FOCUS PAPER

Light-regulated nucleo-cytoplasmic partitioning of phytochromes

Eva Kevei1,2,*, Eberhard Schafer1 and Ferenc Nagy2

1 Biologie II/Institute für Botanik, University of Freiburg, D-79104 Freiburg, Germany
2 Institute of Plant Biology, Biological Research Center, H-6726 Szeged, Hungary

Received 12 April 2007; Revised 25 May 2007; Accepted 30 May 2007

Abstract

Phytochrome photoreceptors regulate development, growth, and fitness throughout the entire life-cycle of plants, from seed germination to flowering, by regulating expression patterns of ~10–30% of the entire plant transcriptome. Identification of components and elucidation of the molecular mechanisms underlying phytochrome-controlled signal transduction cascades have therefore attracted considerable attention. Phytochrome-controlled signalling is a complex cellular process; it starts with the light-induced intramolecular conformational change of the photoreceptor and includes regulated partitioning and degradation of signalling components and of the photoreceptors themselves. In this review, the data available about light quality- and quantity-dependent nucleo-cytoplasmic partitioning of phytochromes is summarized and the possible function of phytochrome-containing nuclear complexes, termed nuclear bodies, in red/far-red light-induced signalling is discussed.

Key words: Light signalling, nuclear import, phytochromes.

Introduction

Plants are sessile organisms which have established a considerable plasticity of development to respond to changes in the natural environment. The highly variable environmental factor light is used not only as the main energy source but also as an environmental cue to respond to daily light/dark cycles and to compete with neighbouring plants. To monitor changes in the ambient light environment, plants have evolved several classes of photoreceptors: the as yet unidentified UV-B photoreceptors (Beggs and Wellman, 1994), the blue/UV-A sensing cryptochromes (CRY1 and CRY2) controlling plant development (Lin and Shalitin, 2003), the phototropins regulating directional growth, chloroplast re-orientation, and stomatal opening (Briggs and Christie, 2002), and the red/far-red-absorbing photoreceptors phytochromes, controlling plant growth and development (Quail, 2002).

Phytochromes are synthesized in the cytosol as ~125 kDa monomers in their inactive, P_r form (red-light-absorbing conformer, \( \lambda_{\text{max}}=660 \) nm). Each monomer covalently binds one molecule of phytochromobilin, a linear tetapyrrole chromophore. Phytochromes exist in vivo as dimers. Red light (R) induces an intramolecular conformational change of the molecule resulting in the formation of the physiologically active P_fr, conformer (far-red light-absorbing form, \( \lambda_{\text{max}}=730 \) nm). The photoreceptor reverts back into its inactive P_r form with subsequent far-red light (FR) treatment and this light quality- and quantity-dependent conformational change enables phytochromes to act as light-sensitive molecular switches.

Higher plants contain various phytochromes, which differ in amino acid sequence by 50%. These different types of phytochromes are selectively responsible for sensing various light qualities. In the model plant Arabidopsis thaliana this gene family consists of five genes, designated PHYA, PHYB, PHYC, PHYD, and PHYE (Clack et al., 1994). Due to the overlapping absorption spectra, a light quality-dependent photo-equilibrium is established, making this photoreceptor system a very effective light sensor, especially in the red/far-red range of the spectrum (Smith, 2000).

Phytochrome proteins can be divided into two classes on the basis of their mode of action and light stability. Type I phytochromes show rapid proteolytic degradation of the P_fr form, controlling Very-Low-Fluence-Responses...
(VLFR) and far-red High-Irradiance Responses (FR-HIR). Type II phytochromes are light-stable and control Low Fluence Responses (LFR) and red light High Irradiance Responses (R-HIR). Analysis of mutants deficient in various phytochromes showed that (i) type I phytochromes are encoded by the PHYA gene and type II phytochromes by PHYB–E genes (Quail, 2002) and that (ii) different members of the family have differential as well as overlapping physiological roles in controlling plant development (Smith et al., 1997; Franklin et al., 2003; Monte et al., 2003).

The phytochrome molecule folds into two major domains separated by a protease-sensitive, flexible hinge region: the N-terminal photosensory domain of ~70 kDa and a C-terminal domain of ~55 kDa (for a recent review see Rockwell et al., 2006). Recent studies established that in all phytochromes the N-terminally located bilin-lyase-domain (BLD) is responsible for binding the chromophore (for a review see Rockwell and Lagarias, 2006). It has recently been shown that the very N-terminal part of phytochromes plays a role in regulating the stability of the Pfr conformer (Jordan et al., 1997; Sweere et al., 2001; Casal et al., 2002; Ryu et al., 2005; Trupkin et al., 2007). The phytochrome domain (PHY) is located toward the C-terminal part of the molecule, adjacent to the BLD. The PHY domain was shown to contribute to the integrity of Pfr (Montgomery and Lagarias, 2002), thus deletion of this domain resulted in substantial alterations in the spectral properties of phytochromes (Cherry et al., 1993). The first domain of the C-terminal part of the molecule contains a core regulator region, termed Quail-box, which is flanked by the so-called PAS1 and PAS2 subdomains (Quail, 1997; Ponting and Aravind, 1997). The PAS1-Quail-box-PAS2 region is adjacent to the dimerization domains (Edgerton and Jones, 1993), and is followed by the histidine-kinase related domain (HKRD) at the end of the C-terminus. The HKRD domain displays homology to bacterial two-component sensor kinases (Yeh and Lagarias, 1998). Figure 1 shows a schematic view of phytochrome structure.

