Running title: Structure/function analysis of phy/cry interaction

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Title: Roles for the N- and C-terminal domains of phytochrome B in interactions between phytochrome B and cryptochrome signaling cascades.

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Abbreviations: CG, phyB C-terminal domain fused to green fluorescent protein; CG-NES, phyB C-terminal domain fused to green fluorescent protein and a nuclear export signal; cry1, cryptochrome 1; cry2, cryptochrome2; GFP, green fluorescent protein; GUS, β-glucuronidase; Ler, Landsberg erecta accession; MS, Murashige & Skoog, NES, nuclear export signal; NG-GUS-NLS, phyB N-terminal domain fused to green fluorescent protein, β-glucuronidase, and a nuclear localization signal; NLS, nuclear localization signal; PBG, full-length phyB fused to green fluorescent protein; phyA, phytochrome A; phyB, phytochrome B; PI, propidium iodide.

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Abstract

Plants fine-tune light responses through interactions between photoreceptors. We have previously reported that the greening of Arabidopsis thaliana roots is regulated synergistically by phytochromes and cryptochromes. In the present study, we investigated the functions of the N-terminal and C-terminal domains of phytochrome B (phyB) in the interactions between phytochrome B and cryptochrome signaling cascades. Transgenic Arabidopsis expressing the phyB N-terminal domain fused to GFP, GUS, and NLS showed intense root greening under blue light, indicating that the C-terminal domain was dispensable for the synergistic interaction in the induction of root greening. However, root greening under red light was substantially reduced in the absence of the C-terminal domain. This effect was opposite to the previous observation that removal of the C-terminal domain enhanced the signaling activity of phyB in the inhibition of hypocotyl elongation. In addition, we found that over-expression of the isolated C-terminal domain of phyB enhanced the blue light response not only for root greening but also for the inhibition of hypocotyl elongation. Analysis of this activity on various photoreceptor mutant backgrounds demonstrated that the isolated C-terminal domain enhanced cryptochrome signaling. In summary, these results demonstrate that different domains of phyB can play various roles which are dependent on light conditions as well as on the specific physiological response.

Keywords: cryptochrome, hypocotyl elongation, photomorphogenesis, photoreceptor interaction, phytochrome, root greening
Introduction

Light is one of the most important environmental cues for plants to make developmental decisions. Plants have evolved multiple photoreceptors for sensing environmental light conditions. To date, the red/far-red absorbing phytochromes and the blue/UV-A absorbing cryptochromes and phototropins have been identified as plant photoreceptors. Utilizing these photoreceptors, plants show various physiological responses to light stimuli that increase their fitness (Briggs and Huala 1999, Briggs and Christie 2002, Sullivan and Deng 2003).

Individual photoreceptors function independently of other photoreceptors in most cases. However, evidence indicates that complex interactions between signaling cascades of different photoreceptors exist (Mohr 1994, Casal 2000, Nemhauser and Chory 2002, Wang and Deng 2002). These interactions are believed to enable plants to fine-tune their response to light. For example, synergistic interactions between blue light photoreceptors and phytochromes have been reported repeatedly based on data from classical physiological experiments. A typical example is anthocyanin production. The maximal induction of anthocyanin in sorghum requires the activation of both a phytochrome and a blue light photoreceptor (Mohr 1994). These interactions are thought to contribute to the fine-tuning of the light responses.

Photoreceptor mutants have been instrumental in the analysis of the interactions between photoreceptors at the molecular level. Photoreceptor mutants of Arabidopsis have been used to demonstrate synergy between cryptochrome 1 (cry1) and phytochrome B (phyB) in the regulation of hypocotyl elongation and cotyledon unfolding under specific light conditions (Casal and Boccalandro 1995, Casal and
Mazzella 1998). In similar experiments, the lack of any one of three photoreceptors, phytochrome A (phyA), phyB or cry1, has been shown to result in a substantial reduction in anthocyanin accumulation under blue light (Poppe et al. 1998).

We have recently analyzed the induction of root greening by light in Arabidopsis (Usami et al. 2004). We found that blue light was far more effective at inducing chloroplast development in Arabidopsis roots than was red light. Analyses with photoreceptor mutants have revealed that either phyA or phyB, in addition to cryptochrome, is required for this response to blue light. Hence, phytochromes and cryptochromes act synergistically to regulate root greening under blue light. These responses are organ autonomous because excised root responded to light in a qualitatively similar manner (Usami et al. 2004), although the potoperceptive site for the regulation of root growth is the shoot (Canamero et al., 2006).

