*cry1-321*). Some of the *cry1* mutations (*cry1-375* and *cry1-344*) may affect the functionality common to photolyase and cryptochromes, such as chromophore binding or energy transfer, because these mutant residues are within or adjacent to a motif (WRWG) that is well conserved among photolyases and cryptochromes (Ahmad and Cashmore, 1993; Kanai et al., 1997).

#### Blue Light-Dependent *cry1* Phosphorylation in Vitro

In an attempt to establish an in vitro cryptochrome phosphorylation assay, we found that *cry1* was phosphorylated in vitro without the addition of a protein kinase. In these experiments, the His-tagged *cry1* protein was expressed using the baculovirus expression system in Sf9 insect cells and purified using nickel affinity chromatography (Lin et al., 1995) (Figure 6A). The *cry1* protein was incubated with  $\gamma$ -<sup>32</sup>P-ATP in vitro in the presence or absence of blue light, fractionated by SDS-PAGE, and examined using autoradiography (Figures 6B and 6D) or immunoblot analysis (Figure 6E). Figure 6 shows that although little *cry1* phosphorylation was detected in the dark, *cry1* phosphorylation clearly was detected in the reactions exposed to blue light (Figure 6B). A control protein (*ubc9*) expressed similarly in the baculovirus system and purified using nickel affinity chromatography was not phosphorylated, regardless of the blue light treatment (Figure 6B). The *cry1* phosphorylation was dependent on ATP, because *cry1* was readily labeled by  $\gamma$ -<sup>32</sup>P-ATP but not by  $\gamma$ -<sup>32</sup>P-GTP (Figure 6D). Light-dependent in vitro phosphorylation also has been reported for both Arabidopsis *cry1* and human *cry1* (Bouly et al., 2003). These results are consistent with the hypothesis that cryptochromes may pos-

sess blue light-dependent autophosphorylation activity. However, this hypothesis needs to be tested further.

#### DISCUSSION

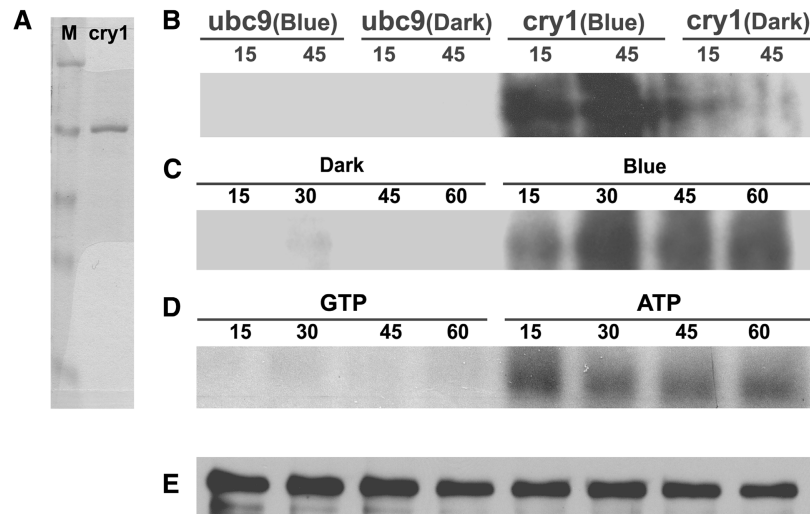
We have shown here that Arabidopsis *cry1* is phosphorylated in response to blue light under both in vivo and in vitro conditions. In Arabidopsis seedlings exposed to blue light, multiple isoforms of phosphorylated *cry1* were detected, suggesting that *cry1* is phosphorylated at multiple residues. The relative amount of all of the phosphorylated *cry1* isoforms increased in response to increased fluence of blue light, whereas *cry1* was dephosphorylated rapidly in the absence of light. The *cry1* phosphorylation seems specific to blue light because little *cry1* phosphorylation was detected in seedlings exposed to similar fluence ranges of red light or far-red light. Moreover, the blue light-dependent phosphorylation of *cry1* was largely independent of phytochromes, and the phosphorylation of *cry1* appears not to be *cry2* dependent and vice versa. Together, the previous study of *cry2* phosphorylation (Shalitin et al., 2002) and these results demonstrate that blue light-dependent phosphorylation is a general light reaction associated with plant cryptochromes.

