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Chimeric Proteins between cry1 and cry2 Arabidopsis Blue Light Photoreceptors Indicate Overlapping Functions and Varying Protein Stability

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A blue light (cryptochrome) photoreceptor from Arabidopsis, cry1, has been identified recently and shown to mediate a number of blue light–dependent phenotypes. Similar to phytochrome, the cryptochrome photoreceptors are encoded by a gene family of homologous members with considerable amino acid sequence similarity within the N-terminal chromophore binding domain. The two members of the Arabidopsis cryptochrome gene family (*CRY1* and *CRY2*) overlap in function, but their proteins differ in stability: cry2 is rapidly degraded under light fluences (green, blue, and UV) that activate the photoreceptor, but cry1 is not. Here, we demonstrate by overexpression in transgenic plants of cry1 and cry2 fusion constructs that their domains are functionally interchangeable. Hybrid receptor proteins mediate functions similar to cry1 and include inhibition of hypocotyl elongation and blue light–dependent anthocyanin accumulation; differences in activity appear to be correlated with differing protein stability. Because cry2 accumulates to high levels under low-light intensities, it may have greater significance in wild-type plants under conditions when light is limited.

INTRODUCTION

Plant growth and development are regulated by interactions between the environment and endogenous developmental programs (Terzaghi and Cashmore, 1995). Of the various environmental factors controlling plant development, light plays an especially important role in photosynthesis, in seasonal and diurnal time sensing, and as a cue for altering developmental pattern (McNellis and Deng, 1995; Chory et al., 1996; von Arnim and Deng, 1996; Barnes et al., 1997). The most well-characterized class of photoreceptors is the phytochromes, which absorb primarily in the red and far-red regions of the visible spectrum and are encoded by an evolutionarily conserved gene family (Furuya, 1993; Vierstra, 1993; Smith, 1994; Quail et al., 1995; Furuya and Schaefer, 1996). An interesting feature of the phytochrome gene family is that the different members are expressed at varying levels and have somewhat distinct (albeit overlapping) functions (Quail, 1991, 1994; Reed et al., 1994). In higher plants, the most striking example of this is phytochrome A (phyA) of higher plants, which mediates the far-red high-irradiance response and is rapidly degraded when activated by red light (Somers et al., 1991; Somers and Quail, 1995). Phytochromes are thought to undergo light-dependent conformational changes when activated; the instability of phyA has been shown to be mediated by targeting of the active form by a ubiquitin-based degradation system (reviewed in Vierstra, 1996).

Recently, the gene encoding a distinct blue light photoreceptor, cry1, has been isolated by cloning the *hy4* gene of Arabidopsis (Ahmad and Cashmore, 1993). cry1 has been shown to mediate blue light responses, including inhibition of hypocotyl elongation, anthocyanin accumulation, leaf and cotyledon expansion, elongation of petioles, responsiveness to night breaks, and expression of blue light–regulated genes (Short and Briggs, 1994; Ahmad et al., 1995; Jackson and Jenkins, 1995; Ahmad and Cashmore, 1996, 1997; Bagnall et al., 1996; Lin et al., 1996b). The cry1 photoreceptor consists of an N-terminal chromophore binding domain, with homology to the microbial DNA repair enzyme photolyase, and a C-terminal domain that may be involved in protein–protein interactions and signal transduction (Ahmad and Cashmore, 1993, 1996). Despite its homology to photolyases, the cry1 photoreceptor mediates no DNA repair (Lin et al., 1995a; Malhotra et al., 1995). In plants, this function is assumed by an entirely distinct enzyme (Ahmad et al., 1997). The overall domain structure of cryptochrome is similar to that of phytochrome, which also consists of an N-terminal chromophore binding domain and a C-terminal extension that is implicated in signaling (Quail et al., 1995). The C-terminal domain of cry1 has been shown to be essential for photoreceptor function (Ahmad et al., 1995).

In addition to *CRY1*, a homologous gene encoding a cryptochrome protein has been identified in Arabidopsis and designated as *CRY2* (Hoffman et al., 1996; Lin et al., 1996a). cry2 contains an N-terminal chromophore binding domain similar to that of cry1; however, the C-terminal extension encoded by *CRY2* is shorter than and bears little homology to that of cry1. Interestingly, the homolog of *CRY2* from a closely related species, *Sinapis alba*, had been identified

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previously and shown to lack a C-terminal extension altogether (Batschauer, 1993). This gene was originally thought to encode a plant photolyase, but subsequent studies indicated no DNA repair activity and >90% amino acid sequence similarity with Arabidopsis cry2 (Malhotra et al., 1995; Lin et al., 1996a).

Overexpression studies of cry2 in wild-type Arabidopsis plants suggested that cry2 mediates inhibition of hypocotyl elongation and cotyledon expansion (Lin et al., 1998). These are phenotypes already to a large part mediated by cry1, as shown by their marked reduction in the cry1-deficient *hy4* mutant (Koornneef et al., 1980; Liscum and Hangarter, 1993; Ahmad et al., 1995; Jackson and Jenkins, 1995), suggesting considerable similarity in function of the two gene family members. Interestingly, the cry2 protein, unlike cry1, was shown to be unstable in green, blue, and UV-A light (all wavelengths that activate the receptor) but not in dark and red light (wavelengths that do not activate the receptor) (Lin et al., 1998). In this respect, the cry2 photoreceptor appears to be more similar to the phytochrome family member phyA, which undergoes light-activated degradation (Somers et al., 1991).

In this study, we constructed a series of cry1 and cry2 fusion proteins and analyzed their expression characteristics in transgenic Arabidopsis plants. By using this approach, we were able to address two specific questions. First, can the light-unstable phenotype of the cry2 photoreceptor be assigned to any specific domain in cry2? Similar studies have been highly informative in the case of phyA. In these studies, gene fusions between light-labile and light-stable phytochromes were used to show that the N-terminal chromophore binding domain is responsible for the light-mediated instability of phyA (Wagner et al., 1996). Second, are the respective N- and C-terminal domains of the cryptochrome photoreceptors functionally interchangeable? Answers to these questions should provide important insights into the functional diversity and mechanism of action of the cryptochromes.

