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The Arabidopsis blue light receptor cryptochrome 2 is a nuclear protein regulated by a blue light-dependent post-transcriptional mechanism

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Summary

Cryptochrome 2 is a flavin-type blue light receptor mediating floral induction in response to photoperiod and a blue light-induced hypocotyl growth inhibition. cry2 is required for the elevated expression of the flowering-time gene *CO* in response to long-day photoperiods, but the molecular mechanism underlying the function of cry2 is not clear. The carboxyl domain of cry2 bears a basic bipartite nuclear localization signal, and the cry2 protein was co-fractionated with the nucleus. Analysis of transgenic plants expressing a fusion protein of CRY2 and the reporter enzyme GUS (GUS–CRY2) indicated that the GUS–CRY2 fusion protein accumulated in the nucleus of transgenic plants grown in dark or light. The C-terminal domain of cry2 that contains the basic bipartite nuclear localization signal was sufficient to confer nuclear localization of the fusion protein. Phenotypic analysis of transgenic plants expressing the fusion protein GUS–CRY2 demonstrated that GUS–CRY2 acts as a functional photoreceptor *in vivo*, mediating the blue light-induced inhibition of hypocotyl elongation. These results strongly suggest that cry2 is a nuclear protein. Although no obvious light regulation was found for the nuclear compartmentation of GUS–CRY2 fusion protein, the abundance of GUS–CRY2 was regulated by blue light in a way similar to that of cry2.

Introduction

The plant blue/UV-A light receptor cryptochromes and red/far-red light receptor phytochromes are the major photosensory receptors of plants which mediate light-regulated growth and development from seed germination to flower initiation (Kendrick and Kronenberg, 1994; Quail *et al.*, 1995). Phytochromes are generally known to mediate light-regulated gene expression (Kendrick and Kronenberg, 1994). However, except for phyB, the cellular localization of most phytochrome species remains unclear. phyB has been shown to be a nuclear protein and the nuclear localization of phyB was dependent on light (Sakamoto and Nagatani, 1996). Dark treatment of light-grown plants before the nuclear isolation reduced the level of phyB in the isolated nuclei; irradiation with far-red light before the dark treatment accelerated the dark-induced decline of the nuclear phyB level, suggesting that the nuclear localization of phyB was a light-regulated process (Sakamoto and Nagatani, 1996).

Arabidopsis cryptochrome 1 (cry1) was the first cryptochrome characterized at the molecular level (Ahmad and Cashmore, 1993; Lin *et al.*, 1995b). The *CRY1* gene was isolated using a T-DNA insertion mutant allelic to *hy4* (Ahmad and Cashmore, 1993; Koornneef *et al.*, 1980). The amino acid sequence of CRY1 apoprotein shares significant amino acid sequence similarity to that of the type I microbial DNA photolyases (Ahmad and Cashmore, 1993; Sancar, 1994). cry1 is a soluble protein and its expression is not apparently regulated by blue light (Lin *et al.*, 1996a). Mutants impaired in the *CRY1* gene result in decreased sensitivity to blue light (Ahmad and Cashmore, 1993; Bruggemann *et al.*, 1996; Koornneef *et al.*, 1980), whereas overexpression of *CRY1* in transgenic plants results in increased sensitivity to blue light (Lin *et al.*, 1995a; Lin *et al.*, 1996a). cry1 mediates not only the blue light-dependent inhibition of hypocotyl elongation, but also blue light-induced anthocyanin accumulation via the activation of genes encoding anthocyanin biosynthetic enzymes (Ahmad *et al.*, 1995; Chory, 1992; Jackson and Jenkins, 1995; Lin *et al.*, 1996a). The cellular localization and molecular mechanism of cry1-mediated blue light regulation of gene expression are not clear.

CRY2 was isolated by a low stringency hybridization screening using *CRY1* cDNA as the probe (Lin *et al.*, 1996b; Lin *et al.*, 1998). The sequence similarities between CRY2 and CRY1 are largely concentrated in the amino-terminal region of about 470 residues of CRY2 (58% identical), which is homologous to photolyases. The C-terminal regions of CRY2 (about 140 residues) and CRY1 (about 190 residues) show less than 15% amino acid sequence identity. The abundance of CRY2 protein in Arabidopsis is down-regulated in blue light (Ahmad *et al.*, 1998a; Lin *et al.*, 1998). Because the *CRY2* mRNA level is not

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apparently affected by blue light and the blue light regulation of CRY2 protein level is independent from the promoter and the 5′ untranslated region of the *CRY2* gene, a protein degradation mechanism has been proposed for the CRY2 regulation (Ahmad *et al.*, 1998a; Lin *et al.*, 1998). The function of *CRY2* has been studied using both *cry2* mutant plants and transgenic plants overexpressing *CRY2*. Transgenic Arabidopsis plants overexpressing *CRY2* were hypersensitive to blue light and developed short hypocotyls under blue light or white light (Lin *et al.*, 1998). Arabidopsis mutants impaired in the *CRY2* gene have been isolated recently (Guo *et al.*, 1998). *cry2* mutant seedlings had longer hypocotyls than the wild type under relatively low fluence rates of blue light (Lin *et al.*, 1998), indicating that the function of cry2 in hypocotyl inhibition was limited to low intensity light. This result was interpreted as being the consequence of the blue light-induced degradation of CRY2 protein in high intensities of blue light (Lin *et al.*, 1998).