Recent structure–function studies have begun to reveal the function of the various domains in initiating light-dependent signalling and showed that phyA–E regulate various aspects of plant photomorphogenesis by independent or partially overlapping signal transduction cascades (for recent reviews see Wang, 2005; Franklin et al., 2005; Rockwell et al., 2006). In molecular terms, however, all of these pathways have one common feature, i.e. light in a quantity- and quality-dependent fashion alters the nucleo-cytoplasmic distribution of phyA–phyE by inducing their import into and accumulation in the nucleus. Consistent with this observation, it has been convincingly documented that phytochromes interact both in vivo and in vitro with a number of Phytochrome-Interacting-Factors (PIFs) of nuclear localization in a light and, therefore, conformation-dependent fashion. These data strongly suggest that the various phytochromes transmit the light signal, at least partly, inside the nucleus.

**Intracellular distribution of phytochromes in etiolated seedlings and during the early phase of photomorphogenesis**

**phyA**

In etiolated seedlings the native phyA (McCurdy and Pratt, 1986; Speth et al., 1986; Pratt, 1994) and the 35S:phyA::GFP fusion protein are distributed throughout the cytosol (Kircher et al., 1999, 2002; Hisada et al., 2000). A single, brief (~5 min) FR-, R- or blue-light pulse (B) induces nuclear import of phyA and subsequent formation of phyA-containing nuclear bodies (phyA NBs) (as described by Hisada et al., 2000; Kim et al., 2000; Kircher et al., 2002). In etiolated seedlings, the nuclear import of phyA is a rapid process: it takes place within a few minutes after the inductive light pulse. An R pulse also promotes the rapid formation of phyA-containing cytosolic spots, also referred to as sequestered areas of phytochromes (SAPs) (Speth et al., 1986). The appearance of SAPs precedes nuclear transport of 35S:phyA::GFP and is thought to be the place of ubiquitination and degradation of the photoreceptor. Continuous FR light (cFR) also initiates nuclear transport and formation of phyA NBs in the nuclei. These data suggest that nuclear import of phyA correlates with phyA-mediated VLFRs and HIRs. Figure 2 shows the fluorescence-dependent nuclear accumulation of 35S:phyA::GFP.

In addition to FR, white (W), R, and B light illumination of etiolated seedlings is also effective in inducing
nuclear translocation and rapid formation of 35S:phyA::GFP. Although the pattern of redistribution of 35S:phyA::GFP within the cell is similar in response to all of these light treatments, the dynamics of protein movement is different. The maximum number of phyA-containing nuclear bodies is visible about 10 min after W light illumination, whereas FR light-induced formation of phyA NBs reaches its highest level approximately 2 h after irradiation (Kim et al., 2000; Kircher et al., 2002).

**phyB**

The intracellular localization of phyB in etiolated seedlings is similar to that of phyA, thus the phyB::GFP fusion protein, expressed from the 35S promoter, is also found mainly in the cytosol (Gil et al., 2000; Kircher et al., 2002). It should be noted, however, that in the case of phyB (in contrast to phyA, which is exclusively localized in the cytosol), a weak, diffuse nuclear staining is always visible in etiolated seedlings. Whether this difference has any biological significance for phyB-controlled signalling and is caused by different retention/import mechanisms for phyA and phyB, or is simply due to the relatively higher over-expression of phyB is not yet understood. Apart from this, it is safe to say that light, in a quality- and quantity-dependent fashion, induces translocation of 35S:phyB::GFP into the nucleus. However, import of phyB is at least one order of magnitude slower than that of 35S:phyA::GFP and it was also shown that, in contrast to phyA, single pulses of R, FR or B light cannot induce detectable nuclear transport of 35S:phyB::GFP. The P_{fr}

**phyC, phyD, and phyE**

Similarly to 35S:phyB::GFP, 35S:phyC–E::GFP are mainly localized in the cytosol, but are also found in the nuclei of etiolated seedlings, displaying diffuse, weak staining (Kircher et al., 2002). The intensity of nuclear staining (the amount of fluorescent protein within the nuclei) varied among the different photoreceptors and was obviously affected by the experimental protocol applied (e.g. light or GA-induced germination). The P_{fr} form of phyC–E is thought to be photostable, similarly to phyB.
Thus it is assumed that the low level of these phytochromes in the nuclei of etiolated seedlings may reflect transient activation of the nuclear import mechanism during germination. White light irradiation, however, clearly increases the concentration of these phytochrome species in the nuclei and induces subsequent formation of 35S:phyC–E-containing nuclear bodies. The kinetics of the formation of 35S:phyC::GFP and phyE::GFP NBs is comparable with that of 35S:phyB NBs, i.e. the maximum number of phyC and phyE NBs is detectable within 2 h after white light irradiation. Interestingly, although the 35S:phyD::GFP is obviously detectable in nuclear complexes, the number of these phyD::GFP NBs does not increase significantly during the first 8 h after irradiation with white light (Kircher et al., 2002). In contrast to phyA and phyB, available data about the light quality- and quantity-dependent nuclear import of phyC–E are fairly limited, thus very little is known about the detailed kinetics of R-, FR-, and B-induced changes in the intracellular distribution of these phytochrome species.

