The role of the N-terminal NTE domain of PHYTOCHROMEs in red and far red light perception

Theses of the Ph.D. dissertation

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Introduction

Plants are sessile, photoautotroph organisms. Light plays a dual role during their life. It **i)** provides the energy for their metabolism and **ii)** regulates their light dependent developmental program through the information light carries about the environment. During the evolution of plants a battery of photoreceptors have been evolved, which make it possible to utilise the information light carries. These sense the presence or lack, intensity and periodic changes of different ranges (colours) of the light. *UVR8* senses UV-B, cryptochromes and phototropins percept UV-A and blue and phytochromes sense red (650-700 nm) and far red (700-750 nm) light. (Rizzini et al. 2011; Lin & Shalitin 2003; Liscum et al. 2003; Chen és Chory 2011). Phytochromes are present in every photosynthetic from cyanobaacteria to dicotyledonous plants. (Yeh et al. 1997; Yeh & Lagarias 1998). Plant phytochromes have around 120 kDa molar mass. They consist of an N-terminal (~70 kDa) and a C-terminal domain (~55 kDa). The N-terminal part perceives and transmits the light signal, moreover it carrier the linear tetrapirrol chromphore covalently bound to an evolutionary conserved Cis residue (Li & Lagarias 1992). Phytochromes exists as two conformers, which show distinct absorption properties (Schafer & Bowler 2002). Phytochromes synthesised in the dark are in a so called Pr conformation, which is the biologically inactive form and shows an absorption peak in red (r) light. By absorbing red light (A_{max} = 650 nm) the chromophore undergoes a conformational change, which induces conformational changes in the phytochrome tertiary protein structure. This process results the biologically active form, which has an absorbtion maximum in far red light (A_{max} = 730 nm), therefore it is called Pfr. (Schafer & Bowler 2002). Since the two conformers have partially overlapping absorption spectra, a $P_r \leftrightarrows P_{fr}$ equilibrium is set up, defined by the spectral composition of the absorbed light. The C-terminal domain of phytochromes is essential for the dimerization and modulates the signal transduction. (Matsushita et al. 2003). In the model plant *Arabidopsis thaliana* phytochromes form a small, five-member gene family, distinguished by letters from A-E (*PHYTOCHROME A – E*, or *PHYA – PHYE*) (Sharrock & Quail 1989). PHYA and PHYB are the most important phytochromes of this gene family, which play a dominant role in the regulation of the light dependent development of plants. Despite the high degree of

homology between PHYA and PHYB they play distinct roles during the life of *Arabidopsis* and there are fundamental differences in their signal transduction. PHYA is a type I phytocrome, which is the dominant phytochrome seedlings developing in the dark (e.g. below the soil's surface) (Sharrock & Clack 2002). After activated by light PHYA is rapidly translocated to the nucleus by two specific transporter molecules called FHY1 and FHL (Hiltbrunner et al. 2006). Activated PHYA P_{fr} undergoes rapid degradation, which is the inactivation process of PHYA and ensures the monitoring of the light conditions (Sharrock & Clack 2002). As the seedling grows to the soil's surface the elevated light intensiy depletes rapidly the PHYA protein level and as a consequence PHYB becomes the dominant phytochrome. (Sharrock & Clack 2002). PHYB is a light-stable protein, therefore belongs to type II phytochromes. The activated PHYB Pfr is inactivated through a spontaneous, light independent relaxation to the inactive Pr state, termed dark reversion (Eichenberg et al. 2000).