To explain those interactions, physical interactions between photoreceptors are hypothesized. Indeed, the cry1 and phyA proteins are known to directly interact through their C-terminal domains in the yeast two-hybrid assay. Furthermore, cry1 is phosphorylated by phyA-associated kinase activity in vitro (Ahmad et al. 1998). PhyB and cryptochrome 2 (cry2) have been shown to interact directly with each other in vivo, and they have been shown to be co-localized in nuclear speckles (Más et al. 2000). Although no evidence of a direct interaction between phyB and cry1 has been demonstrated thus far, both phyB and cry1 are known to physically interact with COP1 (Yang et al. 2001) and ADO1/ZTL/LKP1 (Jarillo et al. 2001). It is possible, therefore, that phyB and cry1 are contained in the same complex. However, the physiological significance of the physical interactions between photoreceptors remains obscure.
To understand the molecular mechanism of the interactions between phytochrome and cryptochrome signaling cascades, we have examined the activities of isolated N-terminal and C-terminal domains of phyB in blue light responses in vivo. For this purpose, we analyzed light responses in transgenic lines that expressed these domains separately on various photoreceptor mutant backgrounds. The results indicate that both the N-terminal and C-terminal domains participate in functional interactions during cryptochrome signal transduction in various ways depending on light conditions and on the final physiological responses.

**Results**

*NG-GUS-NLS can interact synergistically with the cryptochrome signaling cascade in the induction of root greening under blue light*

We have reported that phytochromes and cryptochromes synergistically induce root greening under blue light. Specifically, *Arabidopsis thaliana* roots exhibit intense greening only when both phytochromes and cryptochromes are activated (Usami et al. 2004). Using green fluorescent protein (GFP) as a transgene tracking marker, we first examined the activity of the N-terminal domain of phyB fused to GFP (NG), β-glucuronidase (GUS) and a nuclear localization signal (NLS) (NG-GUS-NLS) (Matsushita et al. 2003) (Fig. 1A) in this response. As a positive control, a line expressing the full-length phyB fused to GFP (PBG) (Yamaguchi et al. 1999) on the *phyAphyB* double mutant background was used.

The NG-GUS-NLS protein was expressed on the *phyAphyB* double mutant
background in Arabidopsis (Fig. 1B). The expression level of NG-GUS-NLS was much higher than that of the endogenous phyB but significantly lower than that of PBG, which was consistent with the previous observation (Matsushita et al. 2003).

Seedlings were grown for 10 d under blue or red light and the levels of chlorophyll in roots were determined (Fig. 1C). The NG-GUS-NLS roots accumulated chlorophyll to a much higher level than either PBG or wild-type under blue light. Hence, the NG-GUS-NLS protein could induce a high level of root greening if cryptochromes were activated at the same time. These results further indicate that the C-terminal domain of phyB is dispensable for the synergistic interaction between phyB and cryptochromes.

By contrast, root greening in red light, in which cryptochromes are not activated, was very low in NG-GUS-NLS compared with PBG or wild-type (Fig. 1C). Thus, contrary to the hypocotyl growth inhibition (see below), the C-terminal domain is required for normal root greening in red light. The transgenic plants that expressed the N-terminal 450 amino acid fragment fused to GFP, GUS and nuclear localization signal (Oka et al. 2004) exhibited similar responses (data not shown).

We then examined root greening in transgenic plants expressing the C-terminal domain of phyB fused to GFP (CG) (Matsushita et al. 2003) on the phyAphyB double mutant background (Fig. 1C). As expected, the expression level of CG in this line was much higher than that of the endogenous phyB (Fig. 1B). In contrast to NG-GUS-NLS, no root greening was observed under blue light even in the presence of cryptochromes. Hence, the lack of phyA and phyB was not complemented by CG for
the root greening under blue light. Taken together with the above results, we conclude that the C-terminal domain of phyB is not necessary for the synergistic interaction between phyB and cryptochromes to induce a high level of root greening.