**Intracellular distribution of phytochromes in plants grown under light/dark cycles**

With the exception of phyB, surprisingly little is known about the intracellular distribution of phytochromes in young plantlets or mature plants grown under light/dark cycles. In the past, studies addressing this problem by using classical approaches, such as immunolocalization, faced several technical problems. phyA is known to be photolabile, thus the low level of phyA made detection problematic; expression levels of the photostable phyB–E are generally low, which once again hindered reliable data being obtained. With the advent of fluorescent protein tags these problems could only partially be overcome. Because of the relatively high level of autofluorescence generated by chlorophyll molecules, reliable detection of 35S:phy::YFP, 35S:phy::GFP, and especially 35S:phy::CFP fusion proteins still requires relatively high level expression of these proteins, which in turn makes interpretation of the data somewhat problematic (for example, the detection of low-level diffuse staining in the cytosol and/or nuclei). Thus it is not surprising that in most of these studies the appearance of phytochrome-containing nuclear bodies was used as a tool to detect phytochromes localized in the nucleus. Interestingly, it was reported that in young plantlets grown under short-day conditions (8/16 h light/dark) all phy forms nuclear bodies and the emergence of these nuclear complexes follows a diurnal pattern (Kircher et al., 2002). However, rhythmic appearance of these NBs required irradiation with distinct qualities/intensities of light. Accordingly, the number and appearance of 35S:phyA NBs were monitored in plants grown under FR/dark cycles, whereas 35S:phyB-E NBs were detected in plants grown under WL/dark cycles. More importantly, it was also found that these nuclear complexes appeared before the light-on signal and started to decrease before light-off (Kircher et al., 2002). Anticipation of light/dark transitions indicates that this process is not simply regulated by the change in the environmental light conditions, but is controlled by the circadian clock. Interestingly, Toth et al. (2001) showed that transcription of PHYA–E genes is also regulated by the circadian clock, thus it appears that the circadian clock regulates both the expression and the activity of PHY genes and phy proteins. Since phytochromes have been shown to regulate light input pathways to the clock (Somers et al., 1998) these data indicate the existence of a feedback loop to control phytochrome-mediated photo-transduction to the clock.

**What is the biological significance of phytochrome-containing nuclear bodies?**

The first papers reporting on light-induced translocation of 35S:phyA::GFP and phyB::GFP into the nucleus (Sakamoto and Nagatan, 1996; Nagatan, 1998, 2004; Kircher et al., 1999) documented that (i) formation of phyA::GFP NBs was as fast as the import of the receptor, i.e. they were easily detectable within minutes after light treatments and that (ii) formation of phyB::GFP NBs was detectable only in about 1.5–2 h after irradiation. More importantly, however, it was also reported that native phyA and phyB also accumulated in electrodense subnuclear structures, excluding the possibility that the accumulation of 35S:phy::GFP fusion proteins is mediated by the fluorescence tag (Hisada et al., 2000; Kircher et al., 2002).

With the advance of microscopic techniques, however, it became obvious that not only phyA but also phyB::GFP and phyD::GFP can form nuclear bodies very rapidly (2–3 min) after the light treatment of etiolated seedlings (Kircher et al., 2002). These early 35S:phyB-D NBs were transient, generally smaller in size, but more numerous, as compared with the late appearing 35S:phyB-D NBs, and they dissolved and became undetectable within 10–15 min. Interestingly, after these early NBs disappeared, in cR new 35S:phyB-D NBs formed in 2–3 h, but these were fewer and larger. More importantly, Bauer et al. (2004) reported that PIF3, a bHLH-type transcription factor co-localized with phyA, B, and D only in the early NBs and it was not detectable in the newly appearing, late NBs. These data provided the first evidence that components of the various 35S:phy NBs are likely to be different.