cry2 also regulates floral induction in response to photoperiods. The *cry2* mutant, which flowered late in LD but not in SD photoperiods, is impaired in photoperiod sensing (Guo *et al.*, 1998). *cry2* is allelic to a previously isolated photoperiod-insensitive flowering time mutant *fha* (Guo *et al.*, 1998; Koornneef *et al.*, 1991). The physiological and genetic studies demonstrated that cry2 suppresses the phyB-dependent inhibition of floral initiation (Guo *et al.*, 1998; Mockler *et al.*, 1999). Although the molecular mechanism underlying cry2-regulated floral development is not clear, cry2 is known to affect the regulation of the flowering-time gene *CO*. The *CO* gene encodes a transcription factor controlling the photoperiodic floral induction. The expression of *CO* is regulated by photoperiods: the *CO* mRNA level is higher in plants grown in LD than that in SD (Putterill *et al.*, 1995). Interestingly, the up-regulation of *CO* expression by LD photoperiods was not found in the *cry2* mutant, whereas overexpression of CRY2 resulted in the significantly higher level of *CO* mRNA in both LD and SD photoperiods (Guo *et al.*, 1998). These results suggest that cry2 is a positive regulator of *CO* expression in response to photoperiods. To further understand the mechanism underlying cry2 action, we have studied the cellular localization of cry2 and found that cry2 is a nuclear protein.

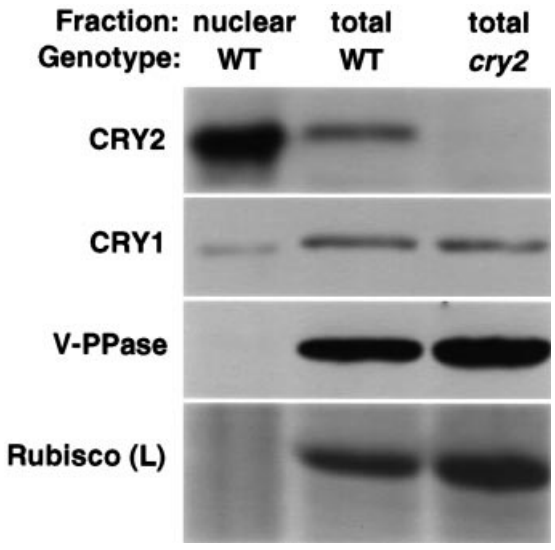

Figure 1. CRY2 protein is enriched in nuclear fraction.
Wild-type and *cry2–1* mutant Arabidopsis plants were grown in continuous white light for 3 weeks and rosettes were collected for nucleus isolation. Samples containing similar amounts of total protein were fractionated in a 10% SDS-PAGE gel and analyzed by immunoblot. The immunoblots showing CRY2, CRY1 and V-PPase were prepared using the same blot by stripping and re-probing technique (Guo *et al.*, 1998). Rubisco large subunit was shown as a Commassie blue stained band.

Results

CRY2 is enriched in the nuclear fraction

To understand the molecular mechanism of cry2 function, we investigated the cellular localization of the cry2 protein by testing whether CRY2 is a nuclear or cytosolic protein. Figure 1 shows that CRY2 protein is greatly enriched in the nuclear fraction (Figure 1, CRY2). In comparison, reprobing the same blot with the anti-CRY1 antibody showed that the relative amount of CRY1 co-fractionated with the nucleus was significantly less than CRY2 (Figure 1, CRY1). We also reprobed the same immunoblot using the antibodies against the vacuolar H+-pyrophosphatase, a tonoplast membrane protein (Zhen *et al.*, 1994); no V-PPase was found in the nuclear fraction (Figure 1, PPase). Similarly, a chloroplast stromal protein (Rubisco large subunit) was not found in the nuclear fraction (Figure 1, Rubisco (L)). In contrast, both V-PPase and the Rubisco large subunit were detected in the total plant extract (Figure 1). The absence from the nuclear fraction of proteins specific to two different organelles indicated that the enrichment of CRY2 in the nuclear fraction was unlikely to be due to a contamination from other organelles.

Nuclear proteins usually possess one or more short peptide motifs, termed a nuclear localization signal (NLS), which are required for nuclear targeting (Raikhel, 1992). One type of NLS that is found in different organisms including plants is the basic bipartite NLS. The bipartite NLS is characterized by two clusters of basic residues separated by a spacer of various lengths of non-conserved sequences (Raikhel, 1992). Because cry2 appeared to be a nuclear protein, we re-examined the amino acid sequence of CRY2 for the presence of the NLS-like sequences.