The downstream components of the phytochrome signal transduction were identified in reverse genetic screens during the past two decades. The identified elements fall into two main cathegories. The loss of some elements cause constitutive photomorphogenesis, while the loss of other elements cause impaired or lost light sensitivity. These elements can be integrated into the signalling model of phytocromes. Seedling growing in darkness accumulate high levels of PIF (PHYTOCHROME INTERACTING FACTOR) transcription factors, which activate the skotomorphogenetic developmental program (elongated hypocoty, closed cotyledons). (Ni et al. 1998; Shin et al. 2009). In a parallel process the COP1 (CONSTITUTIVE PHOTPMORPHOGENIC 1) E3 ubiquitin ligase complexes constantly target the HY5 (ELONGATED HYPOCOTYL 5) and the HYH (HY5 HOMOLOG) transcription factors to the 26S proteosome mediated degradation (Sullivan et al. 2003). The light activated phytochromes interact with both the PIF transcription factors and the COP1 E3 ligase complexes. As a consequence PIFs undergo rapid degradation (Al-Sady et al. 2006). Additionally the substrate specificity of the COP1 E3 ligase complexes change, which lead to the stabilization and accumulation of the HY5 and HYH transcription factors (Holm et al. 2002; Osterlund et al. 2000). The latter transcription factors initiate the light dependent developmental process, termed photomorphogenesis (cotyledons open, greening and true leaf formation initiated).

Objectives

In this study we investigated the role of the N-terminal NTE domain of the PHYA and PHYB photoreceptors in *Arabidopsis thaliana*. During the past two decades numerous genetic screens were carried out in search for the downstream components of the phytochrome signal transduction. The identified numerous mutant *phy* alleles (SNP) proved to be useful tools to reveal the events of the phytochrome signal transduction. We employed random and site directed mutagenesis and subsequent physiological and molecular characterization in order to answer our scientific questions. The scope of this work can be summerised in the following points:

- We characterised the physiology of the phyA-5 mutant.
- We investigated the subcellular dynamics $PHYA^{Ala30Val}$ encoded by the phyA-5 allele.
- We revealed the molecular mechanism caused by the *phyA-5* mutation.
- We identified a phosphoserin residue in the NTE domain of PHYB
- We generated the phosphorylated ($PHYB^{Ser86Asp}$) and the dephosporylated state mimicking ($PHYB^{Ser86Ala}$) forms expressing transgenic *Arabidopsis* plants and characterised the physiological effects of these substitutions.
- We characterised the subcellular dynamics if the mentioned PHYB variants.
- We revealed the molecular mechanism responsible for the altered ligh sensitivity caused by the Ser86 amino acid substitutions.

Methods

- Building of artificial gene constructs.
- Generation of transgenic plant lines.
- Gene expression study by reverse transcription coupled quantitative PCR (RT-qPCR).
- Determination of specific protein levels by Western blot.
- In vivo phosphorylation study by Phos-tag Western blot method.
- Plant potein purification by affinity chromatography.
- Identification of phosphoserin residues by MALDI-TOF-MS.

- Quantification of Phytochromes by spectroscopy (active - P_{fr} and total amounts - P_{tot})
- PHYA degradation kinetics measurement by spectroscopy (decrease of P_{tot} in time).
- Measurement of PHYB dark rversion by spectroscopy (decrease of PHYB P_{fr} in time).
- Measurement of PHYB, PHYB^{Ser86Asp} and PHYB^{Ser86Ala} differential spectra.
- Measurement of protein affinity by yeast two hybrid method..
- Measurement of light dependent hypocotyl elongation inhibition.
- Determination of the action spectrum of PHYA based on hypocotyl elongation test under different wavelength light treatments..
- Measurement of the nuclear import of the PHYB-YFP fusion protein based on the increase of nuclear fluorescence increase followed by microscopy.

Results

The characterization of the *phyA-5* mutant allele

- The *phyA-5* allele encoded PHYA variant carries Ala30Val amino acid substitution, which Ala is evolutionary conserved among type I phytochromes.
- The *phyA-5* mutation does not influence the expression of the PHYA gene.
- The PHYA^{Ala30Val} protein shows impaired degradation under weak far red light treatment. In contrast under intense far red and red light treatment it shows wild type degradation kinetics..
- The microscopic analysis of PHYA-YFP and PHYA^{Ala30Val}-YFP protein variants expressing plants revealed the impaired nuclear import of the PHYA^{Ala30Val}-YFP under weak far red light treatment. In contrast under strong far red light treatment the nuclear import of the PHYA^{Ala30Val}-YFP protein is indistinguishable from that of the wild type (PHYA-YFP).
- In yeas two hybrid experiments we showed, that the PHYA^{Ala30Val} variant binds the FHY1 and FHL nuclear import molecules with reduced affinity.

