Interaction with plant transcription factors can mediate nuclear import of phytochrome B

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Phytochromes (phy) are red/far-red-absorbing photoreceptors that regulate the adaption of plant growth and development to changes in ambient light conditions. The nuclear transport of the phytochromes upon light activation is regarded as a key step in phytochrome signaling. Although nuclear import of phyA is regulated by the transport facilitators far red elongated hypocotyl 1 (FHY1) and fhy1-like, an intrinsic nuclear localization signal was proposed to be involved in the nuclear accumulation of phyB. We recently showed that nuclear import of phytochromes can be analyzed in a cell-free system consisting of isolated nuclei of the unicellular green algae *Acetabularia acetabulum*. We now show that this system is also versatile to elucidate the mechanism of the nuclear transport of phyB. We tested the nuclear transport characteristics of full-length phyB as well as N- and C-terminal phyB fragments in vitro and showed that the nuclear import of phyB can be facilitated by phytochrome-interacting factor 3 (PIF3). In vivo measurements of phyB nuclear accumulation in the absence of PIF1, -3, -4, and -5 indicate that these PIFs are the major transport facilitators during the first hours of deetiolation. Under prolonged irradiations additional factors might be responsible for phyB nuclear transport in the plant.

Arabidopsis | basic helix-loop-helix | signal transduction

Plant development is strictly regulated by the environmental light conditions. Even though light is ubiquitously present, spectral composition, intensity, and light direction varies depending on the local environment. To adapt on local light conditions plants are equipped with a highly developed repertory of photoreceptors (1, 2).

Plant phytochromes are mainly involved in detection of red and far-red light wavelengths of the spectrum. A family of five members in *Arabidopsis thaliana* regulates the major developmental steps in the life of the plant: germination, photomorphogenesis, and flowering (3, 4). Despite the sequence similarities and identical spectroscopic features, the two major phytochromes, phyA and phyB, show overlapping as well as unique photosensory and functional characteristics (5–7). The light-stable phyB mediates red/far-red reversible low fluence response, whereas the light-labile phyA, is involved in far-red light sensing (high irradiance response) and in responses to very weak light (very low fluence response, VLFR) (8).

Phytochromes are cytosolic proteins of 125 kDa that are translocated into the nucleus upon light activation (6, 9–11). Nuclear transport is an essential step in phytochrome signaling and a prerequisite for the interaction with transcription factors in the nucleus (12, 13). Concerning the kinetics of nuclear import, severe differences can be found between the two major plant phytochromes, phyA and phyB (6). PhyA cannot be detected in nuclei of dark-grown plants but is rapidly transported into the nucleus upon light irradiation. In contrast, low levels of phyB are present in the nucleus even in dark-grown seedlings. The nuclear accumulation of phyB does not reach its maximum until 4 h after irradiation, which is at least one order-of-magnitude slower than the nuclear transport of phyA (6). The diverse kinetics of phytochrome

nuclear accumulation suggests two distinct mechanisms responsible for the nuclear transport of phyA and phyB.

Recent studies showed that the nuclear transport of phyA depends on the two paralogous proteins far red elongated hypocotyl 1 (FHY1) and fhy1-like (FHL) (13–16). Both proteins interact in a light-dependent manner with phyA and bear a functional nuclear localization signal (NLS) and nuclear exclusion signal (13, 14, 17–20). In the absence of FHY1 and FHL phyA remains in the cytoplasm and nuclear accumulation of phyA is no longer detected after light irradiation (13). In vitro studies and the fact that an NLS fused to the phyA can complement the *fhy1*-mutant phenotype revealed a “piggy-back” mechanism as basis of the phyA nuclear transport (16, 21). The recognition of the phyA-FHY1/FHL complex by the nuclear import machinery is mediated by the NLS of FHY1 and FHL. It was reported that a functional NLS and a phyA-binding-site of FHY1 are necessary and sufficient to promote the light-dependent nuclear transport of phyA (16). PhyB nuclear transport seems not to be affected in the absence of FHY1 and FHL (13).

