Summary of Ph.D. thesis.

Functional characterisation of a transcription factor component of phytochrome fotoreceptor controlled signalling pathways

András Viczián

Supervisor: Dr. Ferenc Nagy
Biological Research Center of the Hungarian Academy of Sciences
Institute of Plant Biology

Szeged
2004

INTRODUCTION

Light plays a crucial role in the life of the vast majority of higher plants. On the one hand it provides an energy source for photosynthesis and, on the other hand it is an important environmental signal with impact on a wide variety of different physiological and developmental processes from germination to flowering. The effect of light on the development of plants is probably most strikingly demonstrated by a comparison of seedlings grown in light or dark. A dark-grown seedling has a long hypocotyl, ending in a hypocotyl hook, its cotyledons are small and closed, its etioplasts contains no chlorophyll: this way of development is called skotomorphogenesis or etiolation. In contrast, light-grown seedlings have short hypocotyls, no hypocotyl hook, opened cotyledons containing fully developed, green chloroplasts: this way of development is called photomorphogenesis or de-etiolation. Plants can sense the presence or absence, the wavelength, the intensity, the direction, the duration and the diurnal rhythm of light. To monitor the light environment, plants have evolved a series of photoreceptors characterised by the wavelength of light they perceive. Photoreceptors of the widely used model plant *Arabidopsis thaliana* are listed in Table 8.1.

Table 8.1. Photoreceptors and their functions in higher plants

Wavelength of absorbed light/Receptors	**Function**
UV-B region: 280-320 nm Receptor unknown	Induction of gene expression
Blue/UV-A region: 320-470 nm Cryptochromes: CRY1, CRY2 Phototropins: PHOT1, PHOT2	De-etiolation, resetting the circadian clock, induction of flowering Phototropism, chloroplast movement, regulation of stoma opening
Red/Far-red region: 620-750 nm Phytochromes: PHYA PHYB PHYC PHYD PHYE	Seed germination, de-etiolation, shade avoidance, regulation of flowering time, resetting the circadian clock

Plant cryptochromes are chromoproteins with a molecular mass of 70-80 kDa showing significant homology to bacterial DNA photolyases but lacking photolyase activity. They carry pterin and FAD (flavin adenin dinucleotid) chromophores and absorb the blue range of the spectrum (Lin, 2000). Upon illumination with blue light the cry2 protein shows rapid

degradation whereas the level of cry1 does not change, suggesting that cry2 mediates responses to higher and cry1, to lower fluences of blue light.

Phototropins possess two LOV (light oxygen voltage) domains, each of which binds a FMN (flavin mononucleotid) choromophore (Christie et al., 1999). The function of the two phototropins of *Arabidopsis* is redundant in stoma opening and phototropism; PHOT2 however plays a dominant role at lower blue light intensities and this photoreceptor also seems to be responsible for the chloroplast relocation triggered by blue light (Kagawa et al, 2001; Fankhauser and Staiger, 2002).

Phytochromes (PHY) are photoreceptors that perceive red and far-red light. They are functional in homodimers of two ~125 kDa monomers with a covalently attached linear tetrapyrrole chromophore per molecule. There are five different phytochrome genes in *Arabidopsis*, named *PHYA, B, C, D, E* (Clack et al., 1994) with high structural similarity. phyA degrades quickly upon exposure to light. It is the dominant phytochrome of etiolated seedlings and mediates responses to very low red fluences and far-red light. phyB, C, D, E are photo-stable molecules that mediate responses to low and high fluences of red light (Furuya and Schäfer, 1996). phyB proved to be the dominant phytochrome in green seedlings and adult plants (Sharrock and Clack, 2002).

Phytochromes are synthesised in Pr form. This inactive form can be "switched" by a pulse of red light to Pfr form, which is the active conformer of phytochromes and the starting point of signal transduction cascades. The Pfr form can be reverted to Pr form by far-red light illumination. This is the phytochrome photocycle. The Pfr form spontaneously converts to Pr form in darkness: this process is called dark reversion. In case of phyA, the Pfr form is more unstable than the Pr form, it is therefore subject of degradation: this is the third way of removing Pfr forms from the system. The Pr↔Pfr conversion of phytochromes never involves 100% of the available phytochrome pool. Under the effect of the actual light conditions a certain Pr/Pfr ratio of each PHY species is established, which determines the properties of the developing physiological response.

During the past several years it turned out that the subcellular localisation of phytochromes has a functional role: all *Arabidopsis* phytochromes translocate to the nucleus during light illumination (in Pfr form) and forms nuclear speckles in the nuclei with different kinetics, depending on the wavelength and the intensity of light (Kircher et al., 2002). It is probable that these speckles are multiprotein-complexes. The function of these complexes is a matter of debate in the scientific community. Some authors think that the speckles are the scene of phytochrome degradation (Vierstra, 1994), but these speckles are formed only when

phytochromes are in the active (Pfr) form; furthermore, mutant phytochromes defective in light signalling cannot form speckles (but are imported to the nucleus in light) (Kircher et al., 2002).

