Thesis of Ph.D. dissertation

Molecular analysis of the regulator function of phytochrome photoreceptors in transgenic plants

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INTRODUCTION

Light plays a crucial, dual role throughout the entire life cycle of higher plants. It provides the energy source for photosynthesis; however, light also is an environmental signal regulating a wide range of physiological and developmental processes in plants, from seed germination to flowering. Plants monitor the quantity (intensity), quality (wavelength), direction and duration of light in the ambient environment by specialized photoreceptors. These photoreceptors can be classified by their absorption spectra (Table 1.).

Wavelength of absorbed light	Function
UV-B region: 280-310 nm Receptor unknown	Induction of gene expression
Blue/UV-A region: 320-400 nm Cryptochromes: CRY1, CRY2 Phototropin 1,2 NPL1	Photomorphogenesis Phototropism Chloroplast movement
Red/Far-red region: 620-730 nm Phytochromes:PHYA PHYB PHYC PHYD PHYE	Photomorphogenesis: seed germination, de-etiolation, inhibition of hypocotyl/stem-elongation, shade avoidance. Regulation of flowering time.

Table 1. Photoreceptors and their function in higher plants.

Plant cryptochromes are FAD and pterin-containing chromoproteins showing significant homolgy to DNA photolyases, but lacking photolyase activity. Cryptochromes absorb in the blue region of the spectrum. To date, two members of the cryptochrome family, CRY1 and 2, have been identified in *Arabidopsis*. The CRY2 protein shows rapid blue light-induced degradation and functions primary at low light intensities. The CRY1 protein is relatively stable in light and mediates responses to higher fluences of blue light.

Phytochromes are chromoproteins, which contain a covalently linked linear tetrapyrrole chromophore per molecule and exist as homodimers. These photoreceptor molecules absorb red and far-red light which activates and inactivates them, respectively. In higher plants these molecules are encoded by small multigene families. In *Arabidopsis* five genes (*PHYA-E*) have been isolated. PHYA is a photo-labile molecule degraded rapidly upon exposure to light. It is the dominant phytochrome in etiolated seedlings and it mediates responses to very low fluences of red and far-red light. PHYB, C, D and E are relatively photo-stable molecules; in green seedlings PHYB is the dominant phytochrome photoreceptor. They mediate responses to low and high fluences of red light.

According to the general model for phytochrome-mediated signal transduction, phytochromes are synthesized and localized in the cytosol in the dark in their inactive P_r form. Light signals convert the phytochrome receptors into their active P_{fr} form. This latter form activates a signal-transduction cascade, which drives the signal from the cytosol to the nucleus, resulting in the modulation (induction/repression) of the expression of light-responsive nuclear genes at transcriptional level. Changes in the expression pattern of this special subset of genes lead to dramatic changes in developmental and physiological processes, to the induction of photomorphogenesis (light-dependent development). A number of reports supporting the cytosolic localization of phytochromes and describing primary signaling events in this compartment have been published. Phytochromes can activate the plasmamembrane-bound heterotrimeric G-proteins, which in turn activate signal-transduction by modulating the cytosolic cGMP and/or Ca^{2+}/calmodulin levels and finally induce the expression of the *CHS* (chalcone-synthase) and *CAB* (chlorophyll a/b-binding protein) genes and the development of functional chloroplasts. Light-dependent nuclear import of CPRF2 (common promoter/binding factor 2) and GBF (G-box binding factor) transcription factors is mediated by PHYB. Phytochromes are atypical serin/protein kinases and phosphorylate specific substrates such as the cytosolic PKS1 (phytochrome kinase substrate 1) protein. On the other hand, several phytochrome-regulated genes have been described and cis-acting elements in their promoter regions as well as some of the transcription factors binding to these

regions have been identified, representing the final steps of light-induced signaling. However, the signal transduction pathway from the cytosol to the nucleus has remained largely unknown. Recently, it was shown that nuclei of *Arabidopsis* cells contain higher amounts of immunodetectable PHYB protein in the light than in the dark. Very recently it was reported that the nuclear basic HLH protein PIF3, shown earlier to function in phytochrome signaling *in vivo*, binds to PHYB in a light-regulated fashion *in vitro*. Although these findings do not provide unambiguous evidence, they open up a possibility that phytochromes can be imported into the nuclei after illumination and they can act as components of the transcriptional machinery.

