Light Quality–Dependent Nuclear Import of the Plant Photoreceptors Phytochrome A and B

Stefan Kircher,[a] Laszlo Kozma-Bognar,[b] Lana Kim,[a] Eva Adam,[b] Klaus Harter,[a] Eberhard Schäfer,[a] and Ferenc Nagy[b,1]

[a] Institut für Biologie II/Botanik, Universität Freiburg, Schänzlestrasse 1, D-79104 Freiburg, Germany
[b] Institute of Plant Biology, Biological Research Center, P.O. Box 521, H-6701 Szeged, Hungary

The phytochrome (phy) family of plant photoreceptors controls various aspects of photomorphogenesis. Overexpression of rice phyA–green fluorescent protein (GFP) and tobacco phyB–GFP fusion proteins in tobacco results in functional photoreceptors. phyA–GFP and phyB–GFP are localized in the cytosol of dark-adapted plants. In our experiments, red light treatment led to nuclear translocation of phyA–GFP and phyB–GFP, albeit with different kinetics. Red light–induced nuclear import of phyB–GFP, but not that of phyA–GFP, was inhibited by far-red light. Far-red light alone only induced nuclear translocation of phyA–GFP. These observations indicate that nuclear import of phyA–GFP is controlled by a very low fluence response, whereas translocation of phyB–GFP is regulated by a low fluence response of phytochrome. Thus, light-regulated nucleocytoplasmic partitioning of phyA and phyB is a major step in phytochrome signaling.

INTRODUCTION

As sessile organisms, plants have achieved an enormous developmental plasticity. To monitor environmental variations, such as temperature, nutrition, and light, plants have developed several sensory systems. Among these exogenic factors, light is probably the most variable and dominating factor affecting plant development. To perceive changes in the temporal and spatial patterns of the light environment, red/far-red photoreversible phytochromes, blue/UV light photoreceptors, and UV-B photoreceptors have evolved (Kendrick and Kronenberg, 1994). In many cases, the responses have been shown to involve the regulation of the transcription of specific genes (Fankhauser and Chory, 1997).

Members of the phytochrome (phy) family are the best-characterized plant photoreceptors thus far. They are soluble chromoproteins with a monomeric molecular mass of ~120 kD. In vitro and in vivo, they exist as dimers, covalently linked to their chromophore, a linear tetrapyrrole, by a thioether bond (Furuya and Song, 1994). The holoprotein is synthesized in the dark in its physiologically inactive, red light–absorbing Pr form. After absorption of a photon, this inactive form is photoconverted into its physiologically active, far-red light–absorbing Pfr form, which, in turn, is transformed back into the Pr form upon absorption of far-red light (Schäfer et al., 1972). Phytochromes are encoded by a small

multigene family. In Arabidopsis, five members have been described (PHYA to PHYE; Mathews and Sharrock, 1997). The best characterized of these are phyA and phyB. The light-labile phyA molecule is the most abundant phytochrome in dark-grown plants (Clough and Viestra, 1997). It has been shown to be the sensor for very low fluence responses and for absorption of continuous far-red light (Furuya and Schäfer, 1996; Whitelam and Devlin, 1997).

The phyB molecule is responsible for the photoperception of red light and functions as a classical red/far-red light reversible molecular switch (Quail et al., 1995; Furuya and Schäfer, 1996; Whitelam and Devlin, 1997). The abundance of the light-stable phyB protein is ~50 times lower than that of the phyA protein in dark-grown plants. Until recently, immunolocalization studies have yielded ambiguous results regarding the subcellular localization of these photoreceptors (reviewed in Quail et al., 1995). For example, Mösinger et al. (1987) reported that the transcription rate of chlorophyll a/b binding protein genes in isolated barley nuclei is stimulated by the addition of purified oat phyA protein, an observation that contradicts the results of Speth et al. (1986), who found that in oat coleoptiles, phyA is localized exclusively in the cytosol and forms sequestered areas of phytochrome after irradiation. Nagatani et al. (1988) have confirmed this latter observation and showed that association of phyA with nuclei is nonspecific. On the other hand, Sakamoto and Nagatani (1996) have more recently reported that there are higher amounts of immunodetectable phyB in nuclear preparations isolated from light-grown Arabidopsis seedlings

than in nuclei from dark-adapted seedlings. Moreover, these authors also analyzed the localization in transgenic plants of a fusion protein that contained the C-terminal region of the Arabidopsis phyB protein fused to β-glucuronidase. They showed that this fusion protein is imported into the nuclei, an observation that indicates the presence of a functional nuclear localization sequence (NLS) in the C-terminal region of Arabidopsis phyB.

The photoreceptors that have been characterized thus far, namely, phyA, cryptochrome 1 (CRY1), and phototropin (nonphototropic hypocotyl 1; NPH1), with the possible exception of phyB, seem to be localized in the cytosol or associated with the plasma membrane (Speth et al., 1986; Lin et al., 1996; Huala et al., 1997). Therefore, transduction cascades resulting in changes of the expression of light-regulated genes are likely to include at least one cytosol-to-nucleus signaling step. Accordingly, light-controlled translocation of the basic leucine zipper (bZIP) transcription factors, that is, the blue light–mediated nuclear localization of G-box binding factor 2 (GBF2) in soybean cells (Terzaghi et al., 1997) and red/far-red reversible control of the nuclear transport of common plant regulatory factor 2 (CPRF2) in parsley cell suspension cultures (Kircher et al., 1999), have been reported. In addition to light-regulated nuclear import of transcription factors, a light-controlled nuclear depletion of the constitutive photomorphogenic 1 (COP1) protein, which is a central negative regulator of plant photomorphogenesis, also has been reported (Wei and Deng, 1996; Torii and Deng, 1997).

In this study, we provide evidence that light quality–dependent nuclear transport of photoreceptors phyA and phyB is likely to be a critical event in photomorphogenesis. We used the green fluorescent protein (GFP) as a reporter to monitor in transgenic plants the subcellular localization of these photoreceptors under various light conditions. As a central observation, we report that photobiologically functional phyA–GFP and phyB–GFP fusion proteins are transported from the cytosol into the nucleus in a light quality– and chromophore-dependent manner. Furthermore, we suggest that the specific, spotted patterns of phyA and phyB localization in the nucleus are indications of the association of phyA–GFP and phyB–GFP in multiprotein complexes.

RESULTS

phyA–GFP and phyB–GFP Fusion Proteins Are Functional Photoreceptors in Transgenic Tobacco Plants

We first tested the rice phyA–GFP (phyA–GFP) and tobacco phyB–GFP (phyB–GFP) fusion proteins, which were intended to be used in subcellular localization studies, for biological activity in transgenic tobacco. Transgenic plants were produced via Agrobacterium-mediated transformation and contained either the rice *PHYA* cDNA or the tobacco SR1 *PHYB* cDNA fused to the modified *GFP4* (*mGFP4*; Haseloff et al., 1997) reporter gene (Figure 1A). The expression of these transgenes was driven by the cauliflower mosaic virus 35S promoter (Benfey et al., 1990a, 1990b). Expression levels of these transgenes in the transgenic plants were determined by protein gel blot analysis using antibodies specific for phyB or monocotyledonous phyA.