Until now there has been no evidence for a similar mechanism involved in the nuclear import of phyB. Sequence analysis pointed to a bipartite NLS located in the C terminus of the phyB molecule (22). In vivo localization studies showed that the C-terminal half of phyB that lacks the chromophore binding domain indeed has the ability to localize in the nucleus (23, 24). Based on these results, a model of a light-dependent unmasking of an NLS that is located in the C terminus of phyB was proposed as a mechanism of light-dependent nuclear import (24). This hypothesis does not take into consideration that the N-terminal half of phyB also showed in vivo nuclear localization (23). However, no light-dependency has been observed for the nuclear localization of this N-terminal half, although this fragment showed normal spectral activity (23).

We recently showed that a unique in vitro system consisting of isolated nuclei of the green algae *Acetabularia* is suitable to study the nuclear import of the plant photoreceptor phyA (21). This system is independent of exogenously added ATP or cytosolic factors. Nuclear import in as well as nuclear export out of manually isolated nuclei of *Acetabularia* showed the same characteristics as in vivo. Because no functional phytochrome has been identified in *Acetabularia* up to now (25–27), the system brings ideal prerequisites to investigate nuclear transport of phytochromes.

Here we use this system to test the nuclear transport characteristics of full-length phyB as well as N- and C-terminal phyB fragments in vitro and show that the nuclear import of phyB can

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be reconstituted by transport factors that bear an NLS and interact with phyB. We additionally show that in vivo nuclear accumulation of phyB is reduced in the *pifq* background that is lacking the bHLH transcription factors phytochrome-interacting factor (PIF)1, -3, -4, and -5 (28). We thus propose that nuclear transport of phyB requires transport facilitators, as for example the PIF proteins, and is not accomplished by an intrinsic NLS.

Results

To test nuclear transport in vitro, fluorescent-labeled phytochromes were incubated for 1.5 h together with the isolated nuclei of *Acetabularia* at room temperature. The samples were either kept in darkness or were irradiated with red or far-red light. Before microscopic analysis, the nuclei were washed with fresh buffer to remove the proteins that remained in the surrounding buffer.

We first tested whether the ability to accumulate actively in the nucleus is an intrinsic feature of the phyB molecule itself. Therefore, we purified phyB-GFP-TAP fusion protein from 6-wk-old light-grown transgenic plants. Difference spectra of the purified phyB-GFP-TAP showed that it was spectroscopically active (Fig. 1B). Because of its ability to form homodimers, endogenous phyB was copurified by the phyB-GFP-TAP out of the wild-type plants (Fig. 1A).

When phyB-GFP-TAP was added to isolated nuclei of *Acetabularia* and incubated under red light, we could not detect phyB-GFP-TAP import into the nucleus (Fig. 1C). We thus conclude that phyB does not contain a functional NLS, irrespective of whether it is in the Pr- or Pfr-form.

Fig. 1. PIF3 acts as a transport facilitator for phyB nuclear import in vitro. (A) phyB-GFP-TAP was purified from plants. Lane 1 shows the analysis of the purified proteins on a silver-stained SDS-polyacrylamide gel and lane 2 shows the corresponding immunoblot analysis using a polyclonal antibody against phyB. M, Marker bands are denoted in kilodaltons. (B) The purified phyB-GFP-TAP shows spectral activity. The difference absorbance spectra were recorded after irradiation with saturating far-red (Pr-Pfr) and red (Pfr-Pr) light. (C) Isolated *Acetabularia* nuclei were incubated with phyB-GFP-TAP. Nuclear transport of phyB-GFP-TAP was only induced in the presence of PIF3, but not when phyB-GFP-TAP was added solely to the system. Incubations took place either under continuous red light (R), far-red light (FR), or in darkness (D). (DIC images are shown *Left*, fluorescence images are shown *Right*). (Scale bars, 20 μm.)