Examination of light-induced signal transduction cascades is the most extensively investigated area of phytochrome research. Genetical and molecular biological approaches have proved to be the most effective ways of this investigation. Finding and characterizing mutants that defective in phytochrome-specific responses is the main objective of genetic approaches. The used genetic screens implemented have helped to isolate mutants that can be divided into two main groups:

(I) Those, which behave as if grown in dark despite the fact that they grew in light. In these plants positive elements of photomorpogenesis are damaged. Mutants with functionally damaged photoreceptors and damaged chromophore synthesis pathways were isolated. In one of the first screens a mutant was isolated with functional photoreceptors but an etiolated phenotype in light, which was called *hy5* (long hypocotyls 5) (Koorneef et al., 1980). It turned out that the mutation affects a bZIP-type transcription factor in these plants. This protein can bind to a special element (called G-box) of the promoters of light-responsive genes and maintain photomorhogenic development.

(II) Those mutant plants, which show a de-etiolated phenotype even in light, are classified to the second group. In these plants one of the genes which prevent photomorphogenesis is mutated. One set of these mutants is called COP (constitutive photomorphogenesis). Of these, the function of the COP1 protein is the best known: it was shown that COP1 is a protein that confers specificity to an E3 ligase complex thus participates in the ubiquitination of the positive components of photomorphogenic development (Hardtke and Deng, 2000). HY5 is one of the COP1 targets: COP1 ubiquitinates HY5 in dark, preventing photomorphogenic development, whereas in the nuclei of *cop1* mutant seedlings HY5 molecules accumulate, releasing photomorphogenic development independently of the presence of light.

The goal of molecular biological approaches is to find early signalling partners of phytochromes by using them as bait in the yeast two-hybrid system. PIF3 (phytochrome interacting factor 3), one of the factors physically interacting with phytochromes was found using this method (Ni et al., 1998). PIF3 contains a sequence of 524 amino acids which forms (i)a nuclear localisation signal (NLS), directing the protein to the nucleus, and (ii) a conserved motif called basic helix-loop-helix (bHLH) which is responsible for the DNA-binding and the dimerisation of the molecule. Thus, PIF3 belongs to the bHLH family of transcription factors (Bailey et al., 2003).

Molecular properties of PIF3 were examined in the yeast two-hybrid system and *in vitro* binding assays. PIF3 directly interacts with phyA and, with higher affinity, with phyB molecules: the connection is stronger with the Pfr than the Pr forms. The interaction between PIF3 and mutant phytochromes with damaged function is much weaker than that with wild-type forms (Ni et al., 1998; Zhu et al., 2000). PIF3, like most members of the *Arabidopsis* bHLH transcription factor family can bind DNA (Toledo-Ortiz et al., 2003): it binds with high specificity to the G-box, a characteristic sequence motif of light-inducible promoters. In addition it was shown that PIF3 can simultaneously bind to the Pfr form of phyB and to a G-box *in vitro* (Martinez-Garcia et al., 2000).

bHLH transcription factors are functional in dimers and can heterodimerize with other bHLH factors. PIF3 can form heterodimers with HFR1 (long hypocotyls in far-red 1) (Fairchild et al., 2000), PIF4 (phytochrome interacting factor 4) (Toledo-Ortiz et al., 2003) and PIL1 (PIF3 like 1).

These data radically change our view about PHY-specific signalling. The light signal reaches the light-responsive promoter not at the end of a long signal transduction cascade but in one step: the physiologically active phytochrome interacts with PIF3, enabling this complex to bind to the G-box of the promoter of light responsive genes, modifying the activity of their transcription. The specificity of the PIF3-phyB complex can be modified by the heterodimerisation of PIF3 with other bHLH factors, conferring additional fine tuning capability to the system.

The *in vitro* data were supported by the phenotype caused by changed *PIF3* mRNA levels in transgenic *Arabidopsis* plants. PIF3 seemed to act as a positive factor of light induced inhibition of hypocotyl elongation (Ni et al., 1998). These data were further supported by the results of the examination of the *poc1* (photocurrent 1) T-DNA insertion mutant that shows an increased *PIF3* mRNA level (Halliday et al., 1999). The regulation of some genes, which contain G-boxes in their promoters, requires PIF3 for their proper-light induction. Of these *CCA1* (circadian clock associated 1) and *LHY* (late elongated hypocotyl) are of special interest because of their special role in maintaining the plant circadian clock. The circadian clock is an endogenous and autonomous molecular timekeeping mechanism of living organisms. A central oscillator generates oscillations with an approximately 24 h period length, based on negative feedback loops, regulating the expression of the output side of the clock. On the other side of the system, a light signal can entrain the clock via photoreceptor-dependent pathways. This fine-tuning can synchronize the rhythm generated by the central oscillator to the changes in the length of daytime and night. CCA1 and LHY are thought to be

the components of the central oscillator, thus, according to the published model, the binding of the PIF3-phyB complex to their promoter could be the key element of light input to the central oscillator of the circadian clock.