The periodic succession of days and nights is an eternally recurring environmental factor ever since life has appeared on the Earth. It is postulated that (i) organisms possessing the ability to adapt to the predictable changes of the environment have an evolutionary advantage and that (ii) this benefit has promoted the development of timekeeping mechanisms (endogenous clocks). The biological clocks that generate and maintain oscillations of many physiological and molecular processes with a period length close to 24 h are also referred to as circadian clocks. Circadian rhythms persist under constant conditions; however, in order to function reliably and to be useful for the organism, the clocks must operate in harmony with the periodic changes of the outer environment. To achieve this synchrony, the circadian clock is reset to the local time by specific stimuli perceived at dawn and dusk. The most important entraining factors are light and temperature. Light signals are perceived and transduced to the central oscillator via specialized photoreceptors. In plants, the photoreceptor phytochrome and cryptochrome have been shown to be involved in this process. On the other hand, the circadian clock is able to suppress the direct effect of photoreceptor signaling in a phase-dependent fashion by the so-called gating mechanism.

According to the simplest model of the circadian system, the central oscillator generates an oscillation with a period of ~24 h, based on negative feed-back loops formed by the clock genes and proteins, and regulates the expression of genes through the output pathway. On the other side of the system, light signals absorbed by photoreceptors reach the central oscillator through the input pathway and synchronize its

phase to the actual periodic environmental changes. In this model there is a one-way relationship between the input receptors and the oscillator without any feedback mechanisms. However, it has been shown that the oscillator controls the expression of cryptochrome receptors both in *Drosophila* and in mouse. This type of regulation represents an input feedback loop which has not been described in the plant circadian system so far.

RESEARCH OBJECTIVES

The general goal of our research was to study the regulatory role of phytochrome photoreceptors in the photobiological processes and in the circadian system of plants using new ways of approach. The main questions were:

1. Are phytochrome receptors imported into the nuclei? If yes, what are the light-conditions (intensity and wavelength) required for this process and what kinetics characterizes the import?
2. Is the expression of phytochromes regulated by the circadian clock? If yes, at what levels of expression is this effect detectable?

METHODS

- Culturing *Nicotiana tabacum* and *Arabidopsis thaliana* plants under sterile and greenhouse conditions
- Molecular cloning techniques
- Plant genomic D NA extraction
- Plant total RNA extraction
- Northern-blotting, S1 nuclease protection assay
- Western-blotting
- Generation of transgenic plants
- Light, fluorescence and confocal microscopy
- *In vivo* luciferase enzyme activity measurements

RESULTS AND DISCUSSION

1. We have generated transgenic tobacco plants expressing the rice PHYA-GFP or the tobacco PHYB-GFP fusion proteins under the control of the CaMV 35S promoter that ensured high level and constitutive expression of these transgenes. The presence of the fusion proteins was verified by Western-analysis. Specific PHYA and PHYB overexpression phenotypes were observed on transgenic plants expressing the PHYA-GFP or the PHYB-GFP fusion proteins, respectively. This observation indicates that these fusion proteins function as phobiologically active phytochrome receptors, thus the regulation of the intracellular partitioning of these proteins is very likely to reflect the functional properties of the endogenous PHYA and PHYB photoreceptors.

2. Using fluorescence microscopy, we showed that the PHYA-GFP and PHYB-GFP proteins are localized in the nuclei of cells in light-grown plants and in the cytosol of cells in dark-grown or dark-adapted plants. Detailed analysis of the import process showed that three red light pulses given every hour were sufficient to induce a clearly detectable import of PHYB-GFP into the nucleus. The inducing effect of red light pulses could be completely reversed by subsequent far-red light pulses that converted P_{fr} back to P_r. Far-red pulses alone were not inductive. This red/far-red reversibility of the import emphasizes that PHYB-GFP is regulating its translocation through its own photoconversion. This is further supported by the observation that a chromophore-less inactive mutation of full-length PHYB fused to GFP (PHYB*-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 (LF) response and correlates well with the light quality dependence and kinetics of PHYB-triggered light responses. In the case of PHYA-GFP, nuclear import is inducible not only by a single low intesity red light pulse but also by a far-red pulse. Thus, we conclude that nuclear import of PHYA-GFP is controlled by the very low fluence (VLF) response of PHYA.