RESULTS

Genes Encoding cry1/cry2 Fusion Proteins

Sequence alignment of the two photoreceptors cry1 and cry2 shows that the region of homology is maximal in the N-terminal chromophore binding domain, with very little similarity in the C-terminal extension (Figure 1A). However, there is an additional region within the first several hundred amino acids (thought to encode the pterin chromophore binding site) where there is relatively more divergence between the two photoreceptors. In contrast, the presumptive flavin binding domain (amino acids 300 to 500 in both cry1 and cry2) is virtually identical in the two photoreceptors.

To explore the possible functional significance of differences between the two photoreceptors, we constructed a number of protein fusions reflecting this sequence divergence (Figure 1B). Construct C2(366)C1 represents a fusion of the N-terminal 366 amino acids of cry2 fused to the downstream region of cry1. This construct was designed to show the significance (if any) of the small area of divergence within the N-terminal chromophore binding domain of cry1 and cry2. Construct C2(505)C1 represents the fusion of the entire chromophore binding domain of cry2 to the C-terminal domain of cry1. Construct C1C2 consists of the entire N-terminal domain of cry1 fused to the C terminus of cry2 and is thereby the exact opposite of construct C2(505)C1.

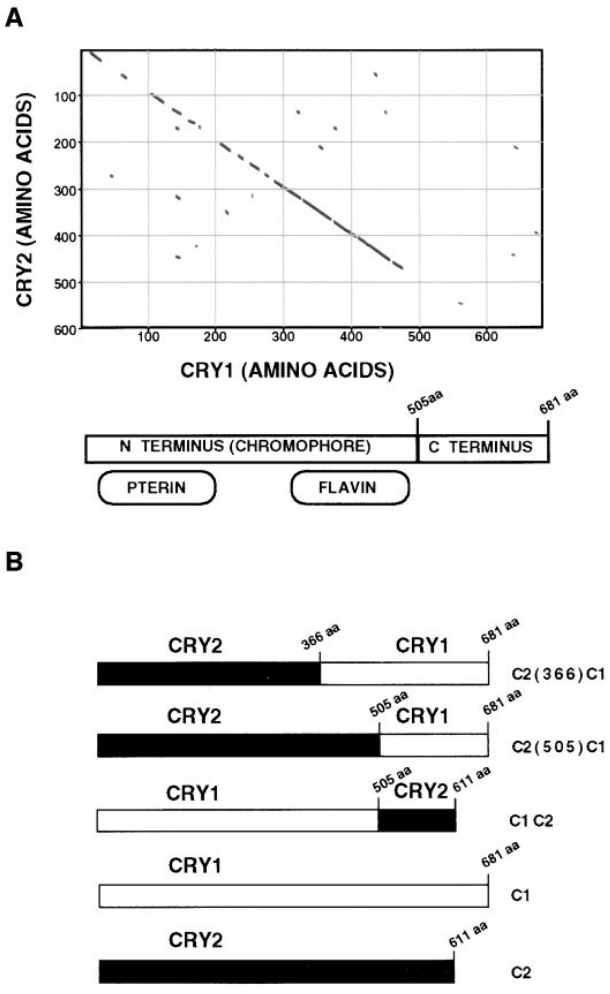

Figure 1. Fusion Constructs.

(A) Amino acid sequence comparison between the cry1 and cry2 blue light photoreceptors generated as a Pustell similarity matrix by MacVector DNA sequence analysis software version 3.5 (International Biotechnologies, New Haven, CT). Regions of similarity are indicated by the solid diagonal line. aa, amino acids.
(B) Diagram of the constructs used in this study.

Full-length cry1 and full-length cry2, designated C1 and C2, respectively, were used as controls. All constructs were cloned behind the high-level expression cauliflower mosaic virus 35S promoter and transferred to Agrobacterium for overexpressing in transgenic plants.

Transgenic Plants Overexpressing Cryptochrome Sequences Complement a cry1-Deficient *hy4* Mutant to Varying Degrees

The constructs indicated in Figure 1B were transformed into Arabidopsis cry1-deficient *hy4-3* (Ahmad and Cashmore, 1993) by the in-the-plant vacuum infiltration method (Bechtold et al., 1993). The *hy4* mutant was used so that weak phenotypes that were not readily apparent in a wild-type (*CRY1*) background could be unmasked. In addition, this mutant lacked the full-length cry1 protein; therefore, protein gel blotting in this genetic background should reveal only the protein expressed from the introduced transgene. Kanamycin-resistant transformants from transgenic plants were identified and allowed to self; the resulting F_2 progeny were analyzed for expression characteristics. At least 40 independent transformants were analyzed for each construct.

In many instances, segregating short hypocotyl seedlings could be clearly distinguished in the selfed lines under continuous blue light (Figure 2). Some of the seedlings were intermediate in hypocotyl length between the wild type and the *hy4* mutant (for instance, lines C2(366)C1, C1C2, and C2), suggesting that the constructs conferred a weaker phenotype than did wild-type cry1 or were perhaps being expressed at lower levels. Representative seedlings from the most strongly expressing transgenic lines derived from each construct are shown in Figure 2. In fact, there was considerable variation in the expression characteristics of the same transgene in different lines (likely due to position effects and variation in levels of protein produced).

To provide a more precise description of the expression characteristics of a given transgene, we present histograms detailing the hypocotyl length of ~100 seedlings from one of the most pronounced overexpressing lines of each construct (Figure 3). Wild-type and homozygous *hy4* seedlings are presented as a control; all transgenic lines presented are F_2 progeny of lines segregating for one T-DNA insertion,

Figure 2. Seedlings Expressing Proteins from the Constructs Outlined in Figure 1B.