Figure 1B shows that in transgenic lines selected for further study, the expression of the phyA–GFP (lane 2) or phyB–GFP (lane 4) fusion proteins is clearly detectable. The expression level of phyB–GFP is comparable to that of endogenous tobacco phyB (Figure 1B, lanes 3 and 4) protein. The majority of transgenic lines exhibited a characteristic phyA or phyB overexpression phenotype, as described for transgenic Arabidopsis plants expressing homologous (Wagner et al., 1991; Quail et al., 1995) and transgenic tobacco plants expressing heterologous *PHYA* or *PHYB* genes (Halliday et al., 1997). Figure 1C shows in detail that a transgenic plant expressing phyA–GFP and irradiated with continuous white light exhibited a strong inhibition of internode elongation compared with wild-type plants, whereas Figure 1D demonstrates a fluence rate–dependent inhibition of hypocotyl growth in transgenic seedlings expressing phyB–GFP under continuous red light irradiation. Taken together, these observations reflect typical physiological changes characteristic for plants expressing phyA and phyB and demonstrate the functional integrity of the rice phyA–GFP and tobacco phyB–GFP fusion proteins as photoreceptors.

phyA–GFP and phyB–GFP Fusion Proteins Are Localized in the Nucleus in Light-Grown Tobacco Plants

Transgenic tobacco plants expressing rice *PHYA–mGFP4* or tobacco *PHYB–mGFP4* transgenes were grown under light and dark cycles in a growth chamber. Intracellular localization of the phyA–GFP and phyB–GFP fusion proteins was first monitored by fluorescence microscopy during the light phase. In the case of phyA–GFP, the green fluorescence indicative of the presence of the fusion protein was detected in the nuclei and, although to a lesser extent, in the cytosol of several cell types, such as epidermal, guard, and trichrome cells (Figures 2A to 2F). Interestingly, distribution of the fluorescence signal was not homogeneous within the nuclei and cytosol but showed a characteristic spotted pattern that was concentrated in numerous small speckled areas.

In the case of phyB–GFP, the green fluorescence also was readily detectable in the nuclei of various cell types, including, once again, stomatal guard and hypocotyl cells as well as root hairs (Figures 2G to 2L). Nuclear localization of phyB–GFP was also evident in epidermal and mesophyll cells of leaves, subepidermal cells of hypocotyls, and various cell types of reproductive organs of mature tobacco plants (data not shown). In general, we demonstrated that in those cells in which signals were detectable, the phyB–GFP
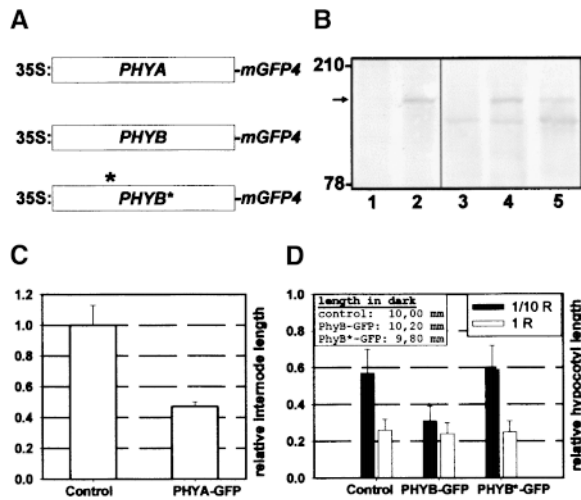
Figure 1. The phyA–GFP and phyB–GFP Fusion Proteins Are Functional Photoreceptors in Transgenic Tobacco Plants.

(A) Diagrams of *PHYA–mGFP4* and *PHYB–mGFP4* gene fusions. Expression of these chimeric genes was driven by the cauliflower mosaic virus 35S promoter (Benfey et al., 1990a, 1990b). The asterisk denotes the relative position of the chromophore-attaching cysteine-encoding codon that was mutated to code for an alanine in the chromophore-less mutant of phyB–GFP (*PHYB**).

(B) Protein gel blot analysis of crude extracts for detection of phy–GFP fusion proteins in transgenic tobacco. Extracts were isolated from leaf tissue of dark-adapted, nontransformed, and phyA–GFP—expressing mature tobacco plants (lanes 1 and 2) or from 8-day-old dark-grown, nontransformed (lane 3), transgenic tobacco seedlings expressing phyB–GFP (lane 4) or phyB*–GFP (lane 5). Each lane contains 10 μg of total protein. phyA–GFP (lane 2) was detected by using the monoclonal antibody mAR14, which specifically detects monocot phyA (Kay et al., 1989). phyB, phyB–GFP, and phyB*–GFP were detected by using the phyB-specific monoclonal antibody mAT1 (López-Juez et al., 1992). The arrow indicates the position of the overexpressed GFP–fusion proteins (lanes 2, 4, and 5). Positions of molecular mass standards are indicated at left in kilodaltons.

(C) Quantitative analysis of the internode elongation of nontransformed (control) and transgenic tobacco plants expressing the phyA–GFP (PHYA-GFP) fusion protein. Plants were grown under long-day conditions (16 hr of light and 8 hr of darkness) and subsequently transferred into continuous white light for 9 days. The relative values of newly developed internodes are given as internode length of phyA–GFP—expressing plants versus those of nontransformed controls. Error bars indicate SD.

(D) Quantitative analysis of the hypocotyl elongation of nontransformed (control) and transgenic tobacco seedlings expressing phyB–GFP (PHYB-GFP) and phyB*–GFP (PHYB*-GFP) fusion proteins. Seedlings were germinated and grown for 6 days either under continuous red light (one-tenth intensity [1/10 R] indicated by black bars; regular intensity [1 R] indicated by open bars) or in darkness. The results shown are relative mean values from at least 30 seedlings (given as hypocotyl length of wild-type [control] and phy–GFP—expressing plants grown in light versus those grown in darkness). The mean values of the absolute lengths of dark-grown seedlings are shown in the upper left corner. Error bars indicate SD.

fusion protein was found in the nuclear compartment. Moreover, in all cell types examined, the fluorescence of phyB–GFP (Figures 2G to 2L), similar to that of phyA–GFP (Figures 2A to 2F), was not homogenously distributed within the nucleus but observed in bright spots. However, in contrast to observations of phyA–GFP, we never detected phyB–GFP—containing speckled areas in the cytosol.

