Detailed Research Summary

Light-induced degradation of phyA

We designed and performed the genetic screen described in our proposal to isolate mutants in which the light-induced degradation of phyA is compromised. Accordingly, we individually determined the degradation rates of the PHYA promoter driven PHYA/LUC fusion protein by *in vivo* luminescence measurements in 250 000 seedlings, and identified 10 mutants in which the degradation rate of the PHYA/LUC reporter was considerably slower than in wild-type seedlings. We back-crossed these mutants 3 times and then analysed the degradation of the native phyA protein. We showed that, similarly to the PHYA/LUC reporter, the degradation of the endogenous phyA was also compromised in 9 out of the 10 mutants. Next we constructed a rough genetic map and identified the chromosomal location of the other 9 mutations. Parallel with genetic mapping, we also characterised the photomorphogenic responses of these mutants. We found that these mutants displayed aberrant photomorphogenic phenotypes both in far-red and red and in white-light-grown seedlings. This observation suggested that these mutations are of pleiotropic nature and affect not only the degradation of phyA, but also other cellular events, for example degradation of phyB. We took advantage of next-generation sequencing and within a short time identified the mutant genes in these 9 lines. We found that all of the mutations result in amino acid substitutions in genes encoding various components of the 26S proteosome. These data explain the phenotypes of the mutant plants, but at present provide only limited information about the molecular machinery mediating extreme rapid degradation of the phyA protein. Independently of this, our results indicate that the rapid degradation of phyA and, to a lesser extent, of phyB is essential for phytochrome signalling, since the mutant lines invariably displayed hyposensitivity to far-red and red light, yet contained significantly higher levels of phyA and phyB than the wild type. Interestingly, we found that none of these mutations affected the degradation of the PIF transcription factors (also known to be degraded in a light-induced fashion), thus we initiated a collaboration on this subject with the laboratory headed by prof. Eberhard Schäfer in Freiburg, Germany. Our goal is to identify additional alleles of the mutant genes which, hopefully, specifically affect the degradation of phyA and/or phyB.

Simultaneously with the above experiments, we sequenced the PHYA/PHYA/LUC reporter in the mutant whose mutation did not affect the degradation of the native photoreceptor. We found that a point mutation in the PHYA/LUC reporter resulted in a V30A amino acid substitution in the N-terminal domain of the photoreceptor. We introgressed the mutant PHYA/PHYA/LUC reporter in the phyA-201 line that lacks functional phyA, and characterised the molecular mechanism by which this amino acid substitution affects phyA degradation. We showed that the mutant displayed conditional hyposensitivity to FR light, reduced interaction with the nuclear import facilitator FHY1, and was degraded considerably slower than the native phyA protein. We demonstrated that the modified nucleo/cytoplasmic partitioning of the mutant photoreceptor is responsible for the reduced degradation of the mutant protein, and the degradation of phyA is faster or more efficient in the nucleus than in the cytoplasm *(Sokolova et al., Plant Phys. 2011*). We also showed that the interaction of FHY1 with phyA is mediated by multiple binding sites, since the short N-terminal fragment of the mutant phyA (1-651), in contrast to the full-length phyA, did not bind FHY1 at detectable levels. To test if the N-terminal region indeed plays a prominent role in phyA signalling, we constructed various fusion proteins containing different domains of phyA, and expressed these fusion proteins in a phyA null background. Our data clearly demonstrated that, in contrast to phyB (*Palagyi et al., Plant Phys, 2010)*, the N-terminal region of phyA does not have biological activity, it cannot even partially complement the phyA mutant but,

somewhat unexpectedly, we found that the phyA N-terminal fragment can still be imported into the nucleus in a light-dependent fashion (*Wolf et al., Plant Cell and Physiology 2010*). We noticed that transgenic seedlings in which the phyA N-terminal fragment was constitutively over-expressed in the nucleus (35S/PHYA-N1-651/NLS) displayed a strong de-etiolation response in darkness. Detailed analysis of these and other lines in which the full-length phyA was constitutively over-expressed in the nucleus indicated that the phyA N-terminal fragment, in contrast to full-length phyA, interacts with COP1, the major negative regulator of photomorphogenesis, in a conformation-independent fashion. Interestingly, we also found that high-level, constitutive nuclear expression of the full-length phyA also induces a weak but detectable de-etiolation response. Taken together, these data again showed that the phyA N-terminal fragment is biologically inactive but, more importantly, also demonstrated that the light-regulated nucleo/cytoplasmic partitioning of phyA is essential for the restriction of phyA action in darkness (*Viczian et al., Mol Plant Biol, 2012*).