A question raised by the blue light-dependent *cry1* phosphorylation concerns the role(s) of this protein modification. To address this question, we isolated and characterized missense *cry1* mutants that showed little blue light inhibition of hypocotyl elongation but still expressed the full-length CRY1 apoproteins. Missense mutations were found in both the N-terminal chromophore binding domain and the C-terminal domain of these *cry1* mutant proteins. Importantly, none of the *cry1* mutant proteins showed the blue light-dependent phosphorylation. It also is interesting that none except one of the missense *cry1* mutations identified affect phosphorylatable residues, such as Ser, Thr, or Tyr. Similarly, no mutation affecting phosphorylatable residues of CRY1 was isolated from a previous screen (Ahmad et al., 1995). These results are unlikely to be attributable to the method of ethyl methanesulfonate mutagenesis, which often results in C/G to A/T transitions affecting several codons for Ser or Thr (data not shown). Moreover, it appears that the mutagenesis is at least partially saturated for *cry1*, because several *cry1* mutants isolated in this study and in a previous study (Ahmad et al., 1995) contain the identical mutation (G347R). The observations that none of the *cry1* mutations isolated showed phosphorylation but few affected phosphorylatable residues may be explained by the notions that *cry1* is

	62	70	343	351	376	384	458	466	504	512	532	540
WT	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---
<i>cry1-305</i>	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---
<i>cry1-375</i>	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---
<i>cry1-388</i>	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---
<i>cry1-321</i>	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---
<i>cry1-344</i>	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---
<i>cry1-349</i>	---WLKNSLAQL---	---	LVDAGMREL---	---	PWRWGMKYF---	---	VLQAAGIEL---	---	NGSEEGGLGD---	---	TEPTRLNPN---	---

**Figure 5.** Lesions of the *cry1* Mutants.

Sequences of the *cry1* mutants were aligned with the deduced amino acid sequence of CRY1 of the Columbia accession (WT; At4g08920). Residue numbers of CRY1 are indicated above the sequence. White and gray boxes highlight mutant residues of the different alleles.



**Figure 6.** Blue Light-Dependent Phosphorylation of cry1 in Vitro.

**(A)** The cry1 protein purified from insect cells (cry1; 1  $\mu$ g) and the molecular mass marker proteins (M; 1  $\mu$ g per band) were fractionated on a SDS-PAGE gel and stained with Coomassie blue.

**(B) to (D)** Autoradiographs showing cry1 phosphorylation in vitro. Arabidopsis cry1 [**B**] and [**C**] and the control protein ubc9 (**B**) were incubated with  $\gamma$ - $^{32}$ P-ATP, and the reaction products were kept in the dark or exposed to blue light ( $33 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for the time indicated (15 to 60 min). cry1 phosphorylation reaction products incubated with  $\gamma$ - $^{32}$ P-ATP or  $\gamma$ - $^{32}$ P-GTP under blue light ( $33 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for the time indicated (15 to 60 min) are shown in (**D**).

**(E)** The immunoblot of the same membrane used in (**C**) was probed with the anti-CRY1 antibody.

phosphorylated at multiple residues and that cry1 phosphorylation may be required for its function. This is because if cry1 activity is dependent on its phosphorylation at multiple residues, a mutation at only one of the phosphorylatable residues may not eliminate its activity and therefore may not be isolated as a strong allele from our function-based genetic screen. On the other hand, a missense mutation that completely abolishes cry1 phosphorylation would inevitably eliminate its activity, regardless of the mutation occurs at a phosphorylatable residue or not.

Which enzyme catalyzes the cryptochrome phosphorylation is another interesting question. The observation that purified cry1 can be phosphorylated in vitro suggests an autophosphorylation activity of the photoreceptor, although the activities of an insect kinase copurified with cry1 cannot be excluded completely. Given that plant and animal cryptochromes share relatively low sequence similarity (Todo, 1999) and the light-dependent nature of the in vitro cry1 phosphorylation (Figure 6) (Bouly et al., 2003), the "contamination" hypothesis has to make an intriguing, albeit not impossible, assumption that the presumed insect kinase copurified with cry1 not only would have recognized Arabidopsis cry1 but also would have distinguished its conformational change in response to light. On the other hand, an obvious difficulty with the "autophosphorylation" hypothesis is that cryptochromes generally lack a well-recognized protein kinase motif. However, a careful examination of the CRY1 sequence revealed a number of putative nucleotide binding P-loop (Saraste et al., 1990; Sinha et al., 1999) motifs (data not shown), and at least one of the putative P-loops is well conserved between Arabidopsis and fern cryptochromes (Imaizumi et al., 2000). Additional investigations are needed to resolve

these competing hypotheses and to fully understand the mechanism and physiological role(s) of blue light-dependent cryptochrome phosphorylation.