In an independent line of experiments, Chen et al. (2003) demonstrated convincingly that the heterogeneity of 35S:phyB::GFP NBs regarding their size and number is fluorescence rate-dependent and that the detectable formation of late phyB NBs (appearing hours after the R treatment)
is not required for phyB signalling at low fluence rates. These authors showed that, at low fluence rates of cR, 35S:phyB::GFP is imported into the nucleus as a Pfr-Pi heterodimer and phytochrome signal transduction is initiated (measured by hypocotyl growth inhibition), but the appearance of phyB NBs cannot be detected. They also showed that by increasing the intensity of the inductive R treatment an increasingly higher percentage of phyB molecules are converted to and exist in Pfr conformation, which is paralleled by the appearance of various 35S:phyB::GFP-containing nuclear bodies. Chen et al. (2003) divided fluence rate-dependent phyB NB patterning into four stages. At stage I, phytochrome is found in both cytoplasm and nuclei, but there is no obvious phyB NB formation within the nuclei. At stage I, no more than 10% of total phyB is in the Pfr form. Gil et al. (2000) reported similar results by studying fluence rate-dependent nuclear translocation of a tobacco 35S:phyB::GFP fusion protein. At stage II, where \(~15\%\) of total phyB is Pfr, numerous small phyB NBs appear in the nuclei after irradiation. The size and number of these 35S:phyB::GFP NBs resemble the so-called early 35S:phyB::GFP NBs detected by Bauer et al. (2004). However, these phyB NBs were monitored 3 d after the onset of R, whereas Bauer et al. (2004) detected the early phyB NBs, in which phyB co-localizes with PIF3, within minutes after the R treatment. Experiments to find answers for these vexing questions, i.e. whether or not these small phyB NBs have identical composition (does PIF3 co-localize with phyB in both of them?) and/or similar function (do both types mediate light-induced degradation of PIF3?) are yet to be performed. Stage III is marked by the presence of about 24% Pfr of total phyB and the presence of larger but fewer nuclear bodies, although a number of small phyB NBs are still visible within the nuclei. When roughly half or more of total phyB is in the active Pfr form, only a few large NBs are observed with little fluorescence in the nucleoplasm. 35S:phyB::GFP NBs detectable at this stage (stage IV) are very similar to the so-called late speckles detected by Bauer et al. (2004) at higher intensities of R. Figure 3 illustrates the fluence rate-dependent formation of phyB NBs. As increasing fluence rates of R not only induced a change in the nuclear patterning of phyB but also enhanced inhibition of hypocotyl elongation, these results also indicated that the formation of phyB NBs plays a role in the regulation of phyB-mediated signal transduction, at least at higher fluence rates (Chen et al., 2003).

These observations by Bauer et al. (2004) and Chen et al. (2003), however, provoked additional questions. First, is the translocation of 35S:phyA::GFP, 35S:phyB::GFP, and 35S:phyD::GFP into the nucleus fast enough to explain co-localization of these phys with PIF3 in the so-called early NBs detected minutes after light treatment? Translocation of phyA into the nucleus is very fast, even a single R pulse induces nuclear import of phyA, thus the appearance of phyA NBs correlates well with the appearance of phyA-PIF3-containing NBs. However, in contrast to phyA, translocation of 35S:phyB::GFP and especially 35S:phyD::GFP into the nucleus is considerably slower even at higher light intensities. In addition, the frequently detected weak, diffuse staining in the nucleus by 35S:phyB::GFP and 35S:phyD::GFP in etiolated seedlings makes it difficult to determine that minimal increase in nuclear levels of phyB and phyD which can be caused by a few minutes of irradiation. A brief R pulse is not sufficient to induce phyB translocation into the nucleus and phyB-mediated signalling. However, at least theoretically, the same short R pulse can convert the nuclear phyB Pfr, visible as weak, diffuse staining in the dark-grown seedlings, into phyB Pfr, thus promoting the formation of early 35S:phyB::GFP NBs in which phyB co-localizes with the PIF3 protein. If so, it is possible that these very early steps of phyB-mediated signalling are mediated by phyB constitutively localized in the nucleus, rather than by the freshly translocated photoreceptor? An equally vexing problem is to explain the relative stability of the large 35S:phyB::GFP NBs in the dark or after FR irradiation. It has been shown that phyB is present in the nuclear bodies in its Pfr form, since PHYB molecules

![Fig. 3. Red light fluence rate dependency of 35S:phyB::GFP speckle formation/morphology. Transgenic Arabidopsis seedlings expressing the PHYB::GFP transgene driven by the constitutive Cauliflower Mosaic Virus promoter (CaMV 35S) were grown at 0.5 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (A), 1 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (B), 2 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (C), or 8 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) (D) of red light for 4 d and then the intracellular distribution of phyB::GFP fusion protein was examined in hypocotyl cells under epifluorescent microscopy. According to calculations by Chen et al. (2003), under 0.5 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) continuous red light 10% of total phyB is in the Pfr form (A), 1 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) eR light induces the formation of 16% Pfr (B), 2 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) induces 24% (C), whereas under continuous red light irradiation of 8 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) R light more than 46% of total phyB is in Pfr form (D). The bar in (A) represents 5 \(\mu\)m.
lacking chromophore never show NB formation (Kircher et al., 1999) and FR alone is also not inductive (Gil et al., 2000; Kircher et al., 2002). Yet the disappearance of phyB NBs in the dark, even after FR treatment, is a slow process that takes hours. The amount of FR applied in these experiments is sufficient to convert all phyB $P_{fr}$ into $P$, thus this FR treatment should result in the rapid decomposition of phyB NBs, which is obviously not the case.

**Molecular mechanism(s) for nucleo-cytoplasmic trafficking of phytochromes**

The transport of proteins from the cytoplasm into the nucleus exclusively occurs through the Nuclear Pore Complexes (NPCs) that span the nuclear envelope and allow the passage of molecules between the two compartments (for reviews see Stöffler et al., 1999; Merkle, 2003; Lim and Fahrenkrog, 2006; Tran and Wente, 2006). Smaller molecules below the size of 40 kDa can diffuse through these pores passively. The transport mechanism by which large proteins or their complexes are moved to the other side of the nuclear envelope is called facilitated translocation; in contrast to diffusion, it can operate against the concentration gradient (Ribbeck and Gorlich, 2001).