The intranuclear localization and subcompartmentalization of phyA–GFP and phyB–GFP were also confirmed by 4′,6-diamidino-2-phenylindole (DAPI) staining and further analyzed by confocal laser scanning microscopy. Figures 2M to 2O show colocalization of nuclear GFP fluorescence with a DAPI-stained nucleus in a transgenic tobacco seedling expressing the phyB–GFP fusion protein. Similar results were obtained upon analysis of transgenic tobacco plants expressing the rice phyA–GFP fusion protein (data not shown). Confocal scanning microscopy makes nuclei within cells readily detectable, permitting an unparalleled demonstration that a fluorescent protein is localized either inside the nucleus or attached to its outer envelope. Figure 3 shows that both the phyA–GFP (Figures 3A to 3D) and phyB–GFP (Figures 3E to 3H) proteins are clearly localized in nuclei but distributed unevenly therein. These observations are based on the distribution of fluorescence. We note that the number of phyB–GFP—containing speckles is lower (Figures 3E to 3H), although they are of a larger size than the phyA–GFP—containing speckles (Figures 3A to 3D).

Intracellular Distribution of phyA–GFP and phyB–GFP Is Regulated by Light Quality

In the nuclei of dark-adapted tobacco plants, in contrast to light-grown ones, the phyA–GFP (Figure 4A) and the phyB–GFP (Figures 5A to 5C) proteins were not detectable. Irradiation of these dark-adapted plants with red light pulses resulted in the reappearance of the green fluorescence signal in the nuclei, albeit with significantly different kinetics. A single 5-min red light pulse given to dark-adapted plants expressing phyA–GFP was sufficient, after a 15-min dark period, to induce accumulation of detectable amounts of green fluorescence in the nuclei (Figure 4B). Red light also promoted nuclear accumulation of phyB–GFP in etiolated seedlings but at a considerably slower rate. Accumulation of phyB–GFP, over the threshold level of detection, required an additional period of ∼2 hr in darkness after the 5 min–inductive red light pulse (Figure 5D). Similar kinetics for reaccumulation of phyB–GFP were observed in dark-adapted mature plants (data not shown). It is important to note that red light also promoted formation of fluorescent speckles in the cytosol of phyA–GFP (Figure 4B) but not in phyB–GFP—expressing plants (Figure 5D).

Irradiation of dark-adapted phyA–GFP and phyB–GFP plant material with far-red light had strikingly different effects. In the case of phyA–GFP, irradiation with 5 min of far-red light was again sufficient to induce nuclear staining within 15
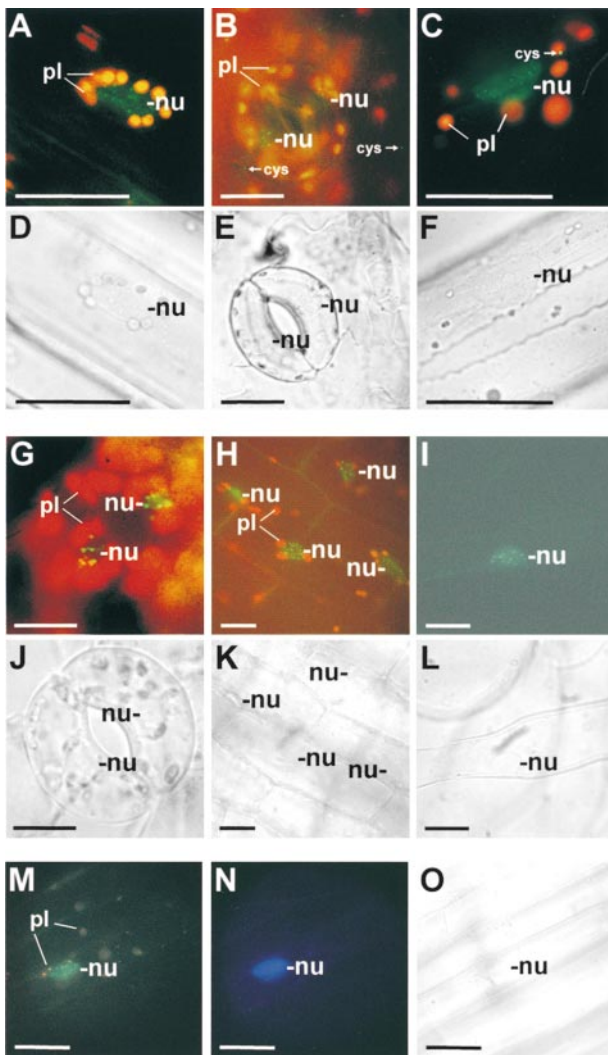
Figure 2. phyA–GFP and phyB–GFP Are Localized in the Nuclei of Different Cell Types of Light-Grown Transgenic Tobacco Plants.

(A) to **(C)** and **(G)** to **(I)** are epifluorescence images. **(D)** to **(F)** and **(J)** to **(L)** are light microscopic images.

(A) and **(D)** Epidermal cell of an adult phyA–GFP—expressing tobacco seedling.

(B) and **(E)** Stomatal guard cell of an adult phyA–GFP—expressing tobacco seedling.

(C) and **(F)** Trichome cell of an adult phyA–GFP—expressing plant.

(G) and **(J)** Stomatal guard cell from a 14-day-old phyB–GFP—expressing tobacco seedling.

(H) and **(K)** Hypocotyl from a 14-day-old phyB–GFP—expressing tobacco seedling.

(I) and **(L)** Root hair cell from a 14-day-old phyB–GFP—expressing tobacco seedling.

(M) and **(N)** Epifluorescence images of GFP and DAPI, respectively, derived from the identical hypocotyl cell of a phyB–GFP—expressing seedling.

(O) A light microscopic image corresponding to **(M)** and **(N)** is shown.

Positions of nuclei (nu), selected plastids (pl), and green fluorescent cytosolic spots (cys) are indicated. Bars = 20 μm.

min but without promoting the appearance of fluorescent spots in the cytosol (Figure 4C). By contrast, treatment of etiolated, phyB–GFP—expressing seedlings (Figure 5E) or dark-adapted mature plants (data not shown), even with repeated pulses of far-red light, was ineffective and never resulted in the accumulation of detectable amounts of fluorescence in the nuclei. In good agreement with these results, our experiments also show that red light–induced accumulation of phyB–GFP (Figure 5F) but not of phyA–GFP (Figure 4D) was reversible by subsequent far-red light treatment. These observations indicate the involvement of phytochrome in regulating subcellular partitioning of phyA and phyB. Moreover, these data strongly suggest that nuclear import of phyA–GFP is controlled by the very low fluence response, whereas nuclear import of phyB–GFP is regulated by the low–fluence response of phytochrome.

Because nuclear import of phyA–GFP is fast, we followed the kinetics of import in more detail under the microscope. For this purpose, we used the actinic light of the microscope as a light source. Figures 4E and 4F show that 2 min of irradiation of dark-adapted plant tissue was sufficient to induce appearance of green fluorescent speckles in the cytosol but not in nuclei. After an additional 20 min, irradiation simultaneously led to cytosolic staining and the accumulation of phyA–GFP in nuclei (Figure 4G).