RESEARCH OBJECTIVES

At the beginning of our work most of the data on PIF3 had been obtained from *in vitro* systems therefore one of our main aims was to obtain more details on the function of PIF3 by using *in vivo* approaches. The main aims were:

1. To express full-length PIF3 and PIF3-GFP proteins in transgenic plants and examine their biological activity.
2. To examine the phenotype of plants with altered PIF3 levels.
3. To examine the localisation of PIF3-GFP molecules under different light regimes.
4. To prove the co-localisation of PIF3 and PHY molecules *in planta*.
5. To examine the role of PIF3 in the light input of the circadian clock.

METHODS

- Molecular cloning techniques.
- Creating transgenic *Arabidopsis thaliana* plants and maintaining them under sterile and greenhouse conditions.
- Plant total RNA extraction.
- RNase protection assay.
- Total plant protein isolation, Western blotting.
- Performing different light treatments and hypocotyl measurement of seedlings.
- Light, fluorescence and confocal microscopy.
- *In vivo* luciferase enzyme activity measurements in intact seedlings.

RESULTS AND DISCUSSION

1. We confirmed a T-DNA-tagged PIF3 mutant line as a null mutant by detecting PIF3 with specific antibody. The light-induced inhibition of hypocotyl elongation was stronger in the *pif3* mutant than in the wild-type seedlings and this effect was observed only during red light treatment but not in far-red or blue light. We could produce real PIF3 overexpressor plants despite the published cloning difficulties (Ni et al, 1998). Red light specific inhibition of hypocotyl elongation was weaker in the overexpressor plants compared to the wild-type controls. This conclusion was further supported by one of our surprising results: we prove that the *poc1* mutant does not contain detectable amounts of PIF3 protein, despite the high *PIF3* mRNA level previously shown in this mutant (Halliday et al, 1999). Our coherent data on a *bona fide pif3* null mutant and on confirmed PIF3 overexpressor plants contradict the published data and suggest that PIF3 is a negative rather than a positive component of phyB-specific photomorphogenic pathways. Our data lead to a contradiction in the proposed model of PIF3 action: a negative factor interacts with the Pfr form of phytochromes and the G-box, therefore it seems that the Pfr form represses photomorphogenic development. Since this function of the Pfr form is not supported by any previous results, the function of PIF3 must be fully revised.

2. We produced transgenic *Arabidopsis* plants expressing PIF3-GFP fusion proteins under the control of constitutive 35S promoter and proved the presence of the fusion proteins by Western-analysis. We observed that these fusion proteins function as biologically active PIF3, thus the regulation of the intracellular partitioning of these fusion proteins is very likely to reflect the functional properties of endogenous PIF3.

3. Using epifluorescent microscopic techniques we have shown that PIF3-GFP molecules are localised in the nuclei of cells after plants have reached a certain age (2 days from the beginning of germination). The presence of the fusion protein was detected in every examined tissue type in etiolated but not in light-grown or light-treated seedlings.

We have observed that the distribution of PIF3-GFP molecules in the nuclei of etiolated seedlings is diffuse; after a short red or far-red pulse, hovever they form nuclear speckles reminiscent of those formed by phytochromes after light-treatment. The PIF3-GFP-specific signal disappears after a 30-min light pulse. This phenomenon is independent of the applied light or dark treatment after the first pulse. The PIF3-GFP-specific signal reappears if

the illuminated plants are incubated in the dark for approximately six hours. By applying a new light pulse, this process (speckle formation – disappearance of the GFP signal – reappearance in the dark) can be repeated.

4. By using Western blotting analysis we proved that the reason of the disappearance of the PIF3-GFP signal is always the disappearance of the PIF3-GFP protein, whose turnover is similar to that of endogenous PIF3. Our results suggest that light induces faster degradation of PIF3 and this degradation is controlled partly by phytochromes. phyA, phyB and phyD photoreceptors are responsible for the red and far-red light-induced degradation of PIF3 and blue light receptor(s) take part in the induction of blue light-specific degradation. Light-induced degradation of PIF3 could represent a new level of regulation of light-specific signal transduction pathways: the appearance of the Pfr forms of phytochromes initiates signal transduction pathways and induces the degradation of a protein that they specifically interact with. The blue and far-red light-induced degradation of PIF3 represents a new possible regulatory mechanism of phyB-specific signalling.