Phytochromes, these large dimeric proteins are transported to the nuclei via this active, energy-consuming process, which requires the action of importins. Nuclear translocation is normally initiated by the recognition of the target protein by the transport receptor (e.g. importin $\beta$-like receptors), followed by docking of the substrate to the nuclear envelope and its translocation through the nuclear membrane. Importins have two subclasses, namely importin $\alpha$-like and importin $\beta$-like proteins (Merkle, 2003). Importin $\beta$-like receptors are components of the nuclear transport that anchor the transportable protein to the NPC by direct binding of the nucleoporins and the target protein itself. Being hydrophobic but soluble proteins, the importin $\beta$-like receptors can facilitate the transport of large proteins or their complexes through the nuclear pores. Within the nuclei importin $\beta$ interacts with the GTP-binding form of RanGTPase and releases the target protein. The importin $\beta$ receptor-RanGTP complex recycles back to the cytoplasm, where GTP is hydrolysed by Ran and the complex dissociates. Import of proteins carrying classical basic nuclear localization signals (NLS) needs the action of another type of importin molecule, importin $\alpha$, which functions as the cytoplasmic NLS receptor (Gorlich et al., 1995). In contrast to the transport receptor importin $\beta$, importin $\alpha$ cannot leave the nuclear compartment on its own. Recycling of importin $\alpha$ to the cytoplasm therefore requires an additional transport receptor, which specifically exports importin $\alpha$ from the nucleus (Kutay et al., 1997).

**Do PHYs have NLS for nuclear targeting?**

If phytochromes contain putative NLS signals, importins can be involved in targeting these photoreceptors into or out of the nucleus. Sakamoto and Nagatani (1996) examined the nuclear localization of 35S:phyB::GUS fusion protein. They showed for the first time that the C-terminal part of phyB is transported into the nucleus, which suggested that an active NLS(s) is present in this part of the molecule. Additional studies showed that, although the carboxy terminus of phyB translocates into the nuclei and forms NBs, yet it fails to complement phyB null mutants (Matsushita et al., 2003). However, it has also been shown that nuclear transport of the C-terminal part of PHYB and the formation of NBs thereof occurs constitutively, irrespective of light conditions (Nagy et al., 2000, 2001; Nagy and Schafer, 2002). These data suggested that the transport machinery is able to recognize and target the PHYB C-terminal part into the nuclei, but light dependence of the nuclear transport requires the presence of the N-terminal region of phyB. On the other hand, it was found that the N-terminal half of phyB does not translocate into the nuclei and fails to form NBs and to complement phyB null mutants, indicating that the nuclear transport machinery is unable to recognize the truncated phyB protein.

If so, what region of phyB is responsible for initiating light-induced signalling? To answer this question, Matsushita et al. (2003) constructed a chimeric gene that contained the N-terminal half of phyB fused to the GUS reporter and a functional NLS or NES (nuclear exclusion signal) and expressed this fusion protein in the phyB-5 null mutant. Their results convincingly showed that (i) the N-terminal part of phyB is sufficient to initiate phyB-dependent light signalling, but only when it is localized in the nuclei and (ii) the C-terminal part is responsible for dimerization, nuclear translocation, and the formation of late, stable phyB NBs. Interestingly, they also showed that a missense mutation located in the C-terminal region prevents efficient translocation of the full-length phyB into the nucleus.

By this time, it was firmly established that translocation of phyB into the nucleus is conformation dependent, that chromophore minus PHYB molecules are not imported into the nucleus, and FR treatment applied immediately after R induction blocks nuclear transport (Nagy and Schafer, 2002), which requires at least the formation of a phyB $P_{fr}$/P$_{fr}$ heterodimer (Chen et al., 2003). One way to interpret these data is to assume that in the inactive P$_{fr}$ conformation the N-terminal half of the phy molecule masks the NLSs of the C-terminal part, whereas in the P$_{fr}$ form the NLS is unmasked and becomes accessible for the transport machinery. Findings reported by Chen et al. (2005) support this hypothesis. These authors showed that the N-terminal and the C-terminal parts of phyB can
interact in yeasts, and this interaction is weakened by light irradiation which induces the conformational change of the molecule, leading to P$_{r}$-P$_{f}$ transition. In vitro pull-down assays showed that, when co-expressed with PCB, the interaction between the N- and C-terminal parts of the molecule is much stronger than in the inactive P$_{r}$ form. Thus, reduction of the intramolecular interaction between the two parts of the molecule may lead to the presentation of a hidden NLS in the C-terminal region and ultimately to nuclear translocation of the photoreceptor.