The possibility that nuclear accumulation of GFP occurred via equilibration of free GFP produced by proteolytic cleavage of the phyA–GFP fusion proteins was also investigated. To this end, total protein extracts isolated from dark-adapted or red light–treated phyA–GFP—expressing plants were analyzed by using protein gel blot assays with a GFP-specific polyclonal antibody. Figure 4H shows relatively high amounts of intact phyA–GFP fusion proteins in extracts derived from either dark-adapted (lane 2) or red light–treated (lane 3) material. In contrast, we were unable to detect any low molecular weight GFP-representing signals in these extracts (lanes 2 and 3) or in extracts derived from nontransgenic tobacco plants (lane 4). These data strongly suggest that light-dependent nuclear accumulation of GFP fluorescence is brought about exclusively by the import of intact phyA–GFP fusion proteins.

A Chromophore-Deficient Form of phyB–GFP Remains in the Cytosol Independent of Light Treatment

It has been shown above that the nucleocytoplasmic partitioning of phyA–GFP and phyB–GFP is regulated by phytochrome itself. The next question addressed was whether the Pr-to-Pfr photoconversion is a necessary prerequisite for
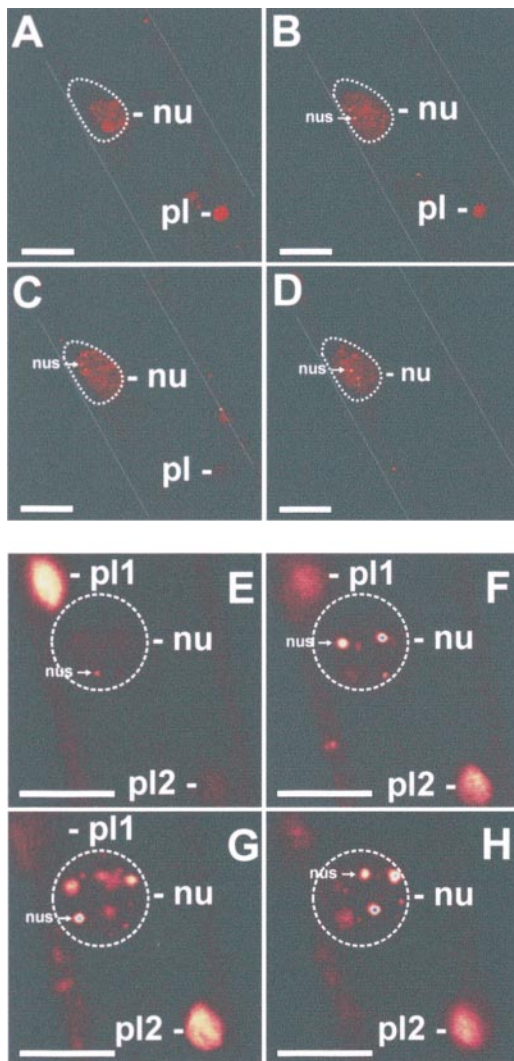
Figure 3. Intranuclear Subcompartmentalization of phyA–GFP and phyB–GFP Analyzed by Confocal Laser Scanning Microscopy.

(A) to **(D)** Serial optical sections taken every 1.5 μm through a trichome cell from a light-grown transgenic tobacco plant expressing phyA–GFP.
(E) to **(H)** Serial optical sections prepared as given in **(A)** to **(D)** but from a light-grown transgenic tobacco plant expressing phyB–GFP. Dashed lines encircle the nuclei (nu). Arrows point to intranuclear spotted areas of phyA–GFP or phyB–GFP (nus). Selected, disappearing, or appearing plastids (pl, pl1, and pl2) are also indicated. Bars = 10 μm.

these phy–GFP molecules to gain nuclear import competence or whether the regulation of subcellular distribution is independent of the photobiological integrity of the phy–GFP transport substrates. For this purpose, an *mGFP4* fusion construct was generated that contained the full-length to-

bacco *PHYB* cDNA mutated in the chromophore attachment site (cysteine-to-alanine exchange; see Figure 1A). Thus, the chromophore-less form of phyB–GFP is not able to undergo light-inducible photoconversion to the physiologically active Pfr form. The mutant *PHYB–mGFP4* transgene was overexpressed in transgenic tobacco plants by using the 35S promoter (Figure 1A). Protein gel blot analysis (Figure 1B, lane 5) shows that the mutant fusion protein was expressed at a level similar to that of the endogenous phyB (lane 3) or the nonmutated phyB–GFP (lane 4). However, in contrast to transgenic plants expressing wild-type phyB–GFP, transgenic plants containing the mutant phyB–GFP never exhibited a characteristic phenotype (Figure 1D). Furthermore, the mutated phyB–GFP could not be detected in the nuclei. In most cells, including trichome cells, irrespective of irradiation, we were able to detect faint yet visible fluorescence signals in the cytosol (Figures 6A to 6B). These results demonstrate that phyB–GFP must be a functional photoreceptor to obtain nuclear import competence.

DISCUSSION

The transition of plant development from skotomorphogenesis to photomorphogenesis at the molecular level includes perception of light by cytosolic photoreceptors, transduction of the signal to the nucleus, and inactivation of the CONSTITUTIVE PHOTOMORPHOGENIC (COP)/DEETIOLATED/FUSCA repressor system (Wei and Deng, 1996; Torii and Deng, 1997). In contrast to this generally accepted model, Sakamoto and Nagatani (1996) have recently suggested that a light-dependent nucleocytoplasmic partitioning of the photoreceptor phyB itself might take place and that the translocation of phyB into nuclei can be regarded as an integral component of the signaling cascade. These authors showed, using cell fractionation and immunocytochemical analyses, that the Arabidopsis phyB protein colocalizes with the nuclei in a light-dependent manner. However, they did not provide unambiguous evidence that the phyB molecules are localized inside the nuclei. It is well known that phytochromes are very "sticky" molecules, and there is considerable debate whether cell fractionation can be used to unequivocally demonstrate their subcellular localization. For instance, a previous report about the nuclear function of phyA (Mösinger and Schäfer, 1984; Mösinger et al., 1987) was challenged after it was shown that nonspecific association of phyA with nuclei occurred during cell fractionation (Nagatani et al., 1988).