5. We have observed co-localisation between PIF3 and phyA, phyB, phyC, phyD but not phyE photoreceptors *in planta*. This interaction takes place after light illumination in nuclear speckles, but these protein complexes rapidly disintegrate, possibly because of the degradation of PIF3. Continued irradiation again induces speckle formation of phytochromes, but these speckles do not contain PIF3. We termed the former “early” and the latter, “late” speckles and observed that the presence of PIF3 is essential for phyB “early” speckle formation, whereas it is not necessary for the formation of “late” speckles.

6. We have observed that the presence of functional COP1 protein is essential for the accumulation of PIF3 in dark but not necessary for its light-induced degradation. Our results also indicate that COP1, a negative regulator of photomorphogenesis, regulates proteolysis of two key transcription factors involved in light signalling, namely PIF3 and HY5, in an apparently antagonistic fashion. COP1, physically interacting with HY5, directs this protein to the proteosome but inhibits, most likely indirectly, degradation of PIF3 in dark. We provided evidence that PIF3 degradation is light-induced, thus we postulate that COP1 is required to degrade one of the components of this yet unknown pathway in the nucleus during skotomorphogenesis. Independent of the molecular mechanism, COP1 action promoting PIF3 accumulation in the nucleus in the dark can be interpreted as follows: COP1 as a negative

regulator of photomorphogenesis maintains skotomorphogenic development by inducing selective degradation of signalling components required at later stages of photomorphogenesis (HY5) but promotes accumulation of signalling molecules utilised early at the dark to light transition (PIF3), thus prepares the etiolated seedling for adapting to a new environment.

7. We found that the role of PIF3 in the light induction of *CCA1* transcription is minor and the altered PIF3 levels do not disturb significantly the function of the central oscillator of the circadian clock. Thus, the previously suggested role of PIF3 in the light input of the circadian clock must be revised.

8. We designed and tested a system for the examination of light-induced degradation pathways of PIF3. Measuring the luminescence emitted by transgenic seedlings expressing the PIF3-LUC fusion protein under the control of the 35S promoter, we can follow changes in the protein level without damaging the plants. After generating mutations in these transgenic plants, we can screen for mutants with damaged light-induced PIF3 degradation pathways: the emitted luminescence of these seedlings will not decrease after light irradiation, indicating an unchanged PIF3-LUC protein level.

CONCLUSIONS

Isolation and functional examination of PIF3 radically changed our view about phytochrome-controlled signalling. According to the new model, PIF3 specifically interacts with the Pfr form of phyB and binds to the G-box of light-responsive promoters, conveying the light signal to the transcriptional complex in one step. We have found that the effect of PIF3 on the photomorphogenic development of seedlings is negative rather than positive as shown previously. Functional PIF3-GFP stays in the nucleus of etiolated and dark-adapted seedlings, forms nuclear speckles rapidly after light illumination and co-localises with phytochromes. PIF3 degrades quickly after light-induced speckle formation. This degradation is controlled mainly by different phytochromes and seems to be an important regulation step in light signalling. Our results basically contradict the previously published model of PIF3 action and invalidate the previous and widely accepted hypothesis on the function of PIF3. Our observation regarding the importance of the COP1 protein in the dark accumulation of PIF3 could be the starting point of further functional analysis of PIF3.

LIST OF PUBLICATIONS

Publication used in the thesis is underlined.

Bauer, D.,[#], **Viczián, A.**[#], Kircher, S., Nobis, T., Nitschke, R., Kunkel, T., Panigrahi, K., Ádám, É., Fejes, E., Schäfer, E., Nagy, F. (2004) COP1 and multiple photoreceptors control degradation of PIF3, a transcription factor, required for light signalling in *Arabidopsis.* (közlésre elküldve: Plant Cell)

[#] These authors contributed equally to this work.

Casal, J.J., Davis, S.J., Kirchenbauer, D., **Viczián, A.**, Yanovsky, M.J., Clough, R.C., Kircher, S., Jordan-Beebe, E.T., Schafer, E., Nagy F., Vierstra, R.D. (2002): The serine-rich N-terminal domain of oat phytochrome A helps regulate light responses and subnuclear localization of the photoreceptor. Plant Physiology, 129:1127-1137.

Viczián, A., Máté, Z., Nagy, F., Vass I., (2000): UV-B induced differential transcription of *psbD* genes encoding the D2 protein of Photosystem II in the cyanobacterium *Synechocystis* 6803. Photosynthesis Research, 64:257-266.