**NG-GUS-NLS efficiently inhibits hypocotyl elongation under blue light**

Cryptochromes are primary photoreceptors that mediate light-induced inhibition of hypocotyl elongation under certain intensities of blue light (Casal and Mazzella 1998, Lin et al. 1998, Neff and Chory 1998, Poppe et al. 1998). In addition, phytochromes contribute to this response independently from cryptochromes (Casal and Mazzella 1998, Neff and Chory 1998). This is not surprising because phytochromes, which are generally considered to be receptors for red light, also absorb blue light.

We examined how NG-GUS-NLS responded to blue light for the inhibition of hypocotyl elongation. Hypocotyl lengths were determined under blue light of two different intensities, 2.6 and 13 µmol m⁻² s⁻¹ (Fig. 2A). In the absence of phyA, phyB mediated the hypocotyl response. This was particularly evident in the response to strong blue light (phyAphyB vs phyA). The PBG response was similar to that of phyB (phyA vs PBG 18-5 (phyAphyB)). NG-GUS-NLS was much more effective in inhibiting hypocotyl elongation under blue light (phyA or PBG 18-5 (phyAphyB) vs NG-GUS-NLS 4-1 (phyAphyB)). The NG-GUS-NLS seedlings were very short even under weak blue light. This difference could not be explained as a function of transgene expression levels because PBG protein accumulated to a higher level than NG-GUS-NLS protein in
the lines used (Fig. 1B). These results indicate that the C-terminal domain was not required for the inhibition of hypocotyl elongation by phyB under blue light.

**CG enhances the blue light response in both roots and hypocotyls**

The isolated C-terminal domain expressed on the wild-type background inhibits the function of endogenous phyA in the inhibition of hypocotyl elongation under continuous far-red light, but does not affect phyB mediated hypocotyl growth inhibition under red light (Sakamoto and Nagatani 1996, Wagner et al. 1996). We examined whether CG affected root greening on the wild-type background. Under red light, the level of root greening was reduced in CG seedlings compared with the wild-type (Fig. 1D). Nevertheless, the level was increased in CG seedlings under blue light. Hence, the over-expressed CG appeared to enhance the synergistic interaction between phyB and cryptochromes in blue light but to suppress the activity of phyB in red light.

We then asked whether or not a blue light response other than root greening was affected by CG expression. Towards this end, we examined the inhibition of hypocotyl elongation by blue light in CG seedlings. The hypocotyl lengths in the CG seedlings under blue light of two different intensities, 3.5 and 17 µmol m$^{-2}$ s$^{-1}$ were determined (Fig. 2B). The CG seedlings were significantly shorter than the wild-type especially under stronger blue light. Hence, CG enhanced the inhibition of hypocotyl elongation by blue light. Similar effects were observed in lines that over-expressed the phyB C-terminal domain without GFP (data not shown).
**Cryptochrome signal is enhanced in CG lines**

The above results suggested that the response to blue light was enhanced by the over-expression of CG. We further investigated whether this enhancement depended on the presence of specific photoreceptors (Fig. 3). We first examined the hypocotyl lengths in the \textit{phyA}, \textit{phyB}, \textit{phyAphyB}, \textit{cry1}, \textit{cry2} and \textit{cry1cry2} mutants of Arabidopsis under a high fluence of blue light (17 \(\mu\text{mol m}^{-2} \text{s}^{-1}\) blue light). Consistent with previous reports (Casal and Mazzella 1998, Neff and Chory 1998, Poppe et al. 1998), the \textit{cry1} single and \textit{cry1cry2} double mutants as well as the \textit{phyAphyB} double mutant exhibited the long hypocotyl phenotype. Hence, both cryptochromes and phytochromes contributed to the response. It should be noted that residual responses were observed even in the \textit{phyAphyB} and the \textit{cry1cry2} double mutants. This observation was in contrast with root greening, in which the response was completely lost in the \textit{phyAphyB} and the \textit{cry1cry2} double mutants (cf. Fig. 1C, Usami et al. 2004).