To overcome these technical difficulties, we developed a new approach to demonstrate the nucleocytoplasmic partitioning of phyB and phyA proteins by monitoring the localization of biologically active full-length phy proteins fused to GFP as a reporter in transgenic tobacco seedlings and mature plants. In contrast to other methods, the use of GFP as a tag for fluorescence allows an easy and fast intracellular
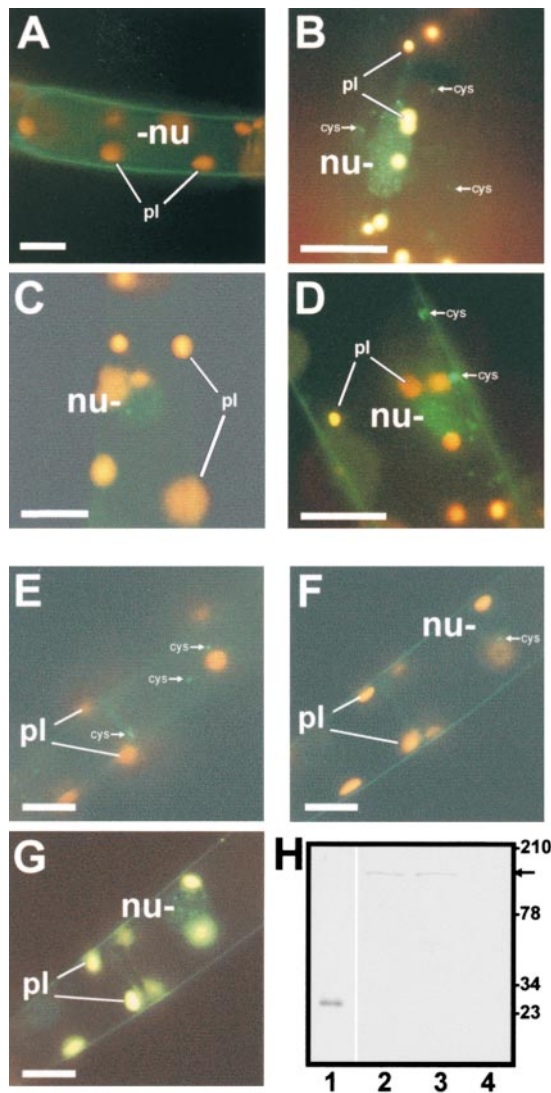
Figure 4. Nuclear Import of phyA–GFP Is Regulated by Phytochrome and Is Preceded by Spotted Cytosolic Aggregation.

(A) to **(D)** Epifluorescence images of trichome cells of a tobacco plant expressing phyA–GFP are shown. Leaf discs, which were dark-adapted for 3 days **(A)**, were irradiated with pulses of red light for 5 min **(B)**, far-red light for 5 min **(C)**, or red light for 5 min followed by 5 min of far-red light **(D)** and transferred to darkness again. Cells were analyzed 20 min after the onset of the light pulses. Alternatively, compartmentalization of phyA–GFP in a trichome cell of a phyA–GFP—expressing plant, which was dark-adapted for 3 days, was analyzed during irradiation with the actinic light of the light source of a Zeiss Axioskop microscope.
(E) and **(F)** Epifluorescence images of the cytosolic and the nuclear cell planes, respectively, are shown after 2 min of irradiation.
(G) Nuclear cell plane after 20 min of continuous irradiation.
(H) Protein gel blot analysis of the phyA–GFP fusion protein. Total protein extracts were prepared and analyzed from a phyA–GFP—expressing (lanes 2 and 3) and from a nontransformed tobacco plant (lane 4). Plants were dark-adapted for 3 days (lane 2) or, after this

detection of protein trafficking without destroying the cell (Haseloff et al., 1997; Köhler, 1998).

Overexpressed phyA–GFP and phyB–GFP Are Functional Photoreceptors

Expression of the phyA–GFP and phyB–GFP fusion proteins under the control of the 35S promoter in transgenic tobacco plants resulted in functional phyA and phyB photoreceptors, as shown by our physiological studies (see Figures 1C and 1D). These data indicate that (1) both phytochrome species tolerate a relative large protein tag attached to their C termini without showing significant alterations in their physiological function in vivo and (2) the regulation of the intracellular partitioning of these GFP fusion proteins is very likely to reflect functional properties of the endogenous photoreceptors phyA and phyB.

Nucleocytoplasmic Distribution of phyB–GFP Is Modulated by Light via a Low-Fluence Response

We used transgenic plants expressing the functional phyB–GFP fusion protein in intracellular localization studies to demonstrate the localization of the full-length phyB protein fused to the GFP protein inside the nuclei of cells from light-grown plants (see Figures 2G to 2I) and the localization of phyB–GFP in the cytosol of dark-grown or dark-adapted plants. Irradiation of dark-adapted plants with continuous red light or red light pulses led to nuclear localization of phyB–GFP, whereas dark adaptation of light-grown plants resulted in the disappearance of phyB–GFP from the nucleus.

As shown in Figure 5, three red light pulses given every hour were sufficient to induce a clearly detectable import of phyB–GFP into the nucleus. The effect of the inducing red light pulses could be completely reversed by subsequent far-red light pulses that transformed Pfr back to Pr. This red/far-red reversibility of the import emphasizes that phyB–GFP is regulating its localization through its own photoconversion ability. This is further supported by the observation that a chromophore-less inactive mutation of full-length phyB

dark period, treated with a 5-min red light pulse followed by 15 min of dark incubation (lanes 3 and 4) before extraction. The phyA–GFP fusion and the recombinant GFP (lane 1) proteins were detected by using a polyclonal antiserum raised against recombinant GFP. Lane 1 contains 20 ng of protein, whereas lanes 2, 3, and 4 contain 20 μg of protein. The arrow indicates the position of the phy–GFP fusion peptide. The positions of molecular mass standards are indicated at right in kilodaltons.
Arrows point to spotted areas of phyA–GFP in the cytosol (cys). Lines indicate the position of the nucleus (nu) and selected plastids (pl). Bars = 10 μm.
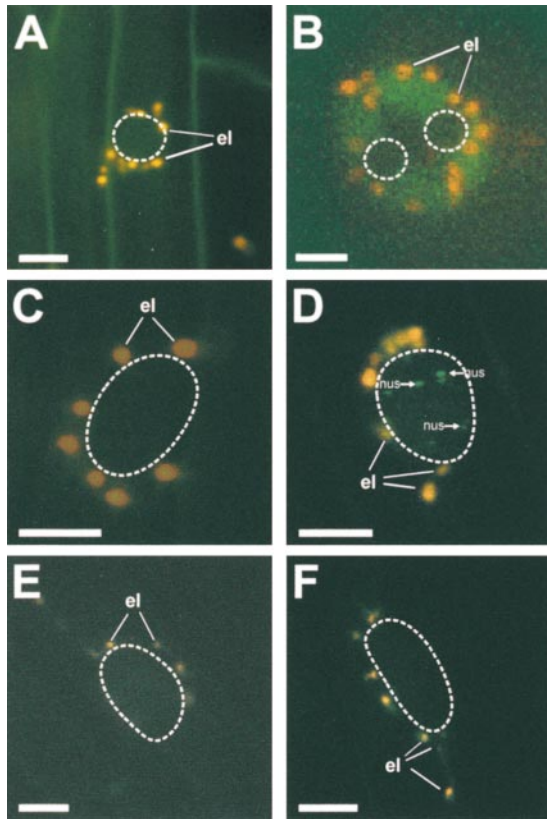
Figure 5. Nuclear Import of phyB–GFP Is Regulated by a Low-Fluence Response of Phytochrome.