Indeed, a basic bipartite NLS-like sequence (KRVKPEEEEERDMKKSR) was found in the carboxyl terminus of CRY2 (Figure 2a,b). The NLS of CRY2, composed of two clusters of basic residues separated by nine amino acids, is very similar to those of other nuclear proteins (Figure 2a), including Arabidopsis phyB (Sakamoto and Nagatani, 1996) and a mouse cryptochrome (Thresher *et al.*, 1998). The presence of a typical NLS in the CRY2 sequence is consistent with the notion that cry2 may be a nuclear protein.

Preparation of transgenic plants expressing GUS–CRY2 and GUS–CRY2C fusion proteins

To further investigate the cellular localization of CRY2, we prepared transgenic plants expressing fusion proteins of the reporter enzyme GUS (approximately 69 kDa) fused to the full-length CRY2 (GUS–CRY2, approximately 136 kDa), or to the C-terminal 133 residues of CRY2 (GUS–CRY2C, approximately 83.6 kDa) (Figure 2b). We prepared all the transgenic lines in both the wild-type and *cry1* mutant (*cry1–301*) background. The *cry1–301* allele accumulates a truncated CRY1 mutant protein and shows a long hypocotyl phenotype under blue light (Mockler *et al.*, 1999). Overexpression of the wild-type CRY2 protein in either the wild-type or *cry1* mutant backgrounds can cause blue light hypersensitivity and a short hypocotyl phenotype under both low and high intensities of blue light, although the endogenous cry2 functions only in low light with respect to hypocotyl inhibition (Lin *et al.*, 1998) (H. Guo and C. Lin, unpublished results). We reasoned that hypocotyl growth inhibition could be a convenient test for the photobiological activity of the GUS–CRY2 fusion protein. Furthermore, the fusion protein GUS–CRY2 may be only partially active due to a change in protein structure, and the partial activity of GUS–CRY2 might be more easily detected and analyzed in the *cry1* mutant genetic background. Another advantage of selecting the wild-type and *cry1–301* mutant rather than mutant lines accumulating no CRY1 or CRY2 protein was that the endogenous cryptochrome proteins can serve as convenient internal controls for the analysis of fusion proteins.

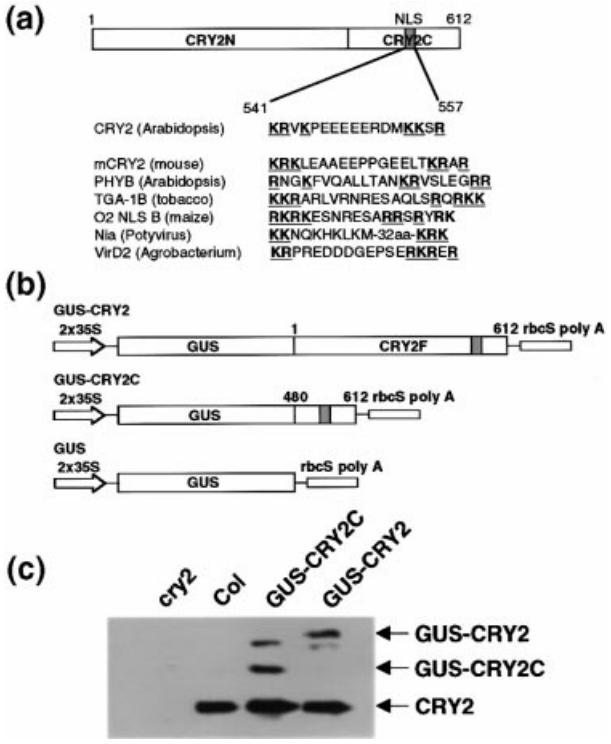

Figure 2. Transgenic expression of fusion proteins of GUS and CRY2 derivatives.
(a) Comparison of the bipartite nuclear localization signal (NLS) of CRY2 and those of other nuclear proteins.
(b) Schematic diagrams showing the constructs of the fusion genes. The hatched region indicates the bipartite NLS.
(c) Immunoblot showing the CRY2 protein in the wild-type (Col) and fusion proteins of GUS–CRY2 and GUS–CRY2C expressed in the respective transgenic plants.

We examined the expression of fusion proteins GUS–CRY2 and GUS–CRY2C in the respective transgenic lines using immunoblot analysis probed with anti-CRY2C antibodies. Figure 2(c) shows that both fusion proteins were expressed in transgenic plants, but at levels lower than the endogenous CRY2 (Figure 2c). This is in contrast to the expression of the wild-type recombinant CRY2 protein, which accumulated at a level significantly higher than the endogenous CRY2 (Lin *et al.*, 1998). There is a protein band (the top band) from the extract of the GUS–CRY2C transgenic plants that appears larger than the calculated molecular mass for the GUS–CRY2C (see also Figure 5b). The nature of this band is not clear. Although we also prepared transgenic plants expressing the fusion protein of GUS to the N-terminal domain of CRY2 (GUS–CRY2N), few GUS–CRY2N transgene products (mRNA or GUS activity) could be detected in any of the transgenic lines tested (data not shown).