To narrow down the region which may contain nuclear targeting signals, a series of different C-terminal truncations of the Arabidopsis phyB molecule were created and the nuclear transport of YFP fusions of these molecules was examined by the same authors. Chen et al. (2005) reported that (i) the very C-terminal HKRD domain alone is not sufficient for mediating translocation of the fusion protein into the nuclei, whereas (ii) the PRD::YFP protein translocates into the nuclei upon light illumination but forms no stable NBs. These data indicated that the PRD domain is required for nuclear transport, but the precise mechanism responsible for importing phyB into the nucleus remained largely elusive for the following reasons. Sequence analysis of phytochromes revealed no canonical NLS within the PHYB. The N-terminal half of the molecule fused only to GUS (which ensures dimerization of the fusion protein) showed limited, although not light-regulated nuclear localization and partial complementation of the phyB null mutant (Matsushita et al., 2003). Simple diffusion can be excluded as the cause of the nuclear localization of the phyB N-terminal-GUS monomer or dimer proteins. Hence these data indicate that (i) the N-terminal region also contains a weak NLS, (ii) the nuclear transport of phyB is mediated by multiple NLSs or (iii) it does not require an NLS or NLS-like domain.

Are FYH1 and FHL involved in mediating nuclear import of PHYA?

The size- and light-dependent, very fast accumulation of phyA in nuclei absolutely requires efficient active transport of the molecule into the nucleus. If so, the transport machineries mediating nuclear import of phyA and phyB are presumably different, since nuclear accumulation of 35S:phyB::GFP is a relatively slow process. Alternatively, it can be hypothesized that phyA contains multiple NLSs with higher affinity for recognition, or unmasking of these NLSs occurs faster in vivo in the cytoplasm than in the case of phyB. Recent observations published by Hiltbrunner et al. (2005, 2006) suggest that phyA is imported into the nuclei as part of a preformed protein complex rather than as a single protein. These authors documented that the nuclear accumulation of phyA requires at least two small, plant-specific proteins: FAR RED ELONGATED HYPOCOTYL 1 (FYH1) and its homologue FYH1 LIKE (FHL). FYH1 and FHL were shown to be positive regulators of phyA signalling during de- etiolation (Desnos et al., 2001; Zhou et al., 2005). Detailed analysis of the fhy1 mutant revealed that nuclear accumulation of phyA during HIR and also VLR is strongly reduced in this mutant background (representative fluorescent microscopic images are shown in Fig. 4A). Recently it was shown that in the fhy1/fhl double mutant the light-induced nuclear accumulation of PHYA:phyA::GFP and or 35S:phyA::GFP is completely abolished, suggesting an important role for these two molecules in the nuclear transport mechanism of phyA (Hiltbrunner et al., 2006).

Interestingly, each of FYH1 and FHL has a functional monopartite NLS and a NES close by and it was shown that FYH1 and FHL can form homo- or heterodimers via binding through the C-terminal part of the molecule (Zhou et al., 2005). Loss-of-function mutations of both molecules lead to a complete loss of sensitivity to FR light, which indicates an indispensable role for these two molecules in FR-light-induced, phyA-mediated responses of Arabidopsis (Zhou et al., 2005) (Fig. 4B shows the hyposensitive growth responses of fhy1 and fhy1/FHL RNAi plants grown in cFR light). In addition, Zeidler and co-workers (2004) showed that in dark-grown seedlings the YFP::FYH1 fusion protein is present in both the nuclei and the cytoplasm. A brief light treatment initiates formation of 35S:YFP::FYH1 NBs, which is reminiscent of the nuclear bodies formed by 35S:phyA::GFP. Similar intracellular distribution was observed for 35S:YFP::FHL (Hiltbrunner et al., 2006). Moreover, the presence of the NLS on the N-terminal half of FYH1 was essential for nuclear targeting of FYH1 and deletion of the NLS resulted in the loss of function of FYH1 in FR-light-induced, phyA-mediated inhibition of hypocotyl elongation (Zeidler et al., 2004).

Based on these observations, Hiltbrunner et al. (2005, 2006) performed a set of experiments that provided evidence for the involvement of FYH1/FHL in facilitating import of phyA into the nucleus. These authors showed by in vitro pull-down assays that phyA interacts both with FYH1 and FHL in a light-dependent fashion, preferentially in its P$_{f}$ form. The 406 amino acid-long N-terminal fragment of phyA is sufficient for binding these two factors and this interaction does not depend on the presence of the very N-terminal region (first 100 amino acid residues) of phyA. These data indicate that FYH1/ FHL binding is mediated by the chromophore-lyase domain of phyA. Moreover, when etiolated plants are irradiated with light, FYH1 and FHL co-localize with phyA in nuclear bodies.

These data indicate that FYH1/FHL may mediate nuclear accumulation of phyA by two different, though not necessarily mutually exclusive mechanisms. Since no obvious NLSs were found in the PHYA sequence, it is
possible that phyA uses the NLS of FHY1/FHL for its nuclear transport, via direct binding to homo- or hetero-dimers of these two proteins. In this case, light unmasks the binding site for FHY1/FHL on phyA, and nuclear transport occurs only after the complex had been formed (Fig. 4C shows the possible mechanism of light-induced nuclear transport of phyA::GFP). Another possible explanation is that FHY1/FHL works as a nuclear anchoring factor rather than a nuclear import factor. In this model, phyA possesses NLS and NES signals and phyA Pr recycles fast between the cytoplasm and nucleus in the dark. After light activation the Pfr form of phyA interacts with FHY1/FHL. This interaction masks the NES signal of phyA, thus the phyA-Pfr/FHY1/FHL complex is retained in the nucleus. Consistently with these models, it is also possible that FHY1 and/or FHL mediate(s) both import and retention of phyA in the nucleus, thus they are also involved in the light-induced degradation of phyA.