Hence, we tested whether the import of phyB is, like in the case of phyA (21), also dependent on transport facilitators. Such a transport facilitator should possess an NLS and should be able to interact with phyB in a light-dependent manner. Because these properties have already been shown for the basic helix-loop-helix transcription factor PIF3 (29), we tested whether PIF3 can induce nuclear transport of phyB in our in vitro test system. A GST-tagged PIF3 was expressed in *Escherichia coli* and purified via glutathione-Sepharose. For nuclear transport studies the GST-tag was proteolytically removed. When PIF3 was added together with phyB-GFP-TAP to the isolated nuclei, a nuclear import of phyB could be detected (Fig. 1C). PIF3-induced nuclear import of phyB occurred under irradiation with red light and far-red light, as well as after preirradiation with RG9 light and subsequent incubation in darkness (Fig. 1C).

Previous studies analyzed the phyB-PIF3 interaction in details and could show that both the N and C termini of phyB can interact with PIF3 (29, 30). We therefore tested if PIF3 is also able to facilitate the import of these two large phytochrome fragments. The phyB-fragments phyBN (amino acids 1–450) and PHYBC (amino acids 594–1172) were expressed in *E. coli* as GST-fusion-proteins and purified on glutathione-affinity columns (*Material and Methods*). PhyBN was reconstituted in vitro with the chromophore phycocyanobilin to obtain spectral activity and in case of PHYBC the GST-tag was proteolytically removed before labeling with fluorochromes. As expected, neither phyBN nor PHYBC accumulated in the isolated nuclei when they were added solely to the in vitro assay (Fig. 2). When we tested the influence of PIF3 on the nuclear transport of the fluorescent-labeled phyBN and PHYBC we observed that PIF3 induced the nuclear import of both phyB-fragments (Fig. 2). Additionally we could detect a clear light-dependency of the nuclear import of phyBN in the presence of PIF3. PIF3 induced nuclear transport of phyBN under red light but not under far-red light (Fig. 2).

To narrow down the requirements for a protein that can promote the transport of phyB into the nucleus, we designed a synthetic protein consisting of the SV40 large T-antigen NLS and phyB-binding domain of PIF3 (APB) (31). When the purified synthetic protein NLS-APB was added together with fluorescent-labeled phyBN and PHYBC to the in vitro nuclear transport assay, we could observe exactly the same results as for PIF3. NLS-APB induced nuclear transport of PHYBC as well as phyBN, the latter in a light-dependent manner (Fig. 2). Regarding the nuclear transport of phyB, the NLS and the phyB-binding domain seem to be sufficient for PIF3 function.

Earlier studies in *Arabidopsis* showed that nuclear complex formation of phyB depends on PIF3 (32). In contrast to the situation in vivo, phyB did not show accumulation in nuclear complexes in any of the analyzed *Acetabularia* nuclei. Although PIF3 and NLS-APB clearly mediated nuclear import of phyB, we never observed nuclear speckles in the isolated nuclei from *Acetabularia* (Figs. 1C and 2).

To further test the specificity of our findings, we used a mutated phyB for the import assays. It was shown previously that the point-mutation G767-to-R in the C-terminal half of phyB, which is impaired in PIF3-binding, does not localize in the nucleus in vivo (23, 30, 33). Surprisingly, this mutated phyB was capable of transducing the phyB signal when fused to an NLS (23). We analyzed whether the ability to bind PIF3 can directly be linked to the loss of nuclear accumulation of the mutated phyB. We therefore expressed a C-terminal fragment of phyB that carried the point-mutation G767-to-R (PHYBC-G767R) as a GST fusion-protein in *E. coli*. The purified protein GST-PHYBC-G767R was labeled with Alexa Fluor 488 and incubated together with isolated nuclei of *Acetabularia*. GST-PHYBC-G767R did not accumulate in the isolated nuclei and nuclear transport could also not be induced in the presence of PIF3, which is in contrast to the wild-type fragment (Fig. 2). Thus, the G767-to-R point-mutation clearly prevents the PIF3 induced nuclear import of the C-terminal phyB fragment.