We then examined the effects of CG expression on hypocotyl elongation under blue light on the photoreceptor mutant backgrounds (Fig. 3). The CG protein effectively reduced the hypocotyl lengths on the \textit{phyA}, \textit{phyB}, \textit{phyAphyB}, \textit{cry1} and \textit{cry2} mutant backgrounds. The hypocotyls were about 20-40\% shorter in the CG lines compared with their respective parental mutant lines. Hence, CG effectively enhanced the response even in the absence of \textit{phyA} and \textit{phyB}. By contrast, CG failed to enhance the response on the \textit{cry1cry2} double mutant background. Therefore, either \textit{cry1} or \textit{cry2} was required for CG to enhance the response to blue light. Hence, CG appeared to enhance the \textit{cry1} and \textit{cry2} signals but not the of \textit{phyA} or \textit{phyB} signals.
Cry2 promotes flowering in response to blue light (Guo et al. 1998, Mockler et al. 1999). Hence, we examined whether CG affected flowering time (Fig. 4). We tested flowering time in the CG 4-4 line, in which the highest CG expressing was observed among the lines established. Contrary to our expectation, neither advancement nor delay of flowering was observed in the CG 4-4 line regardless of the day length. Thus, the enhancement of cryptochrome signals by CG was not observed for all cryptochrome responses.

Since CG enhanced cryptochrome signals, we examined whether the levels of cry1 and cry2 proteins were altered in CG seedlings. Proteins were separately extracted from the aerial parts and the roots of the seedlings and subjected to immunoblot analyses with anti-cry1 and anti-cry2 antibodies. The levels of cry1 were not altered in the CG seedlings (Fig. 5A). In the case of cry2, the protein was not detected in any of the extracts (Fig. 5B). This was probably due to the instability of the cry2 protein under blue light (Lin et al. 1998). Hence, the enhancement of cryptochrome signals was not due to alterations in the cryptochrome levels. We also confirmed that the levels of phyA and phyB were not altered in the CG seedlings (data not shown).

**Neither Nuclear localization nor speckle formation of the CG protein was required for the enhancement of cryptochrome signals**

The CG protein is primarily localized to the nucleus and is only faintly detected in the cytoplasm regardless of the light conditions (cf. Fig. 6B, Matsushita et al. 2003). It forms speckles in the nucleus. Interestingly, cryptochromes are also localized mainly to the nucleus (Cashmore et al. 1999, Guo et al. 1999, Kleiner et al. 1999). To determine
whether nuclear localization was required for the enhancement of cryptochrome signals by CG, we expressed the CG protein fused to a nuclear export signal (NES) (CG-NES) on the wild-type background in Arabidopsis. The protein levels in these lines were comparable to those in the CG lines (Fig. 6A). The CG-NES protein was localized exclusively in the cytoplasm and never formed nuclear speckles under either white (Fig. 6B) or blue light (data not shown). Surprisingly, the hypocotyls of the CG-NES lines were almost as short as those of the CG lines (Fig. 6C). Hence, stable nuclear retention of CG was not required for the enhancement of the cryptochrome signals.

Discussion

The N-terminal domain alone effectively responds to blue light

We have recently shown that phytochromes and cryptochromes act synergistically to induce intense root greening under blue light in Arabidopsis (Usami et al. 2004). As shown in Fig. 1, NG-GUS-NLS induced intense root greening under blue light on the phyAphyB double mutant background. The level of greening was even higher than that in PBG. Hence, the C-terminal domain was totally dispensable for the synergistic interaction of the phyB signaling cascade with that of cryptochromes.

NG-GUS-NLS seedlings efficiently responded to blue light for the inhibition of hypocotyl elongation as well (Fig. 2A). Under continuous blue light, phyA and phyB inhibit hypocotyl elongation independently from cryptochromes (Casal and Mazzella 1998, Neff and Chory 1998). NG-GUS-NLS inhibited hypocotyl elongation more efficiently than did PBG (Fig. 2A). We further confirmed that NG-GUS-NLS could inhibit hypocotyl elongation in the absence of cry1 (data not shown).
Taken together, the data are consistent with the notion that the C-terminal domain attenuates the activity of phyB under blue light in the full-length context. This view is consistent with the previous idea that the C-terminal domain attenuates phyB activity under red light (Matsushita et al., 2003). It should be noted that the root and hypocotyl responses are very different with respect to the synergistic interaction with the cryptochrome signaling cascade. The root response exhibits strong synergy between phytochrome and cryptochrome signaling pathways (Usami et al. 2004), whereas the hypocotyl response is mediated independently by these pathways (Casal 2000). Hence, the enhanced responses observed in NG-GUS-NLS under blue light most likely reflect an increased activity of NG-GUS-NLS per se rather than an increased interaction with the cryptochrome signaling cascade.