These models suggest that, similarly to phyB, the N-terminal half of phyA may play a major role in phyA-mediated signalling. Indeed, it was shown that a chimeric protein containing the N-terminal half of phyA fused to GFP-GUS-NLS can induce VFLR-type signalling but fails to function in FR-HIR mode in a phyA null mutant (Mateos et al., 2006). However, it should be noted that this short N-terminal fragment of phyA apparently generates signals in constant darkness and causes hypocotyl shortening and cotyledon unfolding of etiolated seedlings reminiscent of a weak *Constitutive Photomorphogenesis 1* (*cop1*) allele (Mateos et al., 2006). Thus it is possible that either the Pfr form of the phyA N-terminal fragment localized in the nucleus partially induces photomorphogenesis by an unknown mechanism, or light treatment given on the first day of seedling growth to induce germination is enough to initiate the formation of Pfr and its subsequent transport into nuclei. Independent of
these explanations, this result indicates that, in contrast to phyB, phyA requires additional domains of the molecule, in addition to its N-terminus, to fulfil its role in signalling.

**Does the phosphorylation status of phytochromes play a role in the light-dependent nuclear translocation of phytochromes?**

The phosphorylation status of phytochromes can obviously influence transportability of the molecule. It was shown that phytochromes can act as kinases and they are also subjected to phosphorylation. Indeed, it was shown, that oat phyA (McMichael and Lagarias, 1990) and *Mesotaenium caldariorum* phytochrome are autophosphorylated on Ser/Thr residues (Yeh and Lagarias, 1998) in a light-dependent manner. Therefore it is widely assumed that all phytochromes are light-regulated atypical Ser/Thr protein kinases, which autophosphorylate and phosphorylate other proteins. It has been hypothesized that (auto)phosphorylation of phytochromes may influence the P<sub>I</sub> to P<sub>F</sub> conformational change, therefore changes in the autophosphorylation pattern of phytochromes could be essential for the regulation of the intracellular distribution of these photoreceptors. The Ser<sub>7</sub> residue of phyA was shown to be phosphorylated in vivo, however its phosphorylation occurs in both P<sub>I</sub> and P<sub>F</sub> forms, whereas Ser<sub>17</sub> was shown to be phosphorylated primarily in the P<sub>I</sub> form of the protein in the in vitro experiments. Phosphorylation at these residues induces subtle conformational changes within the molecule (Lapko et al., 1997, 1999). It was shown by Kim et al. (2002) that the Arabidopsis phosphatase 2A (FyPP) interacts with oat phyA in vitro in a light-dependent manner. The over-expression of this phosphatase leads to enhanced responses of plants to light probably by causing hypo-phosphorylation of phyA, whereas decreased expression of FyPP causes reduced phy-A activity. However, cellular distribution of phyA has not been investigated by the authors. In a more recent report, Ryu et al. (2005) convincingly demonstrated that the flux of light signal from P<sub>F</sub>-phytochromes is tightly controlled by phosphorylation/dephosphorylation of the photoreceptors. These authors showed that another phosphatase, designated type 5 protein phosphatase (PAPP5) specifically dephosphorylates biologically active P<sub>F</sub>-phytochromes (phyA and phyB) and enhances phytochrome-mediated responses by modulating phytochrome stability and affinity for downstream transducers. Nuclear import of phyA and phyB, however, was not significantly affected by over-expressing PAPP5. In oat phyA, the Ser<sub>598</sub> residue located in the hinge region of the molecule is preferentially phosphorylated in vivo in the P<sub>I</sub> form. When Ser<sub>598</sub> is mutated, the mutant oat phyA exhibits hypersensitivity to FR light (Kim et al., 2004). These authors also showed that phosphorylation at Ser<sub>598</sub> inhibits interaction of phyA with two well-known signalling partners, NDK2 and PIF3, thus inhibiting the signalling from phyA localized in the nucleus, whereas dephosphorylation of phyA at the same position by a phosphatase could amplify the signals. Ser<sub>598</sub> is not autophosphorylated and is not required for kinase activity of phyA (Kim et al., 2004), thus an as yet unidentified protein kinase could play a regulatory role in phosphorylation of this residue. The same authors reported no difference between the intracellular distributions of the native and the mutant phyA, indicating that the Ser<sub>598</sub> mutation does not affect nucleo-cytoplasmic distribution, i.e. nuclear import of phyA. Taken together, these results indicate that nuclear translocation of phyA is not significantly affected by phosphorylation or dephosphorylation of the photoreceptor.