Characterisation of molecular mechanisms mediating the light-induced import of phyB-phyE into the nucleus

Encouraged by the above results, we also constructed chimaeric genes that contained the N-terminal region of phyC, phyD or phyE, and generated transgenic lines in which the expression of these transgenes was restricted to the nucleus or the cytoplasm. We showed that, in contrast to phyA but similarly to phyB, the N-terminal fragments of phyC, phyD and phyE were biologically active and restored R light induced signalling similarly to the full-length phyC, phyD and phyE photoreceptors. We also demonstrated that the nuclear import of phyE occurs at extremely low light intensities, and it does not require interaction with FHY1/FHL. These observations strongly suggest that the molecular machineries mediating light-induced nuclear import of phyA, phyB and phyE are fundamentally different (*Adam et al., New Phytol, 2013*). We also showed that phyE and phyD do not interact with the PIF transcription factors in Y2H assays, and the nuclear import of phyE and phyD is not affected in the quadruple *pif1/pif3/pif4/pif5* mutant (*unpublished data*). These observations initiated several collaborative projects of which one has already been completed. In collaboration with the group led by Eberhard Schäfer in Freiburg we documented that, similarly to phyA, phyB does not contain an endogenous NLS (nuclear localization motif), and its light-induced import into the nucleus is inhibited in the quadruple *pif1/pif3/pif4/pif5* mutant during the early phase of the dark to light transition. In other words, our data demonstrated that the light-induced import of phyB is mediated at least partially by the PIF transcription factors *in planta (Pfeiffer et. al., PNAS, 2012).* This observation indicates that the generally accepted model explaining light-regulated import of phyB into the nucleus is incorrect and needs to be revised. Our data suggest that translocation of phyB into the nucleus can be mediated by any protein that interacts with phyB in a conformation-dependent fashion and bears an endogenous NLS motif. phyB has been shown to interact with the PIF1-PIF8 proteins, and translocation of phyB into the nucleus is only partially inhibited in the *pif1/pif3/pif4/pif5* quadruple mutant. To find out if any of the remaining PIFs are involved in this process, we initiated an interdisciplinary research project in cooperation with the group led by Wilfried Weber in Freiburg, since construction of an octuple mutant lacking all known PIFs is clearly not feasible. This group performing synthetic biology research was interested in developing light-regulated molecular switches based on the red/far-red reversible interaction of phytochromes with PIFs and other proteins to control expression of genes in mammalian cells by applying red/far-red light treatments. Data obtained so far demonstrated that (i) light induces PIF3-mediated translocation of phyB into the nucleus, (ii) phyC but not phyD and phyE interacts with PIF3 and is translocated in a red/far-red reversible fashion into the nucleus and (iii)

expression of the PIF1-PIF8 proteins is feasible in mammalian cells. On the one hand, these data demonstrate that this approach has the potential to test the functionality of any putative phy-interacting protein in regulating the import of phyB-phyE and thereby provide valuable information for performing targeted experiments in plants. On the other hand, it has the potential to develop novel phytochrome-based molecular switches with superior properties to control gene expression in space and time in mammalian cells. Further research on this subject will be supported by an NK OTKA grant (2014-2017) to F.N.

Characterisation of cell-autonomous and intercellular signalling in phyA- and phyB-regulated photomorphogenesis