**Effects of the C-terminal domain under red light**

An increased sensitivity to red light by NG-GUS-NLS has been observed for the regulation of hypocotyl elongation (Matsushita et al. 2003). Nevertheless, root greening under red light was substantially reduced in NG-GUS-NLS compared with the wild-type (Fig. 1C). Hence, the C-terminal domain appears to act positively in the induction of root greening under red light. This discrepancy might be explained in part by the difference in the organs/tissues in which the response takes place. We have previously shown that root greening caused by both red and blue light is organ autonomous using excised roots (Usami et al. 2004). It is possible that signal transduction mechanisms are different in these two organs, even though gene expression profiles in the root and the whole seedling are similar (Molas et al. 2006). One fascinating possibility here is that cryptochromes modify the phyB function particularly in the root. Namely,
cryptochromes may enhance the phyB function through the interaction with the C-terminal domain under red light in the root.

Interpretation of the results of CG lines is more difficult. The CG fragment can not respond to light by itself because it lacks a chromophore. Accordingly, CG did not complement the loss of phyA and phyB for the induction of root greening (Fig. 1C). Nevertheless, it could interfere with the functions of endogenous phytochromes either positively or negatively. Indeed, expression of CG (or the C-terminal domain of phyB) affected the hypocotyl and the root responses. The CG protein reduced root greening under red light on the wild-type background (Fig. 1D), although the hypocotyl elongation under red light was not affected by the over-expression of the C-terminal domain of phyB (Sakamoto and Nagatani 1996, Wagner et al. 1996). Heterodimerization between CG and the endogenous phytochromes may be involved in such an effect. This hypothesis is tenuous, however, because phyB was not co-immunoprecipitated with CG even in the root extracts (data not shown).

*The isolated C-terminal domain enhances cryptochrome signals*

The C-terminal domain was dispensable for the synergistic interaction between phyB and cryptochromes in root greening (see above). Nevertheless, the responses to blue light were enhanced by the over-expression of CG on the wild-type background (Figs. 1D, 2B). Analysis on different mutant backgrounds indicated that enhancement was observed only if cry1 or cry2 was functional (Fig. 3). Hence, CG most likely enhanced cryptochrome signaling in the regulation of hypocotyl elongation. We confirmed that CG did not alter protein levels of cry1 and cry2 (Fig. 5). However,
flowering was not affected in the same lines (Fig. 4), indicating that the enhancement by CG was observed only for a subset of cryptochrome responses.

It was unexpected that both CG and CG-NES effectively enhanced the blue light response (Fig. 6C). These proteins exhibited markedly different patterns of intracellular distribution (Fig. 6B). One possible explanation is that CG-NES transiently functions in the nucleus, where the endogenous cryptochromes are mainly localized (Cashmore et al. 1999, Guo et al. 1999, Kleiner et al. 1999). Because NES does not prevent protein entry into the nucleus, CG-NES would transiently exist in the nucleus. Consequently, CG-NES might be able to interact with nuclear factors transiently. Alternatively, CG and CG-NES may interact with a factor that localizes both in the cytoplasm and in the nucleus. For example, COP1 protein, which is a key negative regulator of photomorphogenesis, is known to be partitioned into the nucleus and the cytoplasm depending on the light conditions (von Arnim and Deng 1994).

*Does the C-terminal domain of phyB enhance cryptochrome signaling in the full-length context?*

Although the present work suggests that the isolated C-terminal domain of phyB enhances cryptochrome signals, it remains unclear if this activity is in effect in the full-length context. As shown in Fig. 3, under 17 μmol m\(^{-2}\) s\(^{-1}\) blue light, hypocotyls of the $phyApphyB$ double mutant were significantly taller than those of the wild-type. Hence, either phyA or phyB was required for the full response to blue light. Expression of the CG protein on the $phyApphyB$ mutant background partially complemented this phenotype.
However, this does not necessarily mean that the endogenous phyA and phyB enhanced the cryptochrome signals through their C-terminal domains.

It has been proposed that phytochromes and cryptochromes independently inhibit hypocotyl elongation under continuous blue light (Casal, 2000). Indeed, hypocotyl length was reduced by about 35% compared with the dark control in the cry1cry2 double mutant (Fig. 3), which indicated that phytochromes could inhibit the hypocotyl elongation in the absence of cryptochromes. Conversely, hypocotyl length was reduced by about 45% in the absence of phyA and phyB. This inhibition most likely represented the effect of cryptochromes without the aid of phytochromes. In wild-type, the hypocotyl length was reduced by about 80% compared with the dark control. Hence, the inhibition could be explained by additive effects of phytochromes (35%) and cryptochromes (45%).