## METHODS

### Plant Materials

*Arabidopsis thaliana* accession Columbia was used throughout this study. Lights and filters used were as described (Mockler et al., 2003). Mutant lines, except for the newly isolated cry1, were as described previously (Reed et al., 1994; Devlin et al., 1999; Mockler et al., 1999, 2003; Shalitin et al., 2002; Franklin et al., 2003; Halliday et al., 2003). The two cry2 mutant alleles in Col background, which were originally called cry2-1 and cry2-2 (Guo et al., 1998), are renamed as cry2-4 and cry2-5, respectively. Seeds were sown on compound soil, kept in the dark at 4°C for 3 days, treated with white light for 8 h to promote germination, and grown in dark or light conditions as indicated in the figure legends. The cry1 mutants were isolated from the phyA (phyA-211) mutant background (Reed et al., 1994). The phyA seeds were treated with ethyl methane-sulfonate (0.3%) for 11 h, rinsed thoroughly, and grown as 30 subpopulations to prepare independent pools of M2 seeds. M2 seeds ( $\sim 1,000,000$ ) were sown and grown in continuous blue light ( $25 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for 5 days, and individuals that showed long hypocotyls were isolated from independent M2 pools. Putative strong cry1 mutants that expressed CRY1 apoprotein with a molecular mass similar to that of wild-type CRY1 were selected for further study. The cry1 mutations were confirmed and the mutant residues identified by direct DNA sequencing of the cry1 cDNAs amplified from individual lines using reverse transcriptase-mediated PCR. Four independently isolated lines, cry1-315, -320, -375, and -376, later were found to contain the same mutations and were renamed cry1-375.1, -375.2, -375.3, and -375.4, respectively.

### Immunoblot Analysis and Immunoprecipitation Assays

Immunoprecipitation was performed as described previously with minor modifications (Shalitin et al., 2002). Etiolated seedlings were excised from the hypocotyl base under a dim red safelight and homogenized in ice-cold immunoprecipitation buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Triton X-100, 10  $\mu$ M NaF, and 1 mM phenylmethylsulfonyl fluoride) and complete protease inhibitor cocktail (Roche, Mannheim, Germany). Extracts were passed through 0.2-mm filters to remove tissue debris. The filtrates were incubated with anti-CRY1 antiserum (1:1000) on ice for 1 h. Protein A-Sepharose beads (Sigma) were added (1:60) to the mixture, which then was incubated on ice for 1 h. Immunoprecipitated samples were washed three times with ice-cold washing buffer (50 mM Tris-HCl, pH 8, 140 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). The bound proteins were denatured by mixing with SDS-PAGE sample buffer and boiling for 5 min.

Samples were fractionated on a 10% SDS-PAGE minigel for 4 h at constant current (18 mA, up to 110 V), and the protein bands were blotted to a nitrocellulose membrane using a semidry electrophoresis apparatus (Amersham Bioscience, Piscataway, NJ) at a constant voltage (6 V) for 12 h. Immunoblots were probed with the anti-CRY1 antiserum (1:5000 dilution in PBST [7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>PO<sub>4</sub>, 130 mM NaCl, and 0.3% Tween 20]), washed in PBST three times, reacted with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:20,000; Amersham), washed, and exposed to x-ray film using the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham). The same blot sometimes was stripped and reprobed with different antibodies (Lin et al., 1998). Protein signals were digitized by scanning the ECL films and quantified using NIH Image software. The exposure time for ECL of different immunoblots was not controlled precisely, so the signal intensities from different immunoblots are not directly comparable. Anti-CRY1 and anti-CRY2 antibodies were described previously (Lin et al., 1996, 1998).

### Phosphorylation and Dephosphorylation Assays

In vivo labeling analysis was performed as described previously (Shalitin et al., 2002) with minor modifications. Five-day-old etiolated seedlings (~50) were cut above the hypocotyl base and incubated with <sup>32</sup>P (300  $\mu$ Ci of H<sub>3</sub>PO<sub>4</sub> [ICN, Costa Mesa, CA] in deionized water) for 3 h in the dark at room temperature. Excess <sup>32</sup>P was removed by rinsing with water. The tissue aliquots were either exposed to light or kept in the dark before harvesting for immunoprecipitation or immunoblot analyses. For alkaline phosphatase treatment, tissues were homogenized in calf intestinal alkaline phosphatase buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, and 1 mM DTT) and divided into two aliquots. Alkaline phosphatase (New England Biolabs, Beverly, MA) was added to one of the two aliquots (0.25 unit/ $\mu$ L) and incubated at 30°C for 30 min. The dephosphorylation reactions were stopped by adding SDS-PAGE sample buffer and boiling for 5 min. The samples were examined by immunoblot analysis as described above.

The in vitro protein phosphorylation experiments were performed essentially as reported previously (Yeh and Lagarias, 1998) with minor modifications. Arabidopsis cry1 was expressed and purified using the baculovirus expression system as described (Lin et al., 1995). The control protein ubc9 (yeast E2 ubiquitin-like conjugating protein) was expressed and purified similarly. Cry1 or ubc9 (1  $\mu$ g/reaction) was incubated with 100  $\mu$ M  $\gamma$ -<sup>32</sup>P-ATP or  $\gamma$ -<sup>32</sup>P-GTP (2000 to 5000 cpm/pmol) in the phosphorylation buffer (25 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 5 mM MgCl<sub>2</sub>, and 4 mM 2-mercaptoethanol) for 15 to 60 min. The phosphorylation reactions were prepared under dim red light, moved to dark or exposed to blue light, and stopped by adding 4 $\times$  SDS-PAGE sample buffer and boiling for 5 min. The proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes for autoradiography and immunoblot analyses.

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 Chentao Lin, clin@mccdb.ucla.edu.

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