Seedlings were germinated as described in Methods and grown for 4 days under continuous high-fluence blue light. wt, wild-type untransformed Arabidopsis seedlings of ecotype Wassilewskija; *hy4*, the untransformed parental line that was used for transformation with the chimeric constructs. Plants 1 to 6 represent the most pronounced phenotypes observed in transgenic lines segregating for the indicated constructs: plant 1, truncated cry2 N-terminal domain (505 amino acids) fused to the 35S promoter; plant 2, C2(366)C1; plant 3, C2(505)C1; plant 4, C1; plant 5, C1C2; and plant 6, C2.

with the exception of construct C2(366)C1, which has two independent kanamycin-resistant insertions (data not shown).

The constructs that resulted in the most severe short hypocotyl phenotype under continuous light were the intact cry1 overexpressing control (C1) and the fusion between the entire N-terminal chromophore binding domain of cry2 fused to the C-terminal domain of cry1 (C2C1). These resulted in a short hypocotyl phenotype that was even more pronounced than that of the wild-type control (containing the wild-type *CRY1* gene). In each line, approximately one-fourth of seedlings showed the *hy4* mutant long hypocotyl phenotype, which is consistent with segregation of the transgene in the heterozygous population. Fusions containing a smaller portion of cry2 fused to cry1 (C2(366)C1), or consisting of the reciprocal gene fusion of the N terminus of cry1 fused to the C-terminal of cry2 (C1C2), showed only partial complementation of the *hy4* mutant phenotype. This is shown by the fact that virtually none of the seedlings had hypocotyls as short as those of the wild type under blue light, although they were clearly shorter than those of the *hy4* mutant parent. The same was true of the intact cry2 overexpressing seedlings (C2).

Homozygous lines expressing the various transgenes confirmed the expression characteristics observed in the segregating lines. Blue light–dependent inhibition of hypocotyl elongation was most pronounced in homozygous lines containing constructs C2(505)C1 and C1 (Figure 4A). Anthocyanin accumulation was measured as an additional phenotype under the control of the cryptochrome blue light photoreceptors and was highest in the transgenic lines showing the strongest degree of inhibition of hypocotyl elongation (C2(505)C1 and C1 homozygous lines) (Figure 4B). In addition, there was a lesser but measureable increase in anthocyanin accumulation in the remaining transgenic lines (C1C2 and C2), which is consistent with their weaker *hy* response.

Taken together, these data indicate that all of the transgenic lines expressing wild-type or fusion constructs of cry1 and cry2 showed some level of cryptochrome activity and some degree of complementation of the cry1-deficient mutant *hy4.* This was evident by examining both inhibition of hypocotyl elongation and anthocyanin accumulation. The highest level of function in a fusion construct (as high as that of the cry1 overexpresser itself) was mediated by the construct C2(505)C1, with the N-terminal domain of cry2 fused to the C-terminal domain of cry1.

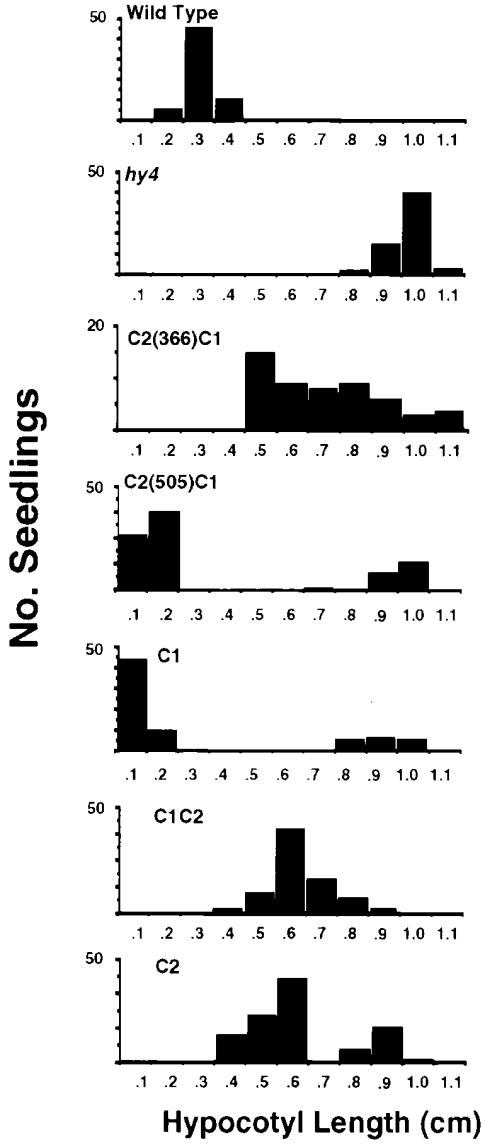

Figure 3. Hypocotyl Length of Seedlings Expressing Transgenes.

Seedlings were germinated as described in Methods and then transferred to continuous high-fluence blue light for 48 hr. Hypocotyl lengths for between 60 and 150 seedlings from each segregating line were measured and rounded off to the nearest millimeter. Wild-type Arabidopsis is of ecotype Wassilewskija; *hy4* is from the untransformed *hy4-3* parent. The remaining histograms represent results from transgenic F_2 lines harboring the indicated constructs in the *hy4-3* mutant genetic background.

Transgenic Lines Overexpressing Cryptochrome Fusion Constructs Express Protein with Varying Degrees of Light Stability

The difference in levels of cryptochrome activity in the transgenic lines could in principle result from differential stability or expression characteristics of the encoded fusion proteins. The cry2 protein is readily detectable in seedlings that have been germinated and grown in continuous dark or red light (Figure 5A). Under these conditions, the photoreceptor is inactive. However, after transfer to high-fluence blue light (30 μmol m^{-2} sec^{-1}), the levels of the cry2 protein decreased to quite low levels in $<$1 hr (Figure 5A). Moreover,

the rate of reduction in levels of the cry2 protein is proportional to the light fluence. Under very high fluences, considerable reduction in protein levels occurred after <1 hr, whereas under lower fluences, protein levels decreased more slowly. At a 3 μmol m^{-2} sec^{-1} fluence rate, there was a marked decrease in the level of cry2 protein after only 6 hr, whereas at even lower light fluences (0.8 μmol m^{-2} sec^{-1}), protein levels did not diminish discernibly (Figure 5A). These data are consistent with protein degradation being triggered by a light-dependent conformational change in the receptor. At higher light intensities, a larger proportion of the receptor molecules would be in the active conformation, and therefore, a higher proportion would be recognized by degradative enzymes.