At present it remains unclear whether, in the full-length context, the C-terminal domain of phyB enhances cryptochrome signaling. As discussed above, it is difficult to prove such an activity because of the intrinsic activity of phytochromes as blue light photoreceptors. Future experiments with mutated phyB, such as a phyB mutant that fails to bind a chromophore (e.g., C357/A, Kircher et al. 1999) might help to answer the question.

Physical interactions between phyB and cryptochromes

The present work demonstrates that both the N-terminal and the C-terminal fragments of phyB affect the cryptochrome induced signaling cascades. In brief, NG-
GUS-NLS synergistically induced intense root greening with cryptochromes (Fig. 1C) where as CG enhanced the endogenous cryptochrome signals (Figs. 1D, 2B and 3). It is intriguing to speculate that direct physical interactions underlie these observations. Indeed, physical interactions between phytochromes and cryptochromes have been reported (Ahmad et al. 1998, Más et al. 2000). However, we could not confirm such interactions. Neither cry1 nor cry2 was co-precipitated with PBG, NG-GUS-NLS or CG proteins in the transgenic lines used in the present study (data not shown). Más et al (2001) have reported that cry2 is co-precipitated with phyB, but they used a cry2 over-expresser in that experiment. It remains possible that the physical interaction between these photoreceptors is relatively weak and difficult to detect by the co-immunoprecipitation assay. Alternatively, the apparent interactions between phyB and cryptochromes may reflect the interactions between downstream components or signaling cascades.

Speckles in the nucleus are formed not only by phytochromes (Kircher et al. 1999, Yamaguchi et al. 1999) but also by signaling factors such as COP1 (Seo et al. 2004) and PIF3 (Bauer et al. 2004). Most relevantly, cry2 and phyB have been shown to co-localize in nuclear speckles when they are over-expressed in the same cell (Más et al. 2000). However, functional relevance of those speckles still remains unclear. Contrary to the above view, NG-GUS-NLS and its derivatives transduce signals but do not form speckles (Matsushita et al. 2003, Oka et al. 2004). Furthermore, NG-GUS-NLS exhibited a strong synergism with cryptochromes (Fig. 1C) without forming speckles in the root cells (data not shown). In addition, CG-NES, which did not form speckles, enhanced the blue light response (Fig. 6). Hence, the speckles might not be important for the interactions between phyB and cryptochrome induced signaling cascades.
Materials and Methods

Plant materials, growth conditions, and growth measurements

The wild-type *Arabidopsis thaliana* used in this study was Landsberg erecta accession (Ler). The mutant alleles used were phyA-201 (Nagatani et al. 1993), phyB-5 (Reed et al. 1993), *cry1-2.23N* (Ahmad and Cashmore 1993), *cry2* (*fha-1*) (Koornneef et al. 1991), *phyA-201phyB-5*, and *cry1-2.23Ncry2* (*fha-1*). The PBG 18-5, NG-GUS-NLS 4-1 and CG 19-2 lines (Matsushita et al. 2003) on the *phyA-201phyB-5* background were established by genetic crossing (Oka et al. 2004). All mutants and transgenic plants were on the Ler background.

Seeds were surface-sterilized and sown on 0.6% agar plates containing Murashige & Skoog (MS) medium with or without sucrose. The plates were kept in darkness at 4°C for 3 to 5 d and then irradiated with white light for 3 h at 23°C to induce germination. For root greening and immunochemical analysis, seedlings were grown on MS agar plates with 2% sucrose for 10 d. Chlorophyll contents in roots were quantified as previously described (Usami et al. 2004). For hypocotyl measurement, seedlings were grown on MS agar plates without sucrose for 5 d at 23°C. Hypocotyl lengths were measured as described (Oka et al. 2004). Flowering time was measured as described (Endo et al. 2005).
**Light sources**

Monochromatic red light was obtained from red fluorescent tubes (FL20S R-F, National, Tokyo) filtered through a 3 mm red acrylic filter (Shinkolite A102, Mitsubishi Rayon, Tokyo). The blue light source was a light-emitting diode array (peak at 470 nm, 25 nm half-width) (KML400300, Moritex Corporation, Tokyo). Far-red light was obtained by a combination of fluorescent tubes (FL20S FR-74, TOSHIBA, Tokyo) and a 3 mm methacrylic plate (Delaglass A-900, Asahi Chemical Industry, Tokyo). The fluence rates were measured with an optical power meter (DataLogger LI-1000, Li-Cor, Lincoln, NE).