Fig. 2. PIF3 as well as the artificial protein NLS-APB facilitated the nuclear transport of phyB-fragments. Isolated *Acetabularia* nuclei were incubated with N- or C-terminal phyB-fragments that had been labeled with Alexa Fluor 488. Neither phyBN nor PHYBC were imported in to isolated nuclei when they were added solely to the system. Nuclear transport of both fragments was induced in the presence of PIF3, as well as the artificial protein NLS-APB. For the nuclear transport of phyBN a light dependency was observed. When a point mutation (G767 to R) was introduced in the C-terminal phyB-fragment, PIF3 did not induce nuclear transport of PHYBC-G767R. Incubations took place either under continuous red light (R), far-red light (FR), or in darkness (D). (DIC images are shown *Left*, fluorescence images are shown *Right*). (Scale bars, 20 μm .)

Even though PIF3 did mediate nuclear transport of phyB in our *in vitro* system, previous *in vivo* localization studies of phyB-YFP fusion proteins in *pif3* mutants could still detect phyB in the nucleus (32). If PIF3 is involved in nuclear import of phyB *in vivo*, the nuclear transport of phyB might not be dominantly regulated by a single factor. We therefore analyzed nuclear

accumulation of phyB-YFP in the *pifq* background that is not only lacking PIF3 but also PIFs 1, 4, and 5 (28). Dark-grown seedlings that expressed the transgene *PHYB-YFP* in the *pifq* and wild-type background were irradiated with red light before we measured nuclear accumulation of phyB-YFP. All pictures were taken with identical exposure time and used for subsequent quantification of the fluorescence intensity (*Material and Methods*). The quantitative data indicated that under nonsaturating conditions (weak or strong R, 4.5 h, 13 or 43 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 656 nm) the nuclear import of phyB-YFP was clearly reduced when we compared two independent lines expressing phyB-YFP in the *pifq* background to the control plants (Fig. 3). In parallel, we performed the quantification of nuclear phyB-YFP with seedlings that were kept for 12 h in white light instead of red light. In contrast to the nonsaturating conditions, the phyB-YFP levels in nuclei of *pifq* showed only a slight reduction of nuclear accumulation compared with wild-type plants (Fig. 3A).

We also followed nuclear accumulation of phyB-YFP over time to analyze whether nuclear import of phyB was not only less sensitive to light intensity but also slower in the absence of PIF1, -3, -4, and -5. Nuclear accumulation of phyB-YFP measured 60 and 90 min after the onset of irradiation with red light was therefore compared with the level of phyB-YFP already present in the nuclei of dark-grown plants (Fig. 3B). Our measurements revealed that the steady import of phyB-YFP was clearly detectable after 60 and 90 min, when we analyzed seedlings expressing the transgene in the wild-type background. In contrast, we could not measure any increase in phyB-YFP accumulation in the nuclei of seedlings that expressed phyB-YFP in the *pifq* background. Even after 90-min irradiation with red light, phyB-YFP levels in the nuclei did not rise above the dark level in the absence of the four PIF proteins.

When we compared the protein level of the transgene in *pifq* and wild-type backgrounds, we could detect that phyB-YFP was expressed at even higher levels in the *pifq* background than in our control line (Fig. S1). The reduced accumulation of phyB in the nuclei of *pifq* seedlings can therefore not be explained by an overall reduction of phyB level in these plants. Nuclear transport of phyB-YFP was therefore clearly reduced—but not completely abolished—in the absence of the bHLH transcription factors PIF1, -3, -4, and -5.

It was hypothesized that FHY1 and FHL specifically evolved to regulate phyA nuclear transport. Because our *in vitro* studies propose that the nuclear transport of phyB could depend on transcription factors that interact with phyB, we were curious whether *in vitro* nuclear transport of phyA can be achieved independently of the already known transport helpers FHY1 and FHL. Such

Fig. 3. Quantification of phyB-YFP nuclear accumulation in the *pifq* and wild-type background. Nuclear import of phyB-YFP was analyzed in 5-d-old etiolated seedlings expressing P35S::PHYB-YFP in the *pifq* (two independent lines *pifq-03* and *pifq-11*) and wild-type background by quantification of epifluorescence microscopy pictures. (A) Mean gray value of nuclei was analyzed after irradiation using either saturating (12-h white light) or nonsaturating (weak R: 4.5 h, 13 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or strong R: 4.5 h, 43 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions. Nuclear fluorescence is given relative to the corresponding wild-type control. (B) Quantification of phyB-YFP accumulation in the nuclei after irradiation with strong red light (43 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) measured over time. $n \geq 18$; error bars, SEM.