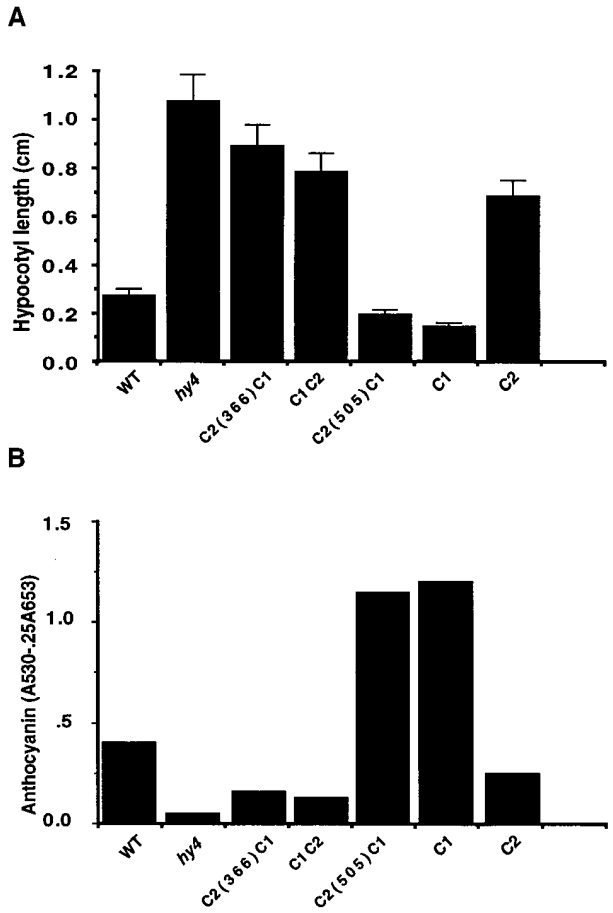

Figure 4. Expression Characteristics of Homozygous Transgenic Lines Expressing Cryptochrome Fusion Proteins.

(A) Hypocotyl lengths of homozygous transgenic lines harboring the indicated constructs under high-fluence blue light (30 μmol m^{-2} sec^{-1}).

(B) Anthocyanin accumulation in transgenic lines harboring fusion constructs.

WT, wild type.

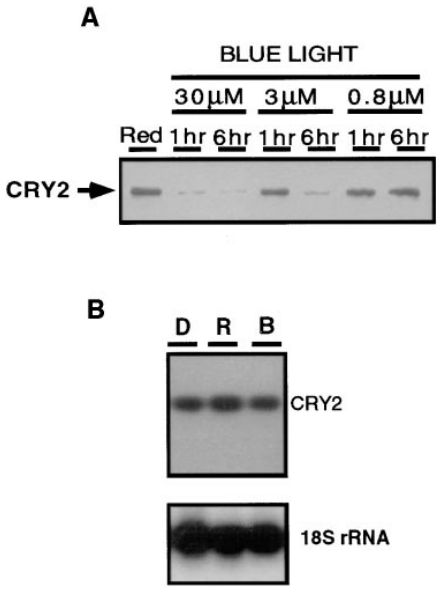

Figure 5. Expression Characteristics of Labile Cryptochrome under Low-Light Intensity.

(A) Gel blot of cry2 protein under differing blue light fluences. Wild-type Arabidopsis seedlings were germinated and grown for 5 days under continuous red light (20 μmol m^{-2} sec^{-1}). Seedlings were subsequently transferred to blue light at the indicated light intensity for 1 and 6 hr, respectively. Proteins were resolved on gel blots and probed with the anti-cry2 antibody. The first lane (Red) represents seedlings not exposed to blue light. Twenty-five micrograms of protein was loaded on each lane.

(B) RNA gel blot analysis of levels of *CRY2* mRNA in darkness (D), red (R), or blue (B) light. Seedlings were germinated as described in Methods and maintained under the indicated light condition for 6 days. As a control of the loading and the integrity of the RNA sample, we used an *18S* rRNA probe (Pruitt and Meyerowitz, 1986).

To help eliminate the possibility that transcriptional mechanisms might result in the observed decrease in levels of the cry2 protein, we performed RNA gel blot analysis and showed that there is no difference in steady state RNA levels of cry2 in red or blue light (Figure 5B; Lin et al., 1998). To rule out the possibility that a translational mechanism may underlie the observed decrease in cry2 protein levels, we monitored the disappearance of the cry2 protein in the presence or absence of cycloheximide (Figure 6A).

Etiolated seedlings were treated with cycloheximide several hours before transfer to red or blue light (Figure 6A). Protein gel blotting was performed to measure levels of the cry1 and cry2 proteins under these conditions. Levels of protein in the dark are identical in both cycloheximide-treated or untreated controls in the case of both cry1 and

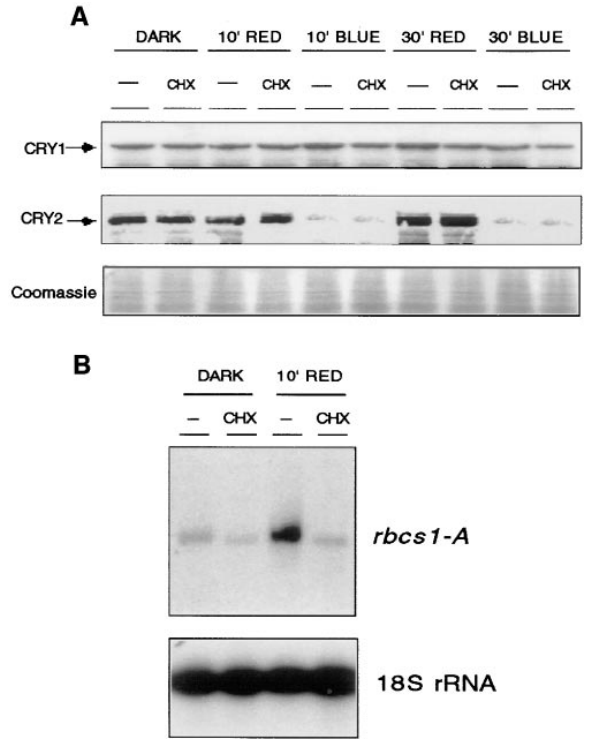

Figure 6. Effect of Cycloheximide on the Stability of the cry2 Protein.