**Mutations within phytochromes alter the subcellular distribution of the photoreceptors, but do they really affect nuclear import?**

Nuclear import and intracellular redistribution of phyA is specifically affected by point mutations identified in various domains of the photoreceptor. For example, it was shown that several missense mutations located in the Quail-box of the *Arabidopsis* PHYA or PHYB genes (Wagner et al., 1996) prevented high-level accumulation and formation of stable nuclear bodies containing the mutant photoreceptor proteins in the nuclei (Kircher et al., 1999). It is worth noting that these mutants also displayed hyposensitivity to cFR or cR, thus suboptimal physiological responses of these mutants correlated with the aberrant intracellular distribution pattern of the photoreceptor. These results indicated that the formation of stable phyA and phyB NBs may affect signalling by these photoreceptors. The phyA-302 mutant, carrying a missense mutation in the so-called PAS2 domain in the C-terminal part of the photoreceptor retained normal VLFR but lacked HIR. The 35S:phyA-302::GFP fusion protein showed normal translocation from the cytosol to the nucleus under cFR, but failed to produce nuclear bodies containing the mutant photoreceptor. These observations suggested that phyA NBs could be involved in mediating HIR signalling (Yanovsky et al., 2002). It is thought that the PAS1-Quail-box-PAS2 region of PHYA and PHYB is involved in mediating the interaction of phyA and phyB with their cognate signalling partners. Therefore, these mutations may have a discernible effect on the nuclear import process itself. The EID4 mutation is localized in the chromophore binding domain (BLD) of phyA (Dieterle et al., 2005). The mutant phyA protein is more stable but displays normal photochemical properties. The *eid4* mutant was shown to form phyA NBs, the appearance of which is transient, i.e. they dissolve in 60–90 min
after the onset of irradiation. In cFR light, a few hours after the lights-on, larger nuclear bodies appear which are stable for 8 h. By contrast with wild-type phyA, phyA<sub>埃德4</sub> can re-accumulate in these NBs even after a single pulse of R. In dark-grown plants, the mutant phyA::GFP showed normal cytosolic distribution. Upon R illumination, however, unlike the native phyA the phyA<sub>埃德4</sub> fails to form cytosolic speckles (SAPs). The <i>eid4</i> mutant is hypersensitive in cFR, displays increased inhibition of hypocotyl elongation and phyA<sub>埃德4</sub> appears in nuclear bodies even after a brief light pulse. This can be explained by the increased stability of the phyA<sub>埃德4</sub> P<sub>r</sub> form. Alternatively, the mutant molecule stays for a longer time in NBs (associates faster and dissociates slower) which results in higher stability of the mutant photoreceptor. In either case, more P<sub>r</sub> is available for a longer time in the nuclei of <i>eid4</i>, which could explain the hypersensitivity of the mutant.

Chen and co-workers (2003) performed a screen to find mutants which show mislocalization of 35S:phyB::GFP protein when seedlings are grown in cR. The mutants were divided into two categories. The first group contained mutants which had missense point mutations within the 35S:PHYB::GFP transgene. The examination of the translocation pattern of these molecules showed that mutations in the hinge region and in the C-terminal PAS-related domain do modify intranuclear localization of phyB. Although all mutant proteins were transported into the nuclei in light-grown seedlings, they were distributed homogenously within the nucleus and none of them formed stable phyB NBs (Chen et al., 2003). The second group of mutants, called <i>Dsf</i> (Deficient in Speckle Formation) also showed nuclear transport of phyB and were able to form early stage II and III but not stage IV type phyB NBs. Since the formation of these late, large, stage IV nuclear bodies is induced by high-fluence R irradiation, the DSF proteins are presumably required to mediate these responses. The identity and molecular nature of these DSF proteins is not yet known.

#### Perspectives

phyA–E-mediated signalling requires nuclear translocation of the photoreceptors and interaction with their cognate signalling partners. Phytochromes interact with a number of proteins and phytochrome-containing nuclear bodies have been described as sites of co-localization of phytochromes with CRY2 (Mas et al., 2000), COP1 (Seo et al., 2004), and PIF3 (Bauer et al., 2004; Al-Sady et al., 2006). The functional importance of these and other phy-containing nuclear bodies is a subject of intense discussion, but relevant experimental data to solve this problem are not available. For example, it is not known whether these apparently different NBs indeed represent structurally and functionally different nuclear complexes and/or whether they contain, beside the few detected proteins, additional specific and common components. Thus it could be concluded that although it is tempting to speculate about the possible function of phy NBs, genetic (specific mutants specifically affecting NB formation) and biochemical data (identification of components present in different NBs) are needed to define the function of these subnuclear complexes in phytochrome-mediated light signalling.

#### Acknowledgements

Work in Hungary was supported by Hungarian Scientific Research Fund (OTKA 60106) and Howard Hughes Medical Institute International Research Scholar grants to FN. Work in Germany was supported by a Humboldt Research Award grant to FN and an SFB 746 grant to ES. Eva Kevei is supported by a grant from the Humboldt Research Foundation. We thank Erzsebet Fejes for critical reading of the manuscript and for providing unpublished results for Fig. 3. Special thanks to Andreas Hiltbrunner for providing the images and the figure describing the function of FHY1 and FHL in light-induced nuclear transport of phyA and to Stefan Kircher and Lana Kim for providing the fluorescent microscopic images showing the 35S:phyA::GFP nuclear localization pattern under different light conditions.

#### References


Clack T, Mathews S, Sharrock RA. 1994. The phytochrome apoprotein family in Arabidopsis is encoded by five genes: the
sequences and expression of PHYD and PHYE. Plant Molecular Biology 3, 413–427.