The C-terminal domain of CRY2 containing the bipartite NLS is sufficient to confer the nuclear localization

We also examined the subcellular localization of the GUS–CRY2 and GUS–CRY2C fusion proteins in the respective transgenic plants using histochemical analysis of the GUS activity. The GUS activity was revealed by staining the whole seedling, and the area of the

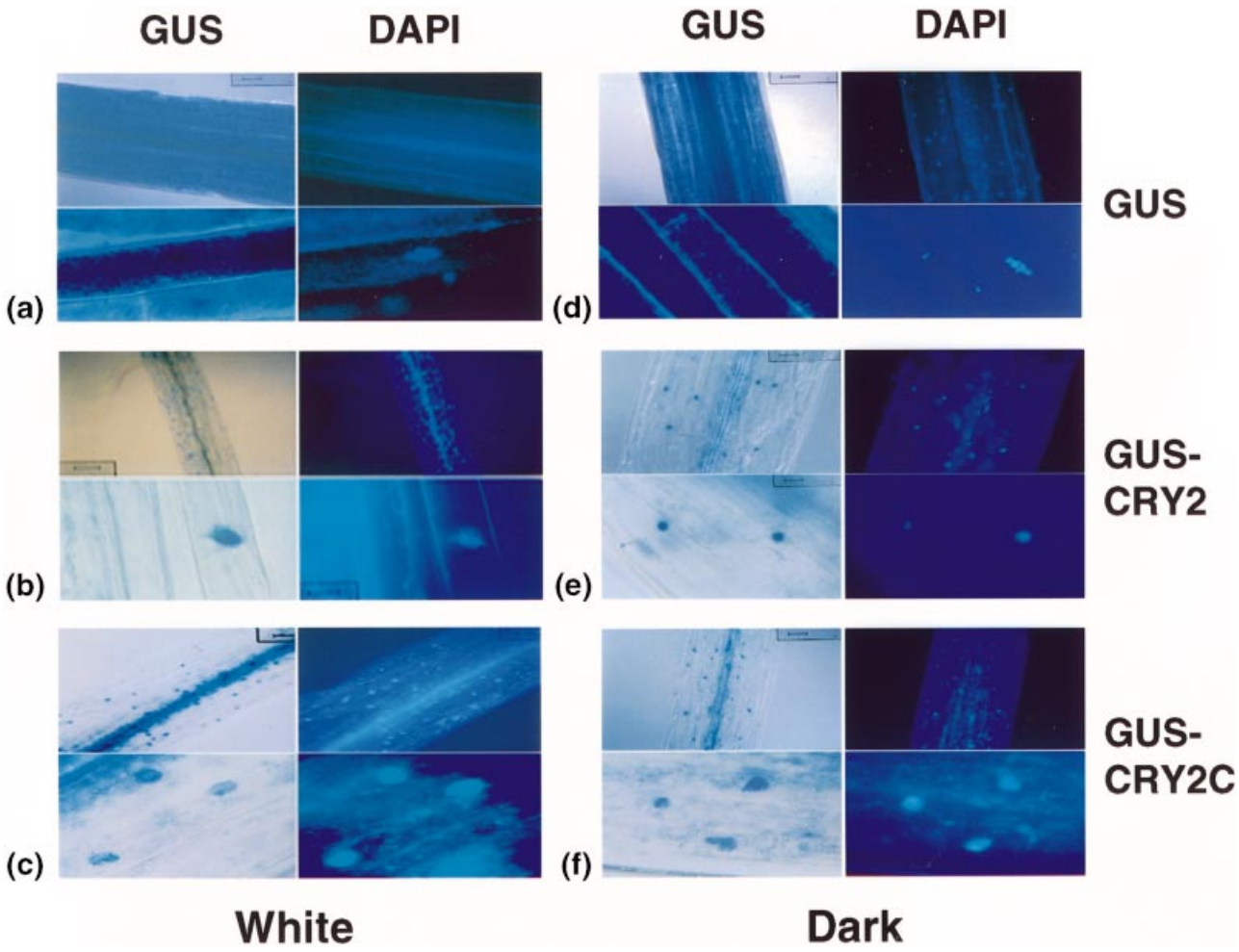

Figure 3. The subcellular GUS staining pattern of hypocotyl cells of transgenic plants (in Col background) expressing GUS, GUS–CRY2C or GUS–CRY2 fusion protein, respectively.
Plants were grown in indicated light conditions for 6 days before GUS histochemical analysis. For each panel (containing four pictures), the left ones were stained with GUS and the right ones were DAPI stained to show the nuclei. The lower ones in each panel (400–1000×) show the same specimen as the corresponding ones in the top of the same panel (100–200×).
(a,d) GUS-expressing transgenic plants grown in continuous white light (a) (100–150 μmole m^{-2}) or dark (d). (b,e) GUS–CRY2-expressing transgenic plants grown in continuous white light (b) or dark (e). (c,f) GUS–CRY2C-expressing transgenic plants grown in continuous white light (c) or dark (f).