(A) Twenty-five micrograms of protein from seedlings treated with cycloheximide (CHX) or mock treated (−) was run on SDS gels, transferred to nitrocellulose, and probed with the anti-cry1 or anti-cry2 antibody as indicated. Identical samples were run on SDS gels and stained with Coomassie Brilliant Blue R 250 to verify equivalent protein load. Light treatments are as indicated in Methods. ′, minutes.
(B) RNA gel blot analysis of red light–inducible *rbcS1-A* transcription in cycloheximide-treated and mock-treated control seedlings. Lanes represent samples at 3 hr subsequent to a 10-min red light pulse (60 μmol m^{-2} sec^{-1}) (RED) or unirradiated controls (DARK). *rbcS1-A* is the ribulose bisphosphate carboxylase probe. An *18S* rRNA probe was used as a control for the loading and the integrity of the RNA sample.

cry2 proteins. When transferred to high-fluence blue light (60 μmol m^{-2} sec^{-1}), cry2 protein levels decreased dramatically within 10 min, but there was no apparent difference in turnover between cycloheximide-treated and untreated controls. Levels of the cry1 protein were not affected by these treatments. To confirm that cycloheximide was indeed taken up and inhibiting protein synthesis in the treated seedlings, red light–induced transcription of ribulose bisphosphate carboxylase small subunit 1-A (*rbcS1-A*) was monitored. This transcriptional response has been reported to require de novo protein synthesis and does not occur subsequent to treatment with cycloheximide (Lam et al., 1989). This transcriptional response was indeed lacking in the cycloheximide-treated seedlings (Figure 6B), confirming that protein synthesis was inhibited. We conclude that the rapid decrease in levels of the cry2 protein under blue light must therefore result from light-dependent proteolysis rather than transcriptional or translational mechanisms.

Protein gel blotting was performed to determine the amount of recombinant protein expressed in the transgenic lines. Levels of fusion protein in constructs C2(366)C1, C2(505)C1, and C1 (all containing the C-terminal domain of cry1) were determined with the anti-cry1 antibody, which specifically recognizes the C-terminal domain of cry1 (Lin et al., 1995b) (Figure 7A). Because the *hy4-3* mutant does not synthesize full-length wild-type cry1 (Figure 7A), all of the protein detected resulted from the introduced transgene. Levels of expression of construct C2(366)C1 were virtually undetectable under blue light. However, seedlings transferred to continuous red light did accumulate protein, suggesting that this construct, like full-length cry2, is unstable under conditions in which the photoreceptor is activated. Construct C2(505)C1 (cry2 N terminus fused to the cry1 C terminus) and the cry1 overexpressing line (C1) showed

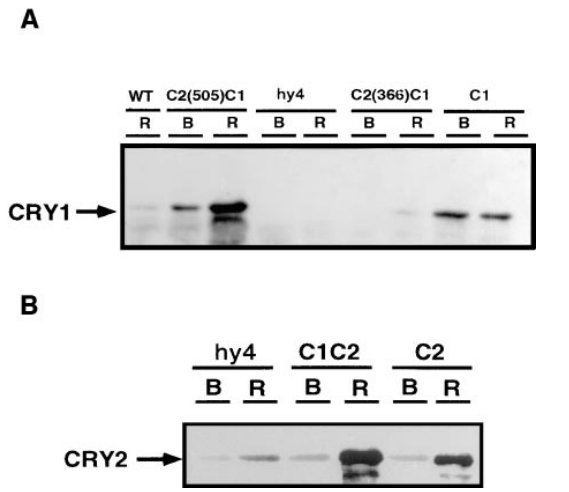

Figure 7. Protein Gel Blots of Seedlings Expressing Cryptochrome Gene Fusion Constructs.

(A) Transgenic seedlings probed with the anti-cry1 antibody. Each lane represents 25 μg of protein and was verified by Coomassie blue staining of duplicate gels (data not shown). Blue light (B) samples represent seedlings germinated as described in Methods and transferred to continuous high-fluence blue light for 5 days. Red light (R) samples represent seedlings germinated as described in Methods and transferred to continuous high-fluence blue light for 4 days. Seedlings were then transferred to red light (fluence of 20 μmol m^{-2} sec^{-1}) for 24 hr to allow the accumulation of labile photoreceptor species. (Levels of protein in etiolated seedlings were identical to those of red light–grown tissue for all chimeric and wild-type species.) WT, wild type.
(B) Protein gel blots of seedlings expressing cryptochrome gene fusion constructs probed with the anti-cry2 antibody. Red and blue light samples were obtained as described for **(A)**.

higher levels of protein expression under blue light than did a wild-type (*HY4*) control.

These levels of protein expression under blue light are consistent with the differences in the phenotypes of the transgenic seedlings, with the most pronounced effects being both on anthocyanin accumulation and the *hy* response resulting from the constructs (C2(505)C1 and C1) that are the most highly expressed. Interestingly, expression levels under continuous red light of C2(505)C1 were higher than under blue light, indicating that this fusion protein is also somewhat light labile. Thus, all fusion constructs containing some fragment of the chromophore binding domain of cry2 fused to the C-terminal domain of cry1 showed protein instability in blue light.