- Transgenic plants expressing the PHYA^{Ala30Val}-YFP-NLS (NLS - nuclear localization signal) protein ectopically, show wild type PHYA degradation and signal transduction. Moreover hey are indistinguishable from the PHYA-YFP and the PHYA-YFP-NLS expressing transgenic plants..

Discussion of the characterisation of the *phyA-5* allele

It has been long known, that the NTE domain is necessary for the full activity of PHYA. Numerous studies demonstrated that deletions and substitutions in the NTE domain lead to dramatically decreased far red light sensitivity. Although the phenomenon was well studied, the underlying molecular mechanism remained unknown (Stockhaus et al. 1992; Cherry et al. 1992; Casal et al. 2002; Trupkin et al. 2007).

The *phyA-5* mutation cause a complex, conditional phenotype. Mutant plants show reduced light sensitivity exclusively under weak far red light treatment. In order to revel the molecular basis of the phenomenon we investigated the effect of the mutation on the PHYA gene expression. Since the phyA-5 gene expression is indistinguishable from that of the wild type PHYA, we tested the degradation of the PHYA variants under different light conditions. Surprisingly only under weak far red light showed the PHYA^{Ala30Val} expressing plant showed impaired degradation. Under strong far red and red light PHYAAla30Val showed wild type degradation. The nuclear import showed similar behaviour, as PHYA^{Ala30Val} protein showed reduced nuclear accumulation under weak far red light treatment. This reduced nuclear import implicated, that the Ala30Val substitution might change the interaction with the FHY1 and FHL nuclear transporters. Therefore we tested the affinity of the PHYA and PHYA^{Ala30Val} proteins to FHY1 and FHL molecules in yeast two hybrid assay. This experiment showed unequivocally, that the Ala30Val substitution indeed changes the binding affinity of PHYA to the transporter molecules, which explains the impaired nuclear import and the reduced sensitivity phenotype of the mutant plant under weak far red light treatment.

Upon light treatment PHYA turn into P_{fr} conformation, which is its the biologically active form and two processes are initiated. The Pfr form **i)** undergoes rapid degradation and through the Pfr form specific interaction with the FHY1 and FHY nuclear transporters a **ii)** rapid nuclear translocation is initiated. In the nucleus the PHYA dissociates from the

transporters, which are transported back to the cytoplasm and transport additional PHYA Pfr into the nucleus. PHYA Pfr is degraded in the nucleus as well, which degradation is significantly faster, than that of in the cytoplasm (Debrieux & Fankhauser 2010). The PHYAAla30Val protein binds FHY1 and FHL transporters, the impaired nuclear import and reduced far red sensitivity under weak far red light. Under these condition the reduced nuclear transport presumably causes reduced degradation, since PHYA Pfr is degraded only by the slower cytoplasmic degradation process. Under intense far red light the elevated Pfr PHYA levels overcome the effect of the reduced FHY1 and FHL biding and the signal transduction and degradation shows wild type behaviour. We showed, that the Ala30 and the neighbouring amino acid residues are likely an integral part of a protein surface, which directly attaches to the FHY1 and FHL nuclear transporters of PHYA.