nucleus was defined by the DAPI stain (Von Arnim and Deng, 1994). As previously reported (Von Arnim and Deng, 1994), the accumulation of GUS protein was detected throughout the cytosol (Figure 3a,d). In contrast, the GUS activity in both the GUS–CRY2 (Figure 3b,e) and GUS–CRY2C (Figure 3c,f) transgenic plants was detected almost exclusively in the nucleus. The fact that the GUS–CRY2C fusion protein was primarily found in the nucleus suggested that the C-terminal domain of CRY2 possessing the bipartite NLS was sufficient to confer the nuclear compartmentation of CRY2.

Light has been shown to regulate subcellular compartmentation for a number of proteins including COP1 (Von Arnim and Deng, 1994) and phytochrome B (Sakamoto and Nagatani, 1996). To examine whether the subcellular compartmentation of CRY2 is regulated by light, we analyzed the location of GUS–CRY2 and GUS–CRY2C fusion proteins in the respective transgenic plants grown in dark or under continuous white light. Figure 3 demonstrates that the GUS activity of both GUS–CRY2 and GUS–CRY2C fusion proteins were confined in the nucleus regardless of whether plants were grown in white light (Figure 3b,c) or in dark (Figure 3e,f). It appears that the nuclear localization activity of the NLS of CRY2 was not affected by light.

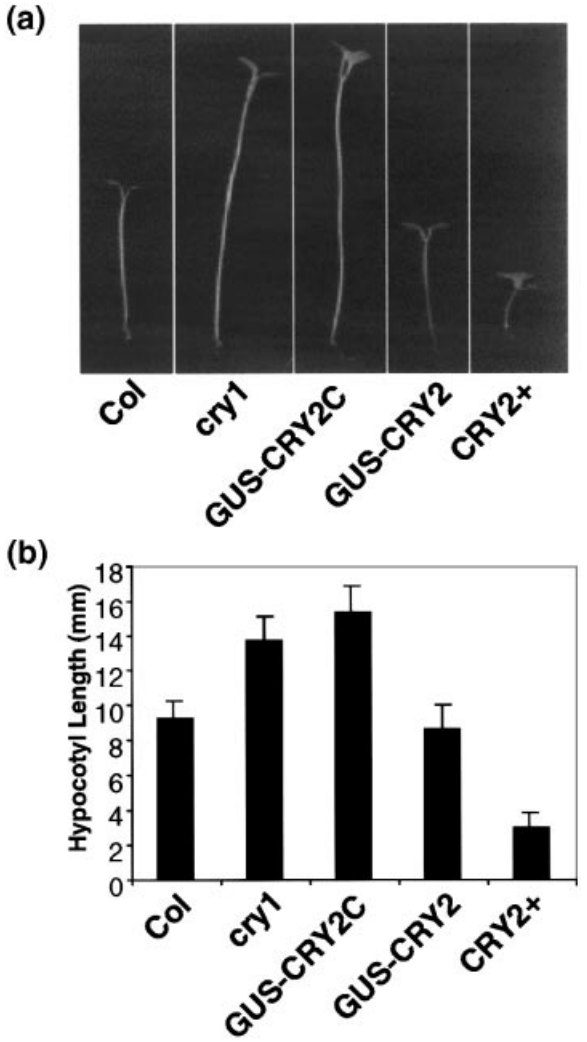

Figure 4. GUS–CRY2 fusion protein can mediate blue light-dependent inhibition of hypocotyl elongation in the transgenic Arabidopsis plants.
(a) A comparison of the seedling phenotype of the wild-type (Col), *cry1* mutant (*cry1*), the transgenic lines (in *cry1* genetic background) expressing GUS–CRY2C, GUS–CRY2 fusion proteins, or CRY2 recombinant protein (CRY2+). 6-day-old seedlings grown under continuous blue light (3 μmole m^{-2} s) were shown.
(b) Hypocotyl lengths of the respective transgenic lines. Seedlings were randomly selected from each line in the same experiment as (a). Means of at least 30 seedlings per line and the respective standard deviations were shown.