We next investigated the expression characteristics and protein stability of fusion proteins containing the C-terminal domain of cry2 (Figure 7B). In the *hy4-3* genetic background, wild-type levels of the cry2 protein were detectable and light labile, with considerably higher levels of protein under red than blue light. The identity of the protein band with cry2 is concluded from its molecular mass (70 kD) and from the marked increase in levels of protein expression in transgenic lines harboring the appropriate overexpression constructs. Both construct C1C2 (the N-terminal domain of cry1 fused to the C-terminal domain of cry2) and full-length cry2 (C2) were expressed at high levels under red light. Under blue light, however, the levels of expression were dramatically reduced, suggesting that these constructs, like the endogenous cry2, encode proteins that were rapidly degraded when activated by blue light. The weak phenotypes of the transgenic lines overexpressing either construct C1C2 or intact cry2 (C2) are therefore consistent with low levels of expression of recombinant photoreceptor under blue light. We observed that cry1 and cry2 function similarly, that their domains are functionally interchangeable, and that the difference in their expression characteristics in the transgenic lines at high-fluence blue light is consistent with differential protein stability.

Transgenic Lines Expressing Light-Labile Cryptochrome Photoreceptors Show Enhanced Activity under Low-Light Intensities

To further correlate the phenotypic characteristics of the transgenic lines with the levels of photoreceptor expression, we determined a fluence–response curve for the *hy* response (Figure 8). Consistent with previous reports, the inhibition of hypocotyl elongation decreased with decreasing light intensities for the cry1 overexpressing transgenic line (C1). However, this fluence dependence was considerably less marked in the case of the C2(505)C1 transgenic line, in which the activity of the receptor remained fairly constant over a broad range of light intensities. This trend was most marked in the case of the C1C2 and the C2 overexpressing lines. In these lines, responsivity to blue light actually increased at lower light fluences, followed by a decrease at the lowest light intensity. The simplest explanation for these anomalous fluence–response curves is that the increased concentrations of photoreceptor in dim light resulted in increased photoreceptor activity that more than compensated for the decrease in light intensity.

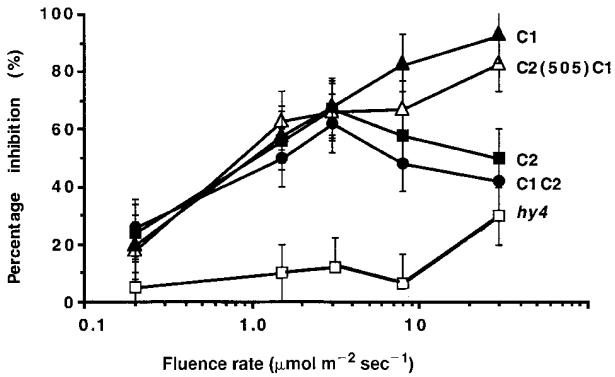

Figure 8. Fluence–Response Curve of Inhibition of Hypocotyl Elongation in Homozygous Transgenic Lines.

Seedlings germinated as described in Methods and grown for 8 days under dim blue light at the indicated light fluences. Twenty seedlings were measured for each point and expressed as the percentage of inhibition of hypocotyl elongation relative to dark-grown controls.

The cry1 Photoreceptor Is Light Labile in Other Plant Species

In the case of the phytochrome gene family, individual members show a high degree of specialization that is evolutionarily conserved. To determine whether protein stability of the individual cryptochrome gene family members also represents a significant specialization among the cry photoreceptors, we assayed the stability of both the cry1 and cry2 proteins in another plant species. Previous experiments with tobacco had shown that levels of the endogenous cry1 protein are higher in etiolated seedlings than in light-grown tissue (Lin et al., 1995b). Protein from tobacco seedlings probed with the anti-cry1 antibody under various light regimes (Figure 9A) was used to demonstrate that a single band of the precise molecular weight of cry1 accumulated to high levels under red light but disappeared in seedlings transferred to green, blue, or UV-A light (conditions in which the photoreceptor is active). The cry1 protein was only observed with the specific anti-cry1 antibody (Figure 9B). A slightly lower band, visible in all of the lanes, also appeared upon probing with the preimmune serum and is thereby a nonspecific cross-reacting band unrelated to cry1.

The time course of the disappearance of the tobacco cry1 protein was investigated under high-fluence blue light and

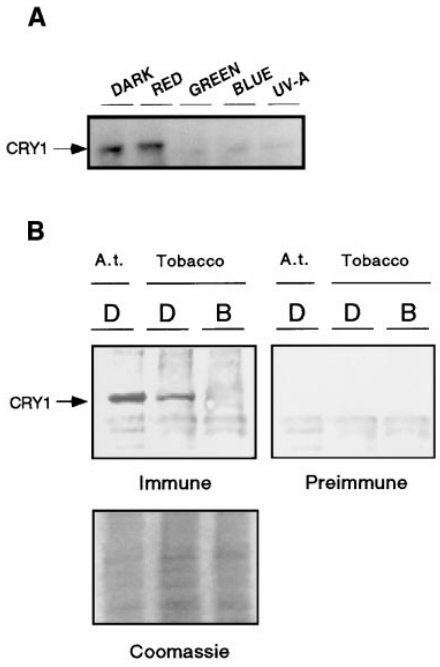

Figure 9. Protein Gel Blot of Tobacco Seedlings Probed with the Arabidopsis Anti-cry1 Antibody.

(A) Tobacco seedlings were irradiated under the indicated light regimes, and protein was prepared as described in Methods.
(B) Tobacco seedlings germinated and grown in continuous darkness (D) for 5 days were transferred to high-fluence blue light (B) for 1 hr. Protein was extracted, and equivalent concentrations were transferred to each lane of an SDS gel, blotted to nitrocellulose, and probed with either preimmune serum or the anti-cry1 antibody. Duplicate samples were run on a separate gel and stained with Coomassie. A.t., Arabidopsis total protein from wild-type 5-day-old seedlings.

was found to decrease dramatically within 1 hr (Figure 9B). Thus, tobacco cry1 appears to have the light-labile characteristics of Arabidopsis cry2. Similar results were observed with endogenous cry1 from tomato (M. Ahmad, G. Guiliano, and A.R. Cashmore, unpublished data). Because tobacco and tomato are dicots and as such relatively closely related to Arabidopsis, it appears that the stability or light lability of the cryptochromes is not evolutionarily conserved.