3. Analysis of the kinetics of light-induced nuclear import of the PHYA-GFP and PHYB-GFP fusion proteins showed that PHYA-GFP accumulation in the nucleus reaches its maximum 15-20 min after the inductive irradiation, whereas in the case of PHYB-GFP this process requires 2 hr. Besides the light-dependent nuclear import of phytochromes, a process responsible for the disappearance of the imported photoreceptors during dark incubation (preceded by a far-red light treatment that converts most of the P_{fr} back to P_r) should also be identified. The disappearance of phytochromes from the nuclei under these conditions is significantly faster than their estimated turnover rate. Therefore, degradation alone is not sufficient to explain the measured depletion kinetics, indicating the effect of an active export mechanism. To explain the mechanism controlling the translocation of the PHY-GFP proteins we suppose the presence of at least one functional NLS (nuclear localization signal) and one NES (nuclear export signal) in the phytochrome proteins. To check this hypothesis for PHYB localization, various mutated forms of PHYB were fused to GFP and their intracellular localization was assayed. Whereas the C-terminal half of PHYB fused to GFP (C-PHYB-GFP) is constitutively localized in the nucleus, the N-terminal PHYB-GFP fusion (N-PHYB-GFP) was always restricted to the cytosol. These results imply that the functional NLS must be located in the C-terminus of PHYB. Since the chromophore-less PHYB*-GFP is also confined to the cytosol in a light-independent manner, we conclude that in the P_r form the NLS of PHYB is masked, abolishing nuclear uptake, whereas in the P_{fr} form its nuclear export is inhibited. On the other hand, cytosolic retention of PHYB-GFP could be effected either by intramolecular masking of the NLS in its P_r form, or by a hypothetical cytosolic retention factor that specifically interacts with the P_r form through a presumed CRS (cytosolic retention signal) motif located at the N-terminus of PHYB.

4. PHYA-GFP and PHYB-GFP transported into the nucleus are not evenly distributed but show spotted localization patterns. The size and number of PHYA-GFP and PHYB-GFP-containing speckles are different. 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 the nuclei. The observed PHYA-GFP and

PHYB-GFP patterns are reminiscent of the spotted nuclear localization of the COP1 protein, which is a member of the genetically defined, heterogenous *COP* gene product group that is assumed to act as a general switch from skotomorphogenesis to photomorphogenesis. In the dark, COP1 (or the COP1-GFP fusion protein) is localized in the nucleus, interacts with the HY5 transcription factor (and probably with other similar bZIP-type transcription factors) that binds to specific cis-acting elements within the promoter of light-regulated genes. As part of a E3-ubiquitin-ligase complex, COP1 targets these transcription factors to the 26S proteosome, facilitating their degradation. In the light, COP1 slowly disappears from the nucleus, transcription factors are allowed to accumulate and induce the expression of their target genes. Because of the similar, spotted staining patterns of COP1-GFP, PHYA-GFP and PHYB-GFP, it is tempting to speculate that PHYA and PHYB in their active P_{fr} form can interact with the COP1-transcription factor complexes, suppress the activity of COP1, and prevent the degradation of the HY5 and HY5-like transcription factors. On the other hand, it was shown recently that the bHLH transcription factor PIF3 (in assotiation with other factors) binds to the promoter region of the *CCA1* gene, but does not induce its transcription. However, PHYB in its P_{fr} form can bind to this inactive complex resulting in the induction of *CCA1* transcription. Therefore, another possible explanation for the nuclear function of phytochromes is that after they have been imported into the nucleus in their P_{fr} form, they can directly activate transcriptional complexes and induce the expression of the corresponding genes. The observation concerning *CCA1* gene activation is especially interesting, because the CCA1 protein is an important component of the plant circadian clock, therefore this process suggests a possible mechanism how the light signals mediated by phytochromes can entrain the circadian oscillator in plants.

5. We have generated transgenic tobacco plants expressing the luciferase reporter gene fused to the promoter of the tobacco *PHYA* or *PHYB* gene (*NtPHYA::LUC* and *NtPHYB::LUC*, respectively) and transgenic *Arabidopsis* plants carrying the *AtPHYB::LUC* (luciferase fused to the promoter of the *Arabidopsis PHYB* gene) transgene. The organ- and tissue-specific expression of these transgenes was consistent

with earlier results concerning the expression of both promoter::GUS fusions and the corresponding endogene genes. Therefore, it is very likely that the temporal changes of the luciferase activity in these plants correctly reflect the temporal regulation of the promoters used.