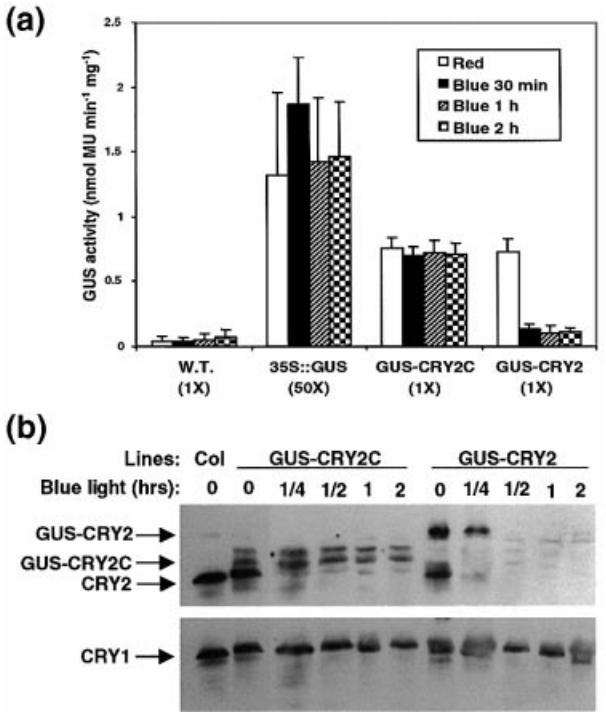

Figure 5. Expression of the GUS–CRY2 and GUS–CRY2C fusion proteins in response to blue light.
(a) GUS enzymatic activity of extracts prepared from different transgenic plants. All plants were grown in continuous red light (50 μmole m^{-2} s) for 10 days. The plants were then transferred to blue light (50 μmole m^{-2} s) for the indicated periods of time before the samples for GUS assay were taken. Specific GUS activities of the extract from different lines were shown.
(b) Immunoblot analysis of fusion proteins from transgenic plants grown under different light conditions. Arabidopsis plants were grown in continuous red light (50 μmole m^{-2} s) for 10 days. Plants were then exposed to blue light (50 μmole m^{-2} s) for 0 min, 15min, 30min, 1 h or 2 h, respectively. Tissues were collected from each sample for immunoblot analysis. The blot was probed first with the anti-CRY2 antibody, stripped and re-probed with the anti-CRY1 antibody.

GUS–CRY2 fusion protein can mediate blue-light induced inhibition of hypocotyl elongation of the transgenic plants

To test whether GUS–CRY2 fusion protein, which accumulated primarily in the nucleus, may be photobiologically active, we analyzed transgenic plants expressing GUS–CRY2 for the blue-light-induced hypocotyl inhibition response. As expected, GUS–CRY2C transgenic plants showed no obvious phenotypic changes in either the wild-type background (not shown) or the *cry1* mutant background (Figure 4). Expression of the GUS–CRY2 transgene in wild-type Arabidopsis also showed little phenotypic alternation (data not shown). However, expression of the GUS–CRY2 fusion protein in the *cry1* mutant background resulted in a short hypocotyl phenotype (compared to the *cry1* parent) in continuous blue light (Figure 4). When the GUC–CRY2 transgenic line and the parental *cry1* mutant line were grown under continuous red light or in the dark, no significant difference was found with respect to hypocotyl length (data not shown). Therefore, the GUS–CRY2 fusion protein can complement a *cry1* mutation. As shown in Figure 4, the 6-day-old GUS–

CRY2 transgenic seedlings grown under continuous blue light developed hypocotyls (approximately 8 mm) significantly shorter than that of the parental *cry1* mutant seedlings (approximately 14 mm). As a control, transgenic plants overexpressing the wild-type recombinant CRY2 protein had hypocotyls significantly shorter (approximately 3 mm) than those expressing the GUS–CRY2 fusion protein (Figure 4). It is not clear whether the difference between CRY2-overexpressing lines and GUS–CRY2 expressing lines was due to the lower photobiological activity of the GUS–CRY2 fusion protein or due to the relatively lower level of expression of GUS–CRY2. Nevertheless, these results clearly demonstrated that the GUS–CRY2 fusion protein could act as an active photoreceptor mediating the blue light inhibition of hypocotyl elongation.

Fusion of CRY2 to GUS confers a blue light regulation of abundance of the fusion protein

It is known that the abundance of CRY2 protein is down-regulated by blue light (Ahmad *et al.*, 1998a; Lin *et al.*, 1998). To test whether either of the fusion proteins is down-regulated in response to blue light, we examined the expression of GUS–CRY2 and GUS–CRY2C fusion proteins in the transgenic seedlings grown in continuous red light followed by a blue light treatment. It was noticed that the GUS activity detected in the GUS–CRY2 and GUS–CRY2C transgenic plants was less than 1/50 of the level detected in the plants expressing GUS (Figure 5a), which could be due to the lower enzymatic activity of the fusion proteins or to the lower level of expression of the fusion proteins. Figure 5(a) shows a significant decrease of the GUS enzymatic activity in the extract prepared from the GUS–CRY2 transgenic plants exposed to blue light for 30 min or longer. In contrast, this light-induced decrease in GUS enzymatic activity was not found in the extract prepared from either GUS or GUS–CRY2C expressing lines (Figure 5a). The immunoblot analysis indicated that the blue light-induced decrease of GUS enzymatic activity in the GUS–CRY2 expressing line was due to the decreased level of the GUS–CRY2 fusion protein (Figure 5b). Similar to that observed for the endogenous CRY2 protein, the abundance of the GUS–CRY2 fusion protein decreased to an undetectable level approximately 30 min after exposure of the transgenic plants to blue light (Figure 5b, GUS–CRY2). In contrast, the abundance of the GUS–CRY2C protein level remained relatively unchanged in response to blue light treatment (Figure 5b, GUS–CRY2C). These results demonstrated that, like the endogenous CRY2 protein, the abundance of GUS–CRY2 fusion protein was down-regulated in response to blue light illumination. It is interesting that CRY2 could confer a blue light-dependent regulation to the GUS–CRY2 fusion protein which was otherwise very stable *in vivo* (with a half-life of 50 h) (Jefferson *et al.*, 1987). The 35S:GUS–CRY2 transgene contains the CRY2-coding sequence and 69 bp of the 3′-untranslated region of *CRY2* (Figure 2b). However, neither the 3′-untranslated region nor the carboxyl domain of *CRY2* is likely to be involved in the regulation of CRY2 expression because these two sequences are also included in the 35S:GUS–CRY2C transgene, for which the expression is not affected by blue light (Figure 5). These results suggest that a protein-degradation mechanism may be responsible for the blue-light-induced down-regulation of CRY2 and that the N-terminal domain of CRY2 may be involved in the degradation of CRY2.