DISCUSSION

In this study, we constructed a series of fusion proteins between two members of the cryptochrome blue light photoreceptor family, cry1 and cry2, and analyzed their expression in transgenic Arabidopsis plants. Transgenic plants overexpressing such fusion constructs as well as overexpressers of intact cry1 and cry2 photoreceptors demonstrate that all of the constructs mediated blue light–dependent inhibition of hypocotyl elongation and anthocyanin accumulation to varying degrees. This suggests that the members of the cryptochrome gene family share considerable similarity in their specificities and modes of action.

The cry1 and cry2 photoreceptors differ primarily in their C-terminal domains; therefore, we found it interesting that these domains were functionally interchangeable in the various fusion constructs. Although the activity of the cry1 N terminus fused to the cry2 C terminus was relatively weak (C1C2), this resulted at least in part from the instability of the fusion protein under blue light and did not differ markedly from the activity of overexpressed intact cry2. An additional fusion construct comprising a smaller region of cry2 fused to the cry1 C terminus (C2(366)C1) showed the weakest phenotype but was also the most poorly expressed (Figure 7A). Based on these results, it is not possible to argue that the C-terminal "effector" domain of cry2 functions in a manner qualitatively distinct from that of cry1. It appears that cry1 and cry2 share considerable overlap in function and that all of the domains identified are to some degree interchangeable.

An interesting feature of the phytocrome family is that one member, phyA, shows pronounced instability in red light, through targeting of the active form by a ubiquitin-associated degradation pathway (Shanklin et al., 1987, 1989; Vierstra, 1996). PhyA is the most abundant form of phytochrome in dark-grown seedlings and has at least one unique function not mediated by other phytochromes—the far-red high-irradiance response (Quail et al., 1995). The functional determinants both of light instability and for the far-red high-irradiance response had been elegantly shown to be localized in the N-terminal chromophore binding domain by using reciprocal gene fusions between *phyA* and *phyB* (Wagner et al., 1996). These findings suggest that the contrasting photosensory information gathered by phyA and phyB through their N-terminal halves may be transduced to downstream signaling components through a common biochemical mechanism involving the regulatory activity of the C-terminal domains of the photoreceptors (Wagner et al., 1996).

Similar to phyA, it has been demonstrated that levels of the cry2 protein, in contrast to cry1, decrease substantially under conditions in which the photoreceptor is active (below a 600-nm bandwidth) (Lin et al., 1998). We showed that this disappearance can occur $<$10 min after the transfer of seedlings to high-fluence blue light, is directly proportional to the degree of activation of the photoreceptors (fluence dependent), does not occur at the level of mRNA abundance, and does not require protein translation. From previous studies and those presented here, we conclude that this light instability results from degradation of the photoreceptor, most likely the result of a light-dependent conformational change.

We have examined whether protein instability could be assigned to any specific domain of the blue light receptor in

the various hybrid constructs. However, in contrast to the situation for phyA and phyB, every combination of fusion protein that was created (whether N-terminal cry1/C-terminal cry2 or vice versa) showed a light-labile phenotype. This was in clear contrast to intact cry1, in which no detectable change in protein expression level was observed under any light regime. It is possible that no readily separable, discrete domain within cryptochrome can be held accountable for overall stability or light lability of the receptor. It is also possible that such determinants do exist but are not readily identifiable by our gene fusion strategy or that there might be multiple regions throughout the photoreceptor required for protein stability. Future studies involving more precise site-specific mutagenesis approaches should help to resolve the question of the precise sequences implicated in the stability or instability of the cryptochrome photoreceptors.

Accumulating evidence indicates that protein degradation in plants is a complex process involving a multitude of proteolytic pathways in the various cellular compartments. The ubiquitin-dependent pathway requires that proteins targeted for degradation become conjugated with chains of multiple ubiquitins (Callis, 1995; Belknap and Garbarino, 1996) and is particularly important for developmental regulation by selectively removing various cell cycle effectors, transcription factors, and cell receptors, such as phytochrome A (Vierstra, 1996). It would be intriguing if such a mechanism were involved in the protein instability of the cryptochromes. It is interesting that the cry2 photoreceptor shows greater protein stability under lower fluences of blue light (Figure 5A), which is consistent with light-dependent conformational change acting as a possible trigger for ubiquitin-dependent protein degradation. If fewer of the photoreceptors are activated (as occurs under dim light), then the rate of degradation of the receptors should decrease in proportion.

If differential light stability of the cryptochrome family members were of major functional significance in higher plants, one would predict that such stability (or lack of stability) would be evolutionarily conserved. This is certainly true for phyA, in which light instability is conserved at least as far as monocots (Quail et al., 1995; Smith, 1995). However, the cry1 protein of tobacco shows light instability typical of cry2 of Arabidopsis (Figure 9A). This does not result from the host plant background, because Arabidopsis cry1 expressed in transgenic tobacco plants is light stable (Lin et al., 1995b). Similarly, the endogenous tomato cry1 protein has been found to be light unstable (M. Ahmad, G. Guiliano, and A.R. Cashmore, unpublished data). Possibly, the stability of cry1 in Arabidopsis may be a recent evolutionary adaptation of the Brassicaceae.

In summary, it appears that the cryptochrome gene family shows considerable overlap in function between the various gene family members. Both cry1 and cry2 affect many of the same responses, and the fact that they do so to varying degrees may be explained at least in part on the basis of differential protein stability. It is also likely, by analogy with the phytochrome gene family, that cry1 and cry2 have distinct functions in addition to the ones that are shared or show altered specificities. Because cry2 accumulates to higher levels in dim light, it may have relatively greater significance in deetiolation or shade avoidance responses and may play the role of a backup system to cry1 under limiting light fluences. Overexpression of the most light-labile photoreceptors (C2 and C1C2) resulted in enhanced responsiveness to blue light under low-light conditions but relatively little difference over background at higher light intensities (Figure 8). In wild-type seedlings, accumulation of cry2 similarly would increase the overall concentration of cryptochrome under low-light conditions, thus allowing the plant an elegant and sensitive mechanism to fine-tune its ability to respond to blue light.