(A) to **(C)** Epifluorescence images of hypocotyl cells from 11-day-old, dark-grown (**[A]** and **[C]**) or stomal guard cells from 14-day-old phyB–GFP—expressing tobacco seedlings dark-adapted for 48 hr **(B)**.
(D) to **(F)** Epifluorescent images of dark-grown phyB–GFP—expressing tobacco seedlings that were irradiated with three consecutive hourly pulses of 5 min of red light **(D)**, 5 min of far-red light **(E)**, or 5 min of red light followed by 5 min of far-red light **(F)**.
The dashed lines encircle the nuclei. Arrows point to spotted nuclear areas of phyB–GFP (nus), and lines indicate selected etioplasts (el). Bars = 10 μm.

fused to GFP is retained in the cytosol under all light conditions. The light-regulated nuclear import of phyB–GFP shows the properties of a photoreversible low-fluence response and therefore is not mediated by phyA, which controls very low fluence and high-irradiation responses (Furuya and Schäfer, 1996). Further experiments are in progress to elucidate whether photoreceptors other than phyB might also influence the import properties of phyB–GFP.

From these data, we deduce that the light-regulated nucleocytoplasmic partitioning of phyB–GFP correlates well with the light quality dependence and kinetics of the phyB-triggered transition from skotophotomorphogenesis to photomorphogenesis in higher plants.

Nucleocytoplasmic Distribution of phyA–GFP Is Modulated via a Very Low Fluence Response

Similar to phyB–GFP, the phyA–GFP fusion protein was also detected in the nuclei of various cell types in light-grown transgenic tobacco plants (Figures 2A to 2C). The apparent nuclear localization of phyA–GFP is in sharp contrast to all previous immunocytochemical studies that indicated a cytosolic localization of phyA (see Kendrick and Kronenberg, 1994). The nuclear import of phyA–GFP is light dependent, its transport is fast, and it is inducible not only by a single red light pulse but also by a far-red light pulse (Figure 4). Thus, we conclude that nuclear import of phyA–GFP is controlled by the very low fluence response of phyA.

A comparison of the kinetics of light-dependent nuclear import of phyA–GFP with those of the phyB–GFP fusion protein yielded additional interesting observations. First, we found that light-driven accumulation of phyA–GFP into nuclei is, by an order of magnitude, faster than that of the phyB–GFP (15 to 20 min versus ~2 hr). This phenomenon cannot be explained by assuming significantly differing amounts of the overexpressed fusion proteins (i.e., different GFP levels), because expression of both transgenes was driven by the same promoter and was comparable in the plants selected for these studies. Second, we found that irradiation of phyA–GFP resulted in the appearance of green fluorescent spots in the cytosol but not in the nuclei 2 min after the beginning of the treatment (see Figure 4). This reaction is similar to the previously described light-dependent formation of sequestered areas of phytochrome in monocotyledonous and dicotyledonous seedlings (Saunders et al., 1983; McCurdy and Pratt, 1986; Speth et al., 1986). Furthermore, we also could demonstrate that this very rapid formation of sequestered areas of phytochrome in the cytosol is
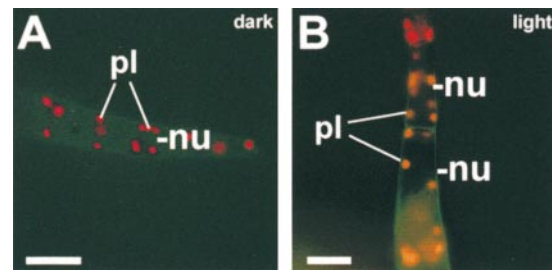

Figure 6. A Chromophore-less Mutant of phyB–GFP Is Retained in the Cytosol.

Epifluorescence images of trichomes from light-grown transgenic tobacco plants are shown.
(A) Trichome from a plant irradiated with a 5-min far-red light pulse and transferred to darkness for 24 hr.
(B) Trichome from plant treated as given in **(A)** but returned to the light. The positions of the nuclei (nu) are shown, and lines point to selected plastids (pl). Bars = 20 μm.

followed by the nuclear transport of phyA–GFP (see Figure 4). However, even after 20 min, cytosolic sequestered areas of phytochrome were still detectable independent of the nuclear accumulation. This latter observation suggests that despite the fast nuclear import process, a significant portion of the phyA–GFP is not imported into nuclei but is retained in the cytosol. By contrast, we have never seen the formation of structures resembling sequestered areas of phytochrome in the cytosol after far-red light treatment of phyA–GFP—expressing transgenic plants and/or after any light treatment of phyB–GFP—expressing plants. Thus, we conclude that the formation of sequestered areas of phytochrome, although their exact physiological role especially in dicotyledonous plants is not well understood, does not play a major role in light-dependent nuclear import of phyA. However, because of the markedly different import kinetics, we speculate that nuclear translocation of phyA and phyB might be mediated by at least partially different molecular mechanisms.

Possible Mechanisms for the Light-Controlled Nucleocytoplasmic Distribution of phy Proteins

As shown for several cellular systems, the amount of protein within the nuclear compartment can be controlled by their import, export, and/or turnover (Corbett and Silver, 1997; Görlich, 1997). Light may possibly control all of these three aspects. As discussed above, nuclear localization of both phyA–GFP and phyB–GFP depends on the physiological state of the molecules. This raises the questions of how the Pr forms of phyA and phyB are retarded in the cytosol and how the Pfr forms are transported into the nucleus. Nuclear import requires at least one functional NLS (Corbett and Silver, 1997; Görlich, 1997). Sakamoto and Nagatani (1996) reported light-independent nuclear import of fusion proteins containing various C-terminal portions of Arabidopsis phyB fused to β-glucuronidase. This observation and a comparative sequence analysis of various phy molecules indicate the presence of multiple NLS-like motifs in the C-terminal regions of phyB as well as of phyA proteins. Wagner and colleagues (1996) reported that fusion proteins encoded by chimeric genes containing the N-terminal region of *PHYA* and the C-terminal region of *PHYB* cDNAs or vice versa function as phyA or phyB photoreceptors when expressed in transgenic plants, respectively. These authors then concluded that the N-terminal regions of these phy molecules define the photobiological properties of the photoreceptors, whereas their C-terminal portions are involved in signal transduction.

Based on our data showing that both phyA–GFP and phyB–GFP can be imported into nuclei, albeit in a different light quality–dependent fashion, we postulate that one of the "signal transductory roles" of the C-terminal regions of chimeric phyA/phyB molecules is to provide the NLS motif(s) required for nuclear import. However, further research should be conducted to verify this hypothesis and to define exactly the localization of a functional NLS(s) within the various phy proteins.

Because the chromophore-less mutant form of phyB–GFP is confined to the cytosol in a light-independent manner, it is conceivable that in the Pr form, the NLS of phyA and phyB is masked intramolecularly, thereby abolishing nuclear uptake. Alternatively, a cytosolic retention of phyA and phyB can be achieved by a hypothetical retention factor that specifically interacts with the Pr forms or by a combination of these two molecular mechanisms. Experiments are in progress to determine to what extent these mechanisms are involved in regulating the intracellular partitioning of the phyA and phyB photoreceptors.