Discussion

We have demonstrated in this report that the Arabidopsis blue/UV-A light receptor cry2 is predominantly a nuclear protein. First, CRY2 is co-fractionated with the nuclear fraction. Second, CRY2 possesses a basic bipartite NLS at the C-terminal domain that is sufficient to target the GUS–CRY2C fusion protein to the nucleus. Finally, the fusion protein of GUS–CRY2, which exhibited the photoreceptor activity in mediating blue light-induced inhibition of hypocotyl elongation, was also found to accumulate in the nucleus. The discovery that cry2 is a nuclear protein is consistent with the function of this photoreceptor in the regulation of floral induction. cry2 has been previously shown to be required for the floral induction and regulation of expression of the flowering-time gene *CO* in response to long-day photoperiods (Guo *et al.*, 1999; Putterill *et al.*, 1995). The result that cry2 is a nuclear protein associated with regulation of gene expression prompts us to speculate that cry2 may act, directly or indirectly, upon the transcriptional apparatus in the nucleus. The action of cry2 has been demonstrated to be antagonistic to the function of phyB in the regulation of floral induction (Mockler *et al.*, 1999). Interestingly, the function of phyB, also a nuclear protein, has been suggested to be involved in the suppression of CO activity (Putterill *et al.*, 1995). The observation that cry2 and phyB are both nuclear proteins that interact antagonistically in the regulation of floral induction and CO expression (or activity) raises an interesting question of how these two photoreceptors might interact with each other. Recently, it has been reported that a different pair of photoreceptors, phyA and cry1, physically interact with one another and that phyA can phosphorylate cry1 *in vitro* (Ahmad *et al.*, 1998b), although the physiological significance of the phyA/cry1 interaction remains to be studied directly. It will be interesting to test whether an analogous interaction may exist between phyB and cry2 to account for the physiological interaction of these two photoreceptors.

In addition to its function in the regulation of floral induction, cry2 also mediates a blue light-induced inhibition of hypocotyl elongation. It has been demonstrated previously that cry1 could mediate a blue light-induced activation of anion channels, which may lead to plasma membrane depolarization and hypocotyl inhibition (Cho and Spalding, 1996; Parks *et al.*, 1998). In this regard, it is interesting that only a small fraction of CRY1 was found in the nuclear fraction prepared from plants grown in continuous white light (Figure 1). Presumably, the non-nuclear cry1 proteins could be in the cytosol, from which it may activate anion channels in the plasma membrane. On the other hand, CRY2 seemed to be predominantly in the nucleus, where it could not directly regulate the activity of plasma membrane proteins such as anion channels. It remains to be shown whether cry2, as well as other nuclear proteins such as HY5, which is also involved in the blue light-induced hypocotyl inhibition response (Koornneef *et al.*, 1980; Oyama *et al.*, 1997), may be associated with other cellular mechanisms regulating hypocotyl inhibition. For example, it would be interesting to know whether changes in gene expression are required for the induction or maintenance of light-induced inhibition of hypocotyl elongation.

The results presented in this report also demonstrated different functions of the two domains of CRY2. The C-terminal non-photolyase domain of CRY2 bears a nuclear localization signal which is sufficient for the nuclear compartmentation of CRY2 protein. We did not find obvious light regulation for CRY2 nuclear localization mediated by the CRY2 C-terminus because both GUS–CRY2 and GUS–CRY2C can be detected in the nucleus in plants grown in light or in dark. However, it remains to be examined whether CRY2 may be exported from the nucleus to be degraded in the cytosol in a light-dependent manner. Our results also indicated that CRY2 N-terminal domain (but not the C-terminal domain) is necessary for the blue-light-induced down-regulation of the abundance of CRY2 protein. Since there is little change in the *CRY2* mRNA level in response to blue light, a protein degradation mechanism has been hypothesized to be responsible for the blue-light-induced decrease of the CRY2 protein level (Lin *et al.*, 1998). The result that the abundance of the GUS–CRY2 fusion protein was down-regulated by blue light in a way very similar to that found for the endogenous CRY2 protein provided additional evidence in support of this hypothesis. Since the transgene encoding GUS–CRY2 and GUS–CRY2C fusion proteins had the identical sequences in the promoter, the 5′-untranslated region and the 3′-untranslated region (Figure 2), protein degradation seems to be the simplest interpretation for why the GUS–CRY2 fusion protein but not the GUS–CRY2C was down-regulated in blue light (Figure 5).