a transport mechanism should only have a minor influence of the *in vivo* situation, because the phenotype of *fhy1/fhl* double-mutants resemble the *phyA* mutant in far-red light (18). However, circumstantial evidence indicated the existence of *phyA*-mediated responses also in the *fhy1/fhl* double-mutants. These responses have been explained by a cytosolic function of *phyA* (15), although the required little amount of active *phyA* in the nucleus could have also been imported by an *FHY1/FHL*-independent mechanism.

To test the hypothesis *in vivo*, we expressed another *phyA* interacting PIF protein, namely PIF1 as GST-tagged fusion protein (Material and Methods) and added GST-PIF1, as well as the already used PIF3 together with labeled *phyAN* to isolated nuclei. *PhyAN*, which was not imported in the absence of *FHY1*, was clearly detected in the *Acetabularia* nuclei when either PIF3 or GST-PIF1 was added to the assay (Fig. 4A). In the presence of PIF3 we could even detect a light-dependent nuclear import of *phyAN* (Fig. S2). Based on our *in vitro* data, the induction of the nuclear transport of *phyA* is not only a privilege of *FHY1* and *FHL*, but can also be obtained by the transcription factors PIF1 and PIF3.

We further analyzed the physiological relevance of a *phyA* nuclear transport independent of *FHY1* and *FHL*. Because localization studies with GFP-tagged *phyA* could not detect *phyA* in the nucleus in an *fhy1/fhl* RNAi line, we decided to use a more sensitive method to study *phyA* responses in *fhy1/fhl* double-mutants. We therefore analyzed gene expression of an early responding light-inducible gene, *PRR9* (pseudoreponse regulator 9). *PRR9* is a member of a small family of genes encoding *Arabidopsis* pseudoreponse regulators and is associated to the circadian clock (34). Expression of *PRR9* is rapidly induced after light activation by the phytochromes (35, 36). Real-time RT-PCR analysis in dark-grown wild-type seedlings detected a maximal induction of *PRR9*-expression around 80 min after a 30-s pulse-treatment with very weak red light (Fig. 4B). As expected, *phyA* mutants showed no reaction to this weak-light pulse treatment concerning the *PRR9* expression. In contrast, *fhy1/fhl* double mutants showed a low but significant induction of *PRR9* expression in response to the weak-light pulse. Even though no nuclear transport of *phyA* into the nucleus was detected in absence of *FHY1* and *FHL* *in vivo* (13), we could detect a *phyA*-dependent gene expression in the *fhy1/fhl* double-mutant. Together with the import assays, these experiments support the presence of an *FHY1/FHL*-independent nuclear import for *phyA*. As in the case of *phyB*, the transport of *phyA* might also be facilitated by the PIF proteins.

Fig. 4. Nuclear transport of *phyAN* and analysis of *phyA*-induced *AtPRR9* expression. (A) Isolated nuclei were incubated with *phyAN* that had been labeled with Alexa Fluor 514. Nuclear transport of *phyAN* was induced in the presence of GST-PIF1 as well as PIF3 when incubated under continuous red light (R). (DIC images are shown Left, fluorescence images are shown Right). (Scale bars, 20 μ m.) (B) Expression levels of *AtPRR9* under VLFR-conditions in wild-type (Col), *phyA*-mutant (*phyA-211*), and *fhy1/fhl* double-mutant were analyzed by quantitative RT-PCR. Transcript levels normalized to *ACTIN1* are given relative to dark levels. Data represent the average of two biological replicates (error bars, SEM).