Independent of the molecular mechanism mediating light-dependent nuclear import of phyA and phyB, a process responsible for the disappearance of the imported photoreceptors from the nuclei during dark incubation should also be found. Depletion of the GFP fluorescence in the nuclei of dark-adapted plants can be explained by postulating an active export mechanism and/or protein degradation. Regarding phyA degradation, Quail et al. (1973) reported that the Pfr form of phyA is extremely unstable (half-life of 1.0 to 2.0 hr), whereas its Pr form is two orders of magnitude more stable (half-life of 100 to 200 hr). By contrast, the Pfr form of phyB is relatively stable (half-life of 5.0 to 8.0 hr; Heim et al., 1981), and its Pr form, although its turnover rate has not been determined, is expected to be equally stable.

We found that in the dark after far-red light irradiation, when the majority of phyA and phyB molecules exist in their Pr forms, the disappearance (i.e., export or degradation) of phyA–GFP and phyB–GFP fluorescence from the nuclei is a relatively slow process. Complete depletion of GFP fluorescence in the dark takes ~24 to 36 hr for both phyA–GFP and phyB–GFP. Light-driven reaccumulation of phyA–GFP and phyB–GFP in the nuclei is significantly faster.

Taken together, these data indicate that (1) nuclear import of both phyA–GFP and phyB–GFP is considerably faster than are their degradation and/or export and (2) protein degradation alone is not sufficient to explain the measured depletion kinetics of the phyA–GFP and phyB–GFP from the nuclei of dark-adapted plants. Although the exact turnover rates of the phyA–GFP and phyB–GFP proteins are not known in tobacco, it follows that an active export mechanism is most likely involved in regulating the nucleocytoplasmic partitioning of these photoreceptors. Finally, we note that kinetics of light-driven nuclear accumulation (import) and/or disappearance (export) of the rice phyA–GFP and tobacco phyB–GFP fusion proteins do not necessarily reflect nucleocytoplasmic partitioning of the endogenous tobacco phyA and phyB proteins, possibly due to the different rates of protein degradation. Further experiments using immunocytochemical analysis coupled with confocal microscopy and cell fractionation are needed to clarify this matter.

Implications of the Role of Phytochromes in Controlling Photomorphogenesis

phyA–GFP and phyB–GFP transported into the nucleus are not evenly distributed but show spotted localization patterns (see Figures 2 to 5). It is interesting that the size and number of phyA–GFP— and phyB–GFP—containing spots are different (see Figure 3). This finding suggests that phyA–GFP and phyB–GFP are members of large but different multiprotein complexes and might interact with different proteins after import into nuclei. This speculation is supported by the fact that the observed phyA–GFP and phyB–GFP patterns are reminiscent of the spotted nuclear localization of the COP1 protein (Ang et al., 1998), which is a member of the genetically defined, heterogeneous *COP* gene product group that is assumed to act as a general switch from skotomorphogenesis to photomorphogenesis (Wei and Deng, 1996; Torii and Deng, 1997). In the dark, COP1 (or the COP1–GFP fusion protein) is localized in the nucleus and interacts with the long-hypocotyl factor HY5, a bZIP factor that binds to specific *cis*-acting elements within promotors of light-regulated genes (von Arnim and Deng, 1994; Ang et al., 1998; Chattopadhyay et al., 1998). When irradiated, COP1 slowly disappears from the nucleus and releases HY5, which in turn controls the transcription of its target genes (Ang et al., 1998; Chattopadhyay et al., 1998). Because of the similar, spotted staining patterns of the COP1–GFP, phyA–GFP, and phyB–GFP, it is tempting to speculate that phyA and phyB in their active Pfr form can interact with the COP1–HY5 complex, releasing COP1 and HY5.

The proposed photoreversible interaction of phyA and phyB with the COP1–HY5 complex may explain how these photoreceptors function within the nucleus. While this manuscript was in preparation, Min et al. (1998) reported isolating a basic helix-loop-helix protein (PIF3) that interacts with the C-terminal regions of Arabidopsis phyA and phyB proteins by using a yeast two-hybrid screen. PIF3 contains a PAS domain and is localized to the nucleus in transient assays. Therefore, these authors postulated that phyA and phyB signaling to photoregulated genes includes a direct physical interaction between these photoreceptors and PIF3, as a transcriptional regulator. Our data strongly suggest that this proposed interaction takes place in the nucleus, and we speculate that PIF3 could be one of those proteins that is recruited by phyA and/or phyB after disassemblement of the repressory COP protein complexes (Nagy and Schäfer, 1999).

Conclusions and Perspectives

From our report, it is conceivable that phyA and phyB each possess specific nuclear functions. This hypothesis raises two questions. First, is the light-regulated nuclear import specific for phyA and phyB or are phyC, phyD, and phyE also imported into nuclei? Second, why are the phyA and phyB proteins retained in the cytosol at all and not immediately imported into the nucleus in their inactive Pr form?

Experiments have been undertaken to provide an adequate answer to the first question. Regarding the second question, we speculate that this phenomenon can be explained by the multifunctional properties of phytochromes that depend on their subcellular compartmentalization. Whereas, in the nucleus, phyA and phyB regulate the transcriptional activity of light-regulated genes, they could also induce cytosolic signaling events before their uptake into the nuclear compartment. Examples for phyB-regulated cytosolic photoresponses are the induction of nuclear import of the bZIP transcription factor CPRF2 in parsley (Kircher et al., 1999) and the functional interaction of phyB with CRY1 in blue light–induced shrinking of Arabidopsis hypocotyl protoplasts (Wang and Iino, 1998). Light-controlled nuclear localization of phyA–GFP is preceded by the formation of sequestered areas of phytochrome, which are believed to be important for phyA degradation (Speth et al., 1987). It is also accepted that in addition to very low fluence responses, phyA mediates the high-irradiance response under continuous irradiation with far-red light (see Furuya and Schäfer, 1996). We speculate that these dual functions of phyA may be reflected by the nucleocytoplasmic partitioning of phyA–GFP described here. To unravel cytosol- and nucleus-specific signaling pathways in more detail, it is obligatory to find out what plant phenotypes emerge when phyA and/or phyB is prevented from being imported into the nucleus or constitutively kept in the nucleus. Experiments aimed at modifying the compartmentalization of these photoreceptors by mutating putative NLS and nuclear export signal motifs are in progress.

In conclusion, the light quality–dependent localization of phyA and phyB in the cytosol and the nucleus opens up a new and fascinating view of the diversity of photoresponses at the molecular level.

METHODS

Light Sources

The white light source was used as described in Frohnmeyer et al. (1992). Red light (regular and one-tenth intensity) as well as far-red (RG9) light were produced as described in Schäfer (1978). Handling of irradiated or dark-grown plants was performed under a dim-green safe light according to Schäfer (1978).

Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum*) SR1 plants were grown on sterile Murashige and Skoog (MS) medium (Sigma, Deisenhofen, Germany), supplemented with 3% sucrose, or in soil in the greenhouse. Selected transgenic tobacco plants were either maintained on the same

MS medium but supplemented with 15 μg/mL hygromycin or transferred to soil and grown to maturation in the greenhouse. Unless otherwise indicated, all plants were grown under light and dark cycles (16 hr of light and 8 hr of dark; intensity of 20 W/m²). Seedling material used in different experiments was always grown, after surface sterilization, under sterile conditions on MS medium supplemented with 15 μg mL^{-1} hygromycin.