We expressed the phyA/YFP and phyB/YFP fusion proteins under the control of ML1, SUC2, CAB3 and PHYA as well as PHYB promoters in phyA null and phyA/phyB null mutants, respectively. The expression patterns of the fusion proteins were characterised by confocal microscopy, and we selected lines that express the photoreceptors exclusively in the epidermis (ML1), in the mesophyll cells (CAB3), in companion cells (SUC2) or ectopically (PHYA and PHYB). Next we determined the expression level of the fusion proteins and chose lines which expressed the phyA/YFP and phyB/YFP proteins at similar levels in the various tissues as compared to PHYA/PHYA/YFP and PHYB/PHYB/YFP, respectively. Homozygous transgenic lines were multiplied and used for further experiments. Next we characterised to what extent phyA and phyB expressed in a single tissue can restore responsiveness to FR and R light, respectively. Our data demonstrated that phyA expressed in the epidermis or companion or mesophyll cells can only partially restore the phenotype of the phyA null mutant. phyA localized in the epidermis partially restored the FR-dependent inhibition of hypocotyl growth; phyA in the mesophyll cells contributed to the regulation of cotyledon expansion, whereas phyA in the companion cells fully restored flowering time of the phyA null mutant. Microarray analysis of the selected phyA-expressing transgenic lines indicated that phyA localized in the different tissues regulated the expression of different sets of genes, thus we concluded that phyA regulates the majority of cellular events in a tissue-autonomous fashion. This conclusion was further supported by the fact that FR light could induce the transcription of HY5 or the degradation of PIF1 and PIF3 transcription factors only in cells which contained phyA. We have submitted a manuscript to Plant Cell reporting the above described observation (Kirchenbauer et al., submitted to Plant Cell, 2014). In contrast to phyA, phyB expressed in the epidermis was sufficient to fully restore R light regulated photomorphogenic responses. Moreover, micro-array analysis demonstrated that, similarly to the ubiquitously expressed phyB photoreceptor, phyB restricted to epidermis cells induced transcription of a large number of genes. Taken together, these data strongly suggest that phyB regulates R light induced photomorphogenesis via generating non-cell-autonomous signal(s). At present we do not yet know the nature of these non-cell autonomous signal(s), but we have initiated a number of different approaches that could be helpful in identifying this elusive factor and publishing our results in top-rated general journals. First, we generated transgenic phyA/phyB/phyD null mutants that ubiquitously express the PIF3 and PIF4/CFP fusion proteins under the control of the 35S promoter or the HY5/HY5/CFP reporter. In this genetic background light-induced transcription and stabilization of HY5/HY5/YFP as well as light-induced degradation of PIF proteins are fully inhibited. Next we introduced into these lines the ML1/PHYB/YFP transgene to express the PHYB/YFP fusion protein exclusively in the epidermis, and used these transgenic lines for further studies. Next we showed that phyB localised in the epidermis can initiate degradation of PIF3/CFP not only in the same cell type but also in mesophyll and vascular cells. These data confirmed results obtained by micro-array analysis and provided direct evidence for the existence of a phyB-generated non-cell-

autonomous signal. Encouraged by these results, we mutagenised these lines and started a genetic screen to isolate mutants in which the degradation of PIF3 is restricted to phyB-containing cells. We were also interested to define whether phyB interacts with different partners in epidermis and mesophyll cells. To this end we isolated phyB by affinity purification from the selected transgenic lines, and analysed the composition of phyB complexes by mass spectrometry. Validation of the results obtained by mass spectrometry, as far as the composition of phyB-containing protein complexes is concerned, is very labour and time consuming and will require approximately 6–8 months' extra time. However, independently of the precise composition of phyB-containing protein complexes, mass-spectrometric analysis established that phyB is phosphorylated and sumoylated in light-grown tissues. These data were absolutely novel, since post-translational modification of phyB has not been reported until recently. We found that phyB was similarly phosphorylated in the various tissues; we experimentally validated the phosphorylation of S86 in the N-terminal region of phyB, and completed the analysis of phyB S86A and S86D mutants to assess the biological significance of this post-translational modification in R light induced photomorphogenesis. We have reported that phosphorylation of S86 accelerates dark-reversion (light-independent thermal relaxation of phyB Pfr into Pr), thereby it negatively regulates phyB signalling. In molecular terms, fast dark-reversion of phyB S86D prevents the interaction of phyB Pfr with PIFs, and thus inhibits the translocation of phyB Pfr into the nucleus, which ultimately leads to attenuated R light induced signalling. Our reports (Adam et al., 2010, Plos One, Medzihradszky et al., 2013, Plant Cell) established a new paradigm for desensitization of phyB signalling, and the significance of the paper published in Plant Cell was highlighted in an Editorial Article. Further research on how post-translational modifications including sumoylation modify phytochrome signalling will be supported by an OTKA NN grant (2014-2017) to F.N.