Discussion

Although the mechanism of the nuclear transport of *phyA* was intensively studied in recent years (13–16, 21), the underlying mechanism of *phyB* nuclear import remained less understood. Until now, an intrinsic NLS in the *phyB* molecule itself was proposed (22–24). The results of our *in vitro* studies strongly indicate that *phyB* does not contain an intrinsic NLS, neither in the C- nor N-terminal half. As has been previously shown, for *phyA*, the import of *phyB* can be reconstituted *in vitro* by the addition of transport-helper proteins. We therefore propose that nuclear transport of all phytochromes is based on the cytosolic interaction with transport facilitators that bear an NLS (e.g., transcription factors). The higher affinity to the Pfr-form could be the driving force for the light-dependent accumulation in the nucleus. For *phyA*, with concentrations almost two orders-of-magnitude higher than those of the other phytochromes (3), the *phyA* specific *FHY1*-transport-system evolved that interacts with N terminus of *phyA* (13, 14, 37).

Based on our *in vitro* and *in vivo* analysis of the nuclear transport of *phyB*, we conclude that members of the PIF family might not only play a role in phytochrome signal transduction but could also be involved in nuclear transport of phytochromes. We showed that PIF3 was able to induce nuclear import of *phyB* and *phyB* fragments *in vitro*. Based on the fact that the synthetic protein NLS-ABP was also able to induce nuclear transport of *phyBN* and *PHYBC* in the *in vitro* assay, additional members of the PIF-family (PIF1, -4, -5, -6, and -7) that interact with *phyB* via the APB-domain (31, 38, 39) are potential candidates for additional factors that could be involved in *phyB* nuclear transport. This hypothesis is additionally supported by our *in vivo* analysis of *phyB*-YFP transport in the *piq* compared with wild-type plants. Nuclear accumulation of *phyB*-YFP was reduced in *piq* when we irradiated for 4.5 h with red light and not measurable at all when we analyzed the nuclear accumulation within the first 90 min of irradiation (Fig. 3). Our measurements of *phyB* nuclear import in the absence of PIF1, -3, -4, and -5 indicate that these PIFs are the major transport facilitators during the first hours of deetiolation. However, under prolonged irradiations the PIFs might not be exclusively responsible for *phyB* nuclear transport in the plant; other *phyB* interacting factors might be involved in this process as well.

The finding that PIF3 and potentially also other PIFs can facilitate the nuclear transport of *phyB* was also strongly supported by the results of the analysis of the G767-to-R mutant of *phyB*. It has previously been shown that this mutation affects PIF3 binding (30). Consistent with this finding, we could not induce nuclear import of the mutated *phyB* fragment by adding PIF3 to our *in vitro* assay. In *Arabidopsis* mutants, the phenotype of the G767-to-R point mutation can be rescued by a fusion of an NLS to the protein (23). It is therefore likely that the mutant *phyB* protein lacks the ability to bind to transport facilitators. It is not known whether the point-mutation G767-to-R also influences the binding to other PIF proteins, but a mutation of the PIF binding site in the *phyB*-molecule would explain the severe phenotype concerning the nuclear import of *phyB* compared with the *piq3* mutant.

The interaction between PIF3 and the phytochromes is not restricted to *phyB*. Several publications showed that *phyA* is also able to interact with members of the PIF-family, namely PIF1 and PIF3 (40–42). Consequently, the results of our *in vitro* transport assay indicate that both PIF proteins were able to induce nuclear transport of the N-terminal *phyA* fragment. The finding of an *FHY1*-independent transport protein for *phyA* is also supported by the *PRR9* expression data. The slight induction of *PRR9* expression observed in the *fhy1/fhl* double-mutant could be because of small amounts of *phyA* in the nucleus that are not detectable by fluorescence microscopy. Because the *fhl* mutant contains no *FHL* transcript and the theoretical protein would lack the *phyA*

binding side (18), we currently assume that FHL is really absent in the *fhyl/fhl* double-mutant. Hence, we suggest that phyA responses, especially those that need only few molecules of active phyA in the nucleus, could be accomplished by a phytochrome import mechanism independent of FHY1 and FHL. However, we cannot principally exclude that residual amounts of both proteins facilitate the import of phyA into the nucleus.