Results of PHYB Ser86 phosphorylation

- By the mass spectrometric analysis of purified PHYB-GFP-TAPc protein we identified a phosphoserin residue in position 86, which is positioned in the NTE domain of PHYB and evolutionary conserved among the type II phytochromes of dicotyledonous plants.
- We generated transgenic *Arabidopsis* lines expressing the PHYB Ser86 phosphorylated state ($PHYB^{Ser86Asp}$) and dephosphorylated state ($PHYB^{Ser86Ala}$) mimicking protein variants using site directed mutagenesis.
- We investigated the PHYB variants expressing lines' red light sensitivity, which revealed, that phosphominic variant ($PHYB^{Ser86Asp}$) shows reduced, while the dephospho mimic variant ($PHYB^{Ser86Ala}$) increased light sensitivity in comparison to the wild type PHYB expressing lines.
- We checked the light induced degradation of the PHYB variants. None of them showed detectable degradation.
- We tested the nuclear import and the nuclear complex formation of the PHYB variants. Under weak read light treatment the phospho mimic ($PHYB^{Ser86Asp}$) version expressing plants needed stronger, while the dephospho mimick variant ($PHYB^{Ser86Ala}$) expressing plants weaker light treatment in comparison to the

wild type PHYB expressing plants to induce similar response. Under strong red light treatment al lines showed similar and equal behaviour.

- PIF3 is the only prooven transport facilitator of PHYB, therefore we tested in a yeast two hybrid experiment the PIF3 binding capacity of the PHYB variants. Under weak red treatment the phosphomimic ($PHYB^{Ser86Asp}$) variant showed higher, the dephosphomimic ($PHYB^{Ser86Ala}$) variant lower PIF3 binding capacity compared to the wild type PHYB. Under strong red light treatment all three forms showed equally high PF3 binding capacity.
- We measured the *in vtro* $P_{fr} \rightarrow P_r$ fotokonverzion antd the differential spectra of all PHYB variants. All variants showed normal, wild type behaviour in these experiments.
- We tested the the dark reversion of the PHYB phosphovariant in *in vivo* and *in vitro* experimental system, which revealed, that phosphomimic variant ($PHYB^{Ser86Asp}$) shows faster, while dephosphomimic variant ($PHYB^{Ser86Ala}$) shows slower relaxation kinetics, than the wild type PHYB.

Discussion of the results of PHYB Ser86 phosphorylation

There is only one study about the phosphorylation of PHYB in *in vitro* experimental system. It has been shown, that PHYB undergoes autophosphorylation in the NTE domain under both red and far red light treatment. So far these results have not benn confirmed in *in vivo* system (Phee et al. 2008).

In this study we identified a phosphoserin residue in the N-terminal NTE region of the PHYB molecule. In order to study the effect of the Ser 86 phosphorylation we generated the phosphorylated state mimicking ($PHYB^{Ser86Asp}$) and dephosphorylated state mimicking ($PHYB^{Ser86Ala}$) PHYB variants expressing transgenic *Arabidopsis* plants. During the physiological characterization of the mentioned lines it turned out that the phosphorylation state of the Ser86 residue has profound effect on PHYB mediated red light sensitivity. The mentioned substitutions do not effect the red light induced degradation of the PHYB. While under intense red light treatment the different PHYB variant showed no difference in hypocotyl elongation, nuclear import, PIF3 binding capacity under weak red light treatment the phosphomimic version ($PHYB^{Ser86Asp}$) needed stronger, while the

dephosphomimic version weaker light treatment to induce similar responses like the wild type PHYB expressing plants. Therfore we measured *in vitro* the differential spectra and the $P_{fr} \rightarrow P_r$ photoconversion of the three PHYB variants. All PHYB variants showed equal and wild type spectral and photoconversion properties, which means on the light perception level all three form behave identically. Therefore we tested the dark reversion of the PHYB variants, which revealed, that the phosphomimic ($PHYB^{Ser86Asp}$) variant shows faster, while the dephospho mimic ($PHYB^{Ser86Ala}$) version shows slower relaxation, than the wild type PHYB.

Taken together our results suggest, that the degree of phosphorylation on the Ser86 residue determines the average sensitivity of the PHYB system through fine-tuning the relaxation properties of the PHYB molecule.

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