METHODS

Construction of Chimeric Coding Sequences

All constructs were fused behind the cauliflower mosaic virus 35S promoter of the *Agrobacterium tumefaciens* binary vector PKYLX7 (Schardl et al., 1987). Construct C2(366)C1 has the first 366 amino acids of *CRY2* that were subcloned from the cDNA clone as an EcoRI-HindIII fragment and fused to the C-terminal 176 amino acids of *CRY1* at the HindIII site of *CRY1*. This HindIII site of *CRY1* and *CRY2* is conserved in the DNA sequence and encodes identical homologous amino acids. Construct C2(505)C1 consists of amino acids 1 to 505 of *CRY2* fused to amino acids 505 to 681 of *CRY1*. The appropriate gene fragments were obtained by polymerase chain reaction amplification from the cDNAs and confirmed by sequencing. Construct C1 is the full-length *CRY1* cDNA (Ahmad and Cashmore, 1993). Construct C1C2 has amino acids 1 to 505 of *CRY1* fused to amino acids 505 to 611 of *CRY2*. Fragments were obtained by polymerase chain reaction amplification over the cDNAs and confirmed by sequence analysis. Construct C2 is the full-length *CRY2* cDNA (Lin et al., 1996a).

Plant Transformation and Phenotypic Analysis of Transgenic Plants

Plasmid constructs were mobilized into the Agrobacterium strain C58 and transformed into transgenic plants by the in-the-plant vacuum infiltration method, as described by Bechtold et al. (1993). After infiltration, T_1 seeds were bulk collected and plated on Murashige and Skoog (MS) medium (Sigma) supplemented with 1% sucrose and 50 μg/mL kanamycin. Antibiotic-resistant F_1 plants were identified after 7 to 10 days in a growth chamber; they were then transferred to soil and allowed to self. T_2 progeny of individual T_1 plants were harvested and analyzed further.

Seeds of transgenic T_2 and T_3 lines were sown on MS plates, cold treated at 4°C for 2 days, and allowed to germinate 2 days under white light before being transferred to the light regime indicated in the legends to Figures 2 to 8. At least 60 seedlings per plate were measured to generate the histogram data in Figure 3. Anthocyanin accumulation was measured as described by Ahmad et al. (1995), except that exactly 100 seedlings for each transgenic line were combined to obtain each value.

RNA Isolation and Transfer

Seeds were sown on MS plates and grown in continuous red or blue light for 6 days. Approximately 200 seedlings were sown per plate and harvested into liquid nitrogen. RNA was then prepared. Forty micrograms of total RNA was separated on a 1% formaldehyde–agarose gel, transferred to a nylon membrane, and probed with Arabidopsis *CRY2* cDNA. As a control of the amount and the integrity of the RNA sample, we used an *18S* rRNA probe (Pruitt and Meyerowitz, 1986).

Protein Gel Blots

Protein gel blot analysis using the anti-cry1 antibody was performed essentially as described by Lin et al. (1995b). Twenty seedlings from each transgenic line were combined, frozen in liquid nitrogen, homogenized, resuspended in SDS sample buffer, and boiled. Twenty-five micrograms of protein was loaded on each lane of an SDS–polyacrylamide gel and transferred to nitrocellulose membrane for probing with the antibody. Gel blotting with the anti-cry2 antibody was performed in the same manner as with the anti-cry1 antibody. The anti-cry2 antibody was generated against a full-length expressed recombinant cry2 protein from *Escherichia coli* (purified by affinity chromatography, using a nickel column and a six-histidine tag introduced into the expressed protein). The antibody was then affinity purified against the C-terminal domain fragment of cry2 (expressed and purified from *E. coli*); the resulting antibody did not recognize cry1 (data not shown). Four experiments were done with each transgenic line under the conditions indicated. The results were quantitatively and qualitatively similar.

Cycloheximide Treatment

Seedlings were plated onto filter paper overlaid onto MS plates, germinated, and grown for 6 days in darkness. Seedlings were then submerged by moving filter papers to Petri dishes containing 10 mL of MS medium with either 0.1% DMSO alone (mock) or 300 μM cycloheximide 2 hr before the light treatments. All manipulations were done in a dark room under a dim, green safelight. Blue and red light treatments were given for 10 and 30 min at 60 μmol m^{-2} sec^{-1}, respectively. Tissue was harvested into liquid nitrogen for the preparation of protein extracts and protein gel blots. A control sample of seedlings was irradiated with red light for 10 min and returned to darkness for an additional 3 hr before harvest for RNA gel blot analysis to verify the effectiveness of the cycloheximide treatment. RNA and protein gel blot analyses were done as described above. The *rbcS1-A* probe used in RNA blot hybridizations corresponds to a 0.4-kb HindIII fragment of the 5′ untranslated region of the gene (Krebbers et al., 1988). As a control of the loading and the integrity of the RNA sample, we used an *18S* rRNA probe (Pruitt and Meyerowitz, 1986).

Tobacco Seedling Protein Gel Blots

Cultivar SR1 tobacco seeds were sown on soil and allowed to germinate and grow for 10 days under continuous white light. Seedlings were then divided into groups and transferred for 24 hr to darkness, red (20 μmol m^{-2} sec^{-1}), green (50 μmol m^{-2} sec^{-1}), blue (30 μmol m^{-2} sec^{-1}), and UV-A (20 μmol m^{-2} sec^{-1}) light or otherwise treated as indicated in the legends to Figures 2 to 9. Subsequently, seedlings were harvested into liquid nitrogen, ground, transferred to SDS sample buffer, and boiled for 5 min. Equivalent protein concentrations for each sample were loaded on SDS–polyacrylamide gels, and protein gel blotting was conducted as described above.

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