Recombinant DNA Techniques and Construction of the *PHY–mGFP4* Fusion Genes

A full-length tobacco phytochrome *PHYB* cDNA clone was isolated from a tobacco leaf cDNA library (Stratagene, La Jolla, CA) by the standard plaque hybridization method using as probe a chemically synthesized (55-mer) single-stranded oligonucleotide fragment, homologous to the last 50 nucleotides of the untranslated leader region of the tobacco *PHYB1* genomic clone described by Adam et al. (1996). To facilitate construction of the fusion genes containing the mutated green fluorescent protein *mGFP4* reporter gene, the 5′ and 3′ regions of the isolated tobacco *PHYB* cDNA clone as well as that of the *mGFP4* gene (Haseloff et al., 1997) were modified by polymerase chain reaction (PCR) as follows. (1) A unique BamHI site was introduced in front of the ATG start codon. To create a version of the *PHYB* gene lacking a stop codon, the last six nucleotides of the *PHYB* coding region were changed to a SmaI site. The resulting product was cloned into the pKS plasmid as a BamHI-SmaI fragment. (2) The *mGFP4* gene was also modified by introducing an SmaI site in front of its ATG and a unique SacI site after the stop codon. The product was then transferred as an SmaI-SacI fragment into pKS plasmid (Stratagene) that already contained the modified *PHYB* gene, resulting in the *PHYB–mGFP4* fusion gene.

To create the fusion gene containing the rice *PHYA* cDNA, the *mGFP4* gene was modified by PCR by adding unique SmaI and EheI sites in front of its ATG and a unique SacI site after its stop codon. The product was cloned into the pKS plasmid as a SmaI-SacI fragment. The *PHYA* cDNA was also modified by PCR, generating a unique BamHI site in front of the start ATG and adding a unique EheI site at the 3′ end. The latter was achieved by changing the last six nucleotides of the coding region to create a version of the *PHYA* gene lacking a stop codon. The PCR product was cloned into the pKS plasmid containing the *mGFP4* gene as a BamHI-EheI fragment.

To create a mutated phyB protein (phyB*) that is unable to bind its chromophore, a point mutation was introduced into the modified *PHYB* cDNA fragment by PCR-mediated mutagenesis that led to a cysteine-to-alanine exchange at amino acid position 342 (downstream from the start methionine). All *PHY* and *mGFP4* clones subjected to PCR amplification were partially sequenced. All DNA manipulations were conducted as described by Sambrook et al. (1989). All PCR reactions were performed by using the ProofSprinter polymerase system (AGS, Heidelberg, Germany).

Plant Transformation and Regeneration of Transgenic Tobacco Lines

Various pKS plasmids containing the above-described fusion constructs were first digested with BamHI-SacI restriction enzymes. The resulting BamHI-SacI fragments were isolated and transferred into a modified pPCV812 binary vector that was originally described by Koncz et al. (1994). The modified pPCV812 binary vector contained unique BamHI and SacI sites sandwiched between the 35S promoter and the nopaline synthase transcription termination region. The pPCV812 binary vectors containing the various *PHY–mGFP4* fusion genes were then transferred from *Escherichia coli* to *Agrobacterium tumefaciens* GV3101. Tobacco plants were transformed via leaf disc–mediated transformation as described by Koncz et al. (1994). Transgenic shoots were then selected on hygromycin (15 μg mL^{-1}) containing MS medium. For each construct, at least 15 independent transgenic lines were raised, grown to maturation in the greenhouse, and selfed.

Protein Extraction, Protein Assay, SDS-PAGE, Protein Gel Blotting, and Immunodetection

Two hundred milligrams of 8-day-old, dark-grown tobacco seedlings or of leaf tissue from dark-adapted mature plants was homogenized in a homogenizer (Braun, Melsungen, Germany) by using 0.5 mL of hot extraction buffer (65 mM Tris-HCl, pH 7.8, 4 M urea, 5% [w/v] SDS, 14 mM 2-mercaptoethanol, 15% [v/v] glycerol, 0.05% [w/v] bromphenol blue), and the homogenate was heated for 5 min at 95°C. The suspension was cleared by centrifugation (10 min at 20,000g at 25°C), and the supernatant was used for further experiments. Protein assays were performed as described by Popov et al. (1975). Ten micrograms of crude protein extract was separated on an SDS–polyacrylamide gel and blotted to a polyvinyldifluoride membrane, as described previously (Harter et al., 1993). Immunodetection of phyB, phyB–GFP, phyB*–GFP, and phyA–GFP was performed using the phyB-specific monoclonal antibody mAT1 (López-Juez et al., 1992) and the phyA-specific monoclonal antibody mAR14, respectively (Kay et al., 1989), as the primary antibodies and an alkaline phophatase–coupled anti-mouse antiserum (Boehringer Mannheim) as the secondary antibody. Immunodetection of phyA–GFP was performed using a GFP-specific antiserum produced against the *mGFP4* gene product and prepared as described in Kircher et al. (1998).

Epifluorescence, Light, and Confocal Microscopy

For epifluorescence and light microscopy, plant material was transferred to glass slides and analyzed in an Axiovert microscope (Zeiss, Oberkochem, Germany). Excitation of GFP was performed with standard fluorescein isothiocyanate filters. Representative cells were documented by photography with an automatic Contax 167 MT (Yashica Kyocera, Hamburg, Germany) camera containing 64T film (Kodak AG, Stuttgart, Germany). Dark-incubated or light pulse–treated plant material was manipulated under dim-green safe light before the onset of microscopy. Photographs were taken during the first 5 min of microscopic analysis. 4′,6-Diamidino-2-phenylindole (DAPI) staining of selected nuclei was performed as described by Sakamoto and Nagatani (1996). For computer processing, the slides were scanned by an LS-1000 scanner (Nikon, Tokyo, Japan). For confocal images, the cells were visualized under a confocal laser microscope (model DM RBE TCS4D; Leica, Bensheim, Germany) using a two-channel scan with an argon–krypton laser at 488 nm excitation, a beam splitter at 510 nm, and a 515-nm filter (Kircher et al., 1999). Scanned slides and confocal images were processed for optimal presentation using the Photoshop 3.0 (Adobe Systems Europe, Edinburgh, UK) and Designer 6.0 (Micrografx Deutschland GmbH, Unterschleissheim, Germany) software packages.

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NOTE ADDED IN PROOF:

While this manuscript was under revision, similar results on phyB–GFP localization to those described here were published (**Yamaguchi, R., Nakamura, M., Mochizuki, N., Kay, S.A., and Nagatani, A.** [1999]. Light-dependent translocation of a phytochrome B–GFP fusion pro-tein to the nucleus in transgenic Arabidopsis. J. Cell Biol. **145**, 437–445).

Light Quality−Dependent Nuclear Import of the Plant Photoreceptors Phytochrome A and B

Stefan Kircher, Laszlo Kozma-Bognar, Lana Kim, Eva Adam, Klaus Harter, Eberhard Schäfer and
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