Our in vitro and in vivo data clearly show that phyB import into the nucleus does not require an intrinsic NLS. Hence, transport facilitators were identified that could accomplish light-dependent import of phyB into the nucleus. In contrast to the nuclear transport of phyA, the identified putative transport facilitators also induced import of the inactive phyB. This finding could explain the nuclear accumulation of phyB in dark-grown plants. A light-independent interaction between PIFs and the C-terminal part of phyB—as we could demonstrate in vitro by analyzing C-terminal phyB fragments and as has been shown by Ni et al. (29)—might be one explanation for light-independent nuclear transport of phyB in the dark-grown plant. However, we cannot exclude that additional, up-to-now unidentified transport facilitators that interact with the inactive phyB, are responsible for this light-independent nuclear accumulation of phyB.

Regarding the role of PIF1, -3, -4, and -5 in the light-dependent nuclear transport of phyB, one would expect that the *pifq* mutant seedlings should react hyposensitively to red light. However, because these four PIFs also function as transcriptional repressors of photomorphogenesis in darkness, *pifq* seedlings actually display a constitutive photomorphogenic (cop)-like phenotype and are therefore shorter in darkness and light compared with wild-type (28, 43). However, on a molecular level, the *pifq* mutant was shown to be less sensitive to red light than the wild-type. Expression of rapidly light-induced genes that are not dependent on PIF1, -3, -4, and -5 in darkness were studied by Leivar et al. using whole-genome microarrays (these genes were denoted as class 3 and 6 genes in their study) (43). When comparing the gene expression in *pifq* to wild-type, these genes show a reduced level of induction in response to 1-h red light but no regulation by PIFs in darkness. Based on our results, the hyposensitivity of class 3 and 6 genes could be explained by the reduced light-dependent nuclear import of phyB in the *pifq* mutant. Even though their function as repressors of photomorphogenesis is dominating the morphological effects, we still can detect the positive role of the PIFs in phyB signal transduction on the molecular level.

Materials and Methods

For the cloning of the constructs, the purification of the proteins and the TaqMan real-time RT-PCR, see *SI Material and Methods*.

Generation of Transgenic Plants. Transgenic plants expressing the P35S::PHYB-YFP in Col 0 and *pifq* (28) were obtained by *Agrobacterium tumefaciens* mediated transformation. Transformation and selection of transgenic lines was performed as previously described (6).

Isolation of Nuclei from *Acetabularia acetabulum*. For the manual isolation of *Acetabularia* nuclei, we used cells of at least 20 mm in length that were grown and cultivated in artificial seawater Ace 27 (44, 45). For further detail, see Pfeiffer et al. (21).

Nuclear Import Assays. The nuclear import assays were done as described previously (21). The nucleus buffer (46) was supplemented with 0.01 $\mu\text{g}/\mu\text{L}$ of the labeled phytochrome fragments or 0.001 $\mu\text{g}/\mu\text{L}$ of phyB-GFP-TAP. Potential transport facilitators were added in a 10-times molar excess to the phytochromes. Incubations for 1.5 h took place either under continuous red light (R, 2.5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 656 nm), far-red light (FR, 13 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 730 nm), or in darkness (D) after a 5 min RG9 pulse (40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 756 nm). Ten to 20 nuclei were incubated in the same drop of buffer for each experiment. Import experiments were counted as positive when repeatedly more than 40% of the nuclei showed a detectable import signal after the incubation. If less than 10% of the nuclei showed such a signal in a recurrent manner, the assay was considered negative. SDS/PAGE analysis of all proteins that were used for nuclear import assays are shown in Fig. S3.

Plant Material and Growth Conditions. The *phyA-211* mutant (5), *phyA-211/phyB-9* double mutant and *fh1/fhy1-3* double mutant (15) are in the Columbia ecotype background and were therefore compared with the Columbia wild-type in the real-time RT-PCR analysis. Transgenic plants expressing the P35S::PHYB-YFP in Col-0 and *pifq*, were used for the microscopic analysis. All seeds were imbibed for 2 d at 6 °C in the dark and germination was induced by 24 h irradiation with white light.

Quantification of Nuclear Import. We took images of at least 18 nuclei per experiment with a specific YFP-filter set using identical exposure time. The images were transformed into grayscale and analyzed using ImageJ software (National Institutes of Health). The mean gray value per area was calculated for each nucleus.

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