**Plasmid construction and plant transformation**

Cauliflower mosaic virus 35S promoter, CG and Nos terminator had been cloned in binary vector pPZP211 (pPZIP211/35S::CG::nosT) (Matsushita et al. 2003). 35S::CG::nosT fragment was transferred to pPZP221. The CG moiety of this plasmid was replaced by a PCR-amplified C-terminal domain of phyB without GFP to obtain pPZP221/35S::C::nosT. NES from PKI (Wen et al. 1995) was fused to CG of pPZP211/35S::CG::nosT (pPZIP211/35S::CG-NES::nosT). Wild-type and photoreceptor mutants were transformed with the plasmids described above by Agrobacterium tumefaciens -mediated floral dip method (Clough and Bent 1998). The transformants were selected on MS agar plates containing 166 mg/liter claforan (Hoechst) and 25 mg/liter kanamycin (pPZP211/35S::CG::nosT and pPZP211/35S::CG-NES::nosT) or 50 mg/liter gentamycin (pPZP221/35S::C::nosT). For each construct, several T3 homozygous lines that showed approximately 3:1 segregations for drag-resistance in the
T2 generation were selected. Protein expression levels in these lines were checked by immunoblotting, and two independent lines with higher levels were selected.

Western blotting

To separate the roots and the aerial parts of seedlings, 10-day-old seedlings were cut using micro-scissors under a stereoscopic microscope. Roots or aerial parts of seedlings were collected, weighed and frozen in liquid nitrogen. Frozen samples were ground on ice in the presence of extraction buffer (90 mM Tris-HCl, pH8.3, 4.5 mM EDTA, 10% glycerol) containing 1/10 (v/v) of protease inhibitor cocktail for general use (Sigma, Saint Louis, MO) at a volume-to-fresh weight ratio of 1:1. Cell debris was removed by centrifugation at 10,000 rpm. These steps were carried out under a dim green safe-light (Nagatani et al. 1989). Total protein concentration of the crude extract was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). The crude extracts were electrophoresed in SDS-PAGE gel and blotted onto nitrocellulose membrane. The monoclonal antibodies used were mBA1 and mBA2, which are specific to N-terminal and C-terminal domains of phyB, respectively (Shinomura et al. 1996). The anti-cry1 and anti-cry2 polyclonal antibodies (Lin et al. 1996, Lin et al. 1998) were kind gifts from Dr. Chentao Lin.

Observation of GFP fluorescence in transgenic seedlings

Seedlings of CG lines and CG-NES lines grown under white light (44 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)) were stained with 20 \( \mu \text{g/ml} \) propidium iodide (PI) (Invitrogen) to visualize cell walls and nuclei. The subcellular localization of GFP and PI fluorescence was observed using a
confocal microscope (FLUOVIEW FV300, Olympus, Tokyo) with EGFP channel and PI channel, respectively. We observed fluorescence in different parts of seedlings in two independent lines and obtained similar results. The root cells are shown as representatives.

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References


Legends to figures

Fig. 1  Root greening in transgenic plants that express phyB derivatives. (A) Diagrams of phyB derivatives. (B) Protein levels of phyB and its derivatives in wild-type and transgenic plants. Total proteins were extracted from seedlings grown under white light for 10 d. The same amount of total protein was loaded to each lane. Immunoblots stained with the antibody against the N-terminal (left) or the C-terminal domain (right) of phyB are shown. Arrows indicate the positions of endogenous phyB. The closed, open and shaded arrowheads indicate the positions of PBG, NG-GUS-NLS and CG proteins, respectively. Relative densities of bands are indicated at the bottom of the panels. (C) Root greening in the wild-type (Ler), the phyAphyB mutant, and transgenic plants expressing PBG, NG-GUS-NLS or CG on the phyAphyB background under continuous blue (12 µmol m\(^{-2}\) s\(^{-1}\)) (left) or red (15 µmol m\(^{-2}\) s\(^{-1}\)) (right) light for 10 d. (D) Root greening in the CG lines on the wild-type background under continuous blue (left) or red (right) light. Averages ± SD are indicated.