6. The activity of all of the reporter constructs showed diurnal oscillations under LD (12 h light/12 h dark photoperiods). However, the rhythmic expression of *NtPHYB::LUC* and *AtPHYB::LUC* continued after transfer to LL (constant light) or DD (constant dark) conditions, indicating that a circadian clock controls their activity. (Based on our very recent results, which do not form part of this thesis, the lack of rhythmicity in the activity of the *AtPHYA* promoter probably is due to its tissue-specific expression pattern: the circadian regulation affects its activity only in the leaves where its expression level is the lowest.) The observed *PHYB::LUC* rhythms are very similar to the *CAB2::LUC* rhythm in LD and LL: both have identical periods and high amplitudes. The *CAB2::LUC* rhythm damps rapidly in darkness, especially in *Arabidopsis*, because of the overall decrease in the level of *CAB* transcription. The persistence of *PHYB::LUC* rhythms in LL and DD probably reflect the relatively light-insensitive transcription of *PHYB* genes, which is consistent with previously published results.

7. The significance of the rhythms observed in *PHYB::LUC* expression is underlined by the observation of circadian rhythms in the accumulation of the *AtPHYB* and *NtPHYB* mRNA and in the synthesis of the NtPHYB protein under LD, LL and DD conditions. The synthesis of new NtPHYB protein was measured by the luminescence generated by the *NtPHYB::NtPHYB-LUC* transgene, which encodes a NtPHYB-LUC fusion protein. The rhythm of NtPHYB synthesis retains similar amplitude to the rhythm of promoter activity and closely follows the accumulation of *NtPHYB* RNA, which indicates that translational control has little effect on PHYB accumulation. A long half-life of the NtPHYB protein is therefore most likely to account for the observed suppression of circadian rhythmicity in the accumulation of bulk NtPHYB. Synthesis of PHYB in the morning would thus

contribute to the daily peak of new PHYB, but the contribution is apparently too small for reliable detection by Western blotting in the presence of the larger, existing PHYB pool. Rhythmic synthesis of PHYB nonetheless might be significant. The phase of maximal PHYB protein synthesis (ZT 0-2) is similar to the phase of maximal light responsiveness of *CAB* expression (ZT 4) and to the maximal inhibition of hypocotyl elongation (ZT 0) in *Arabidopsis*. A 2-fold change in PHYB gene dosage causes well-defined, biological responses, but would have been at the limit of detection in our assays. The daily change in bulk PHYB protein thus might be functionally relevant.

CONCLUSIONS

We have shown that the functional PHYA-GFP and PHYB-GFP fusion proteins are imported into the nucleus in a light-dependent manner in their P_{fr} form. After they have been imported, they form nuclear speckles probably representing multi-protein complexes, which may directly or indirectly regulate the transcription of light-responsive genes. From our data we deduce that the light-regulated nucleocytoplasmic partitioning of phytochromes correlates well with the light quality dependence and kinetics of certain phytochrome-mediated light responses, therefore regulated nuclear translocation may be a key element of light-induced signal transduction. On the other hand, many light responses have been described which are induced primarily by cytosolic events, therefore it is clear that phytochromes have important cytosolic roles as well. To unravel cytosol- and nucleus-specific signaling pathways in more detail, it is obligatory to find out what plant phenotypes emerge when phytochromes are prevented from being imported into the nucleus or are constitutively kept in the nucleus.

We have provided evidence on the circadian regulation of *PHYB* expression at the level of promoter activity, mRNA accumulation and protein syntesis. The apparent lack of rhythmicty at the level of bulk PHYB protein accumulation measured by Western blot is most probably due to the long half-life time of the PHYB protein. However, the rhythmic sensitivity of some PHYB-dependent physiological processes strongly supports the existence of rhythmic changes in the available amount of active PHYB receptors.

(Our very recent results, which do not form part of this thesis, indicate that rhythmic regulation can be detected in the temporal expression patterns of all *PHY* genes in *Arabidopsis*). This observation indicates the presence of an additional regulatory loop within the plant circadian system. It is proposed that this regulatory loop ensures (i) maximal efficiency in the perception of the resetting light signals at the right times and (ii) neutralization of signals from non-predictable environmental cues, which could cause resetting of the circadian clock. In addition, this postulated regulatory loop can also mediate the generation of more robust rhythms with higher amplitude under relatively constant conditions.

LIST OF PUBLICATIONS

Publications used in the thesis are underlined.

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