Experimental procedures

Plant materials

The wild-type Arabidopsis ecotype Columbia (Col) and the *cry1–301* (in Col background) mutant (Mockler *et al.*, 1999) were used for the transformation experiments. Plant growth conditions were as described previously (Guo *et al.*, 1998). The plasmids used for the transformation were prepared using standard procedures (Ausubel *et al.*, 1992). To facilitate preparation of the fusion protein constructs, a *Xho*1/*Xba*1 fragment containing the coding sequence of β-glucuronidase (GUS) was cloned from pRTL2 (Carrington *et al.*, 1991) into pBS(SK-) and referred to as pBS-GUS. Transgenes encoding fusion proteins of GUS and various portions of the *CRY2* gene were constructed in the T-DNA vector pKYLX (Schardl *et al.*, 1987) with the GUS coding region being at the N-terminus for all the fusion proteins. The complete coding region of CRY2 encoding 612 residues, the N-terminal 445 residues of CRY2, and the C-terminal 133 residues of CRY2, were fused in-frame to GUS to create GUS–CRY2, GUS–CRY2N and GUS–CRY2C, respectively. All the transgenes were under the control of a double CaMV 35S promoter. Transgenes do not contain the 5′ untranslated sequence of the *CRY2* gene. GUS–CRY2 and GUS–CRY2C contain the same 69 bp of the 3′ untranslated sequence of the *CRY2* gene. The respective transgene plasmids were transformed into both the wild-type (Col) and *cry1* (*cry1–301*) mutant using the vacuum infiltration method (Bechtold *et al.*, 1993). Seeds were harvested from individual plants and were referred to as T_1 lines. The progenies of the self-pollinated T_1 were referred to as T_2, and so on. The kanamycin-resistant transgenic lines were selected on agar plates containing MS nutrient salts and 50 mg l^{-1} kanamycin. Homozygous lines were identified by the analysis of kanamycin resistance of the progenies of respective T_2 lines. Light conditions and hypocotyl length measurements were as described previously (Guo *et al.*, 1998; Lin *et al.*, 1998). For the hypocotyl growth inhibition analysis, similar results were obtained from at least three independent lines for each construct, although the results from a single line from each are shown.

Nuclei isolation

Arabidopsis plants were grown in continuous white light (120 μmole m^{-2} s) for 3–4 weeks. Nuclei were isolated using the Percoll gradient centrifugation method essentially as described by Sakamoto and Nagatani (1996). Briefly, 30–50 g of leaves were harvested and homogenized in 400 ml of the nucleus isolation buffer (Jensen *et al.*, 1988) with a blender. The crude homogenate was filtered through two layers of Miracloth, and nuclei in the filtrate were collected by centrifugation at 1880 ***g***. The nuclear fraction was purified by Percoll gradient centrifugation (30–60% step gradient) at 5000 ***g*** for 25 min. The nuclear proteins were fractionated in 10% SDS-PAGE and subjected to the immunoblot analysis as described (Guo *et al.*, 1998; Lin *et al.*, 1998). The nuclei isolation was carried out under normal laboratory illumination conditions.

GUS analysis

The GUS enzymatic activity in transgenic Arabidopsis seedlings was determined using the standard protocol (Jefferson *et al.*, 1987). The relative activity was calculated by dividing the enzymatic activity of an individual sample by its total protein content determined using the Bio-Rad protein assay kit according to the manufacturer's instructions. For the histochemical analysis, 5- to 7-day-old transgenic Arabidopsis seedlings (conditions specified in figure legends) were fixed in 100 mM sodium phosphate, 2% formaldehyde, and 1 mM EDTA for 10 min with brief vacuum infiltration. The seedlings were stained for GUS activity and the nucleus was visualized using the DAPI fluorescence optics according to the method described previously (Von Arnim and Deng, 1994).

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Notes added in proof

It was reported recently (Cashmore *et al.*, 1999) that a GFP-CRY1 fusion protein is found in the nucleus in a biolistic transformation experiment on onion epidermal cells.

The paper *Nuclear localization of the Arabidopsis blue light receptor cryptochrome 2* by Oliver Kleiner, Stefan Kircher, Klaus Harter and Alfred Batschauer, which is based on independent research, reached the same conclusion as our work at the same time and is published on pages 289–296 in this issue of *The Plant Journal*.