Fig. 2  (A) Hypocotyl lengths in PBG and NG-GUS-NLS seedlings on the phyAphyB double mutant background under blue light. Seedlings were grown under blue light or darkness for 5 d. Hypocotyl lengths relative to dark controls are shown. Open and shaded bars represent the lengths under weak (2.6 µmol m\(^{-2}\) s\(^{-1}\)) and strong (13 µmol m\(^{-2}\) s\(^{-1}\)) blue light, respectively. Absolute hypocotyl lengths in darkness were 11.06 ± 0.52 mm (Ler), 13.43 ± 0.41 mm (phyAphyB), 10.90 ± 0.03 mm (phyA), 11.04 ± 0.29 mm (PBG 18-5), and 9.77 ± 0.47 mm (NG-GUS-NLS 4-1). Averages ± SE (n = 16-25) are indicated. (B) Hypocotyl lengths in CG lines on the wild-type background under blue
light. Seedlings were grown under blue light or darkness for 5 d. Hypocotyl lengths relative to dark controls are shown. Open and shaded bars represent the lengths under weak (3.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and strong (17 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) blue light, respectively. Absolute hypocotyl lengths in darkness were 8.97 ± 0.03 mm (Ler), 9.25 ± 0.04 mm (CG 4-4), and 9.90 ± 0.02 mm (CG9-5). Averages ± SE (n = 20-25) are indicated.

**Fig. 3** Hypocotyl lengths in CG lines on photoreceptor mutant backgrounds. Seedlings were grown under blue light (17 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) for 5 d. The genetic background of each line is indicated in parenthesis. Hypocotyl lengths are indicated as relative values to those of the dark controls. Averages ± SE (n = 18-26) are indicated.

**Fig. 4** Flowering times in wild-type (Ler), cry2 mutant and CG 4-4 lines. Plants were grown under long day (LD; 16 h light/8 h dark, 52 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) or short day (SD; 8 h light/16 h dark, 105 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). Flowering time was measured by counting the number of rosette leaves upon the opening of first few flowers. Averages ± SE (n = 5-6) are indicated.

**Fig. 5** Protein levels of cry1 and cry2 in wild-type and the CG 4-4 line. Total protein was extracted from the aerial parts (lanes 1 and 3) or roots (lanes 2 and 4) of seedlings grown under blue light (12 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) for 10 d. The same amount of total protein was loaded to each lane. Immunoblots stained with anti-cry1 (A) or anti-cry2 (B) antibody are shown. Lanes 1 and 2, wild-type; lanes 3 and 4, CG4-4. The arrowhead in (A) indicates the specific bands for cry1. Relative densities of bands were indicated at the bottom of the panels. Cry2 was not detected in this condition. nd, not detected.
Fig. 6  Analysis of CG and CG-NES seedlings. (A) Protein levels of CG and CG-NES. Total protein was extracted from 5-day-old seedlings grown under white light (44 µmol m\(^{-2}\) s\(^{-1}\)). The same amount of total protein was loaded to each lane. The immunoblot was stained with the mBA1 monoclonal antibody raised against the C-terminal domain of phyB. The arrow indicates the bands of the endogenous phyB. Closed and open arrowheads indicate CG and CG-NES proteins, respectively. (B) Subcellular localization of CG and CG-NES. Seedlings grown under white light for 5 d were stained with PI to visualize cell walls and nuclei. PI (left) and GFP (right) fluorescence in the same view is σηων. Αρροωηεαδσ ινδιχατε νυχλει. (Χ) Ηψποχοτψλ λενγτησ ιν τηε ΧΓ ανδ ΧΓ−ΝΕΣ λινεσ ον τηε ωιλ−τψπε βαχκγρουνδ. Σεεδλινγσ οερε γροον υνδερ βλυ ιν τηε ωιλ−τψπε υνδερ βλυ ε λιγητ (17 µmol m\(^{-2}\) s\(^{-1}\)) for 5 d. Hypocotyl lengths are presented as relative values to those of dark controls. Averages ± SE (n = 24-25) are indicated.
Figure 1
Figure 2
Figure 3

Figure 4
Figure 6