During the 4 year period of research supported by the present grant we have focussed our work to determine to what extent post-translational modifications (SUMOylation and/or phosphorylation) of various components of the red/far-red absorbing photoreceptor phytochrome controlled signaling cascade modify photomorphogenesis in the model plant Arabidopsis thaliana. Our research unambigously established that reversible phosphorylation and SUMOylation of the photoreceptor phytochrome-B (phyB) as well reversible SUMOylation of the phyB interacting bHLH type transcription factor (PIF3) plays significant role in fine tuning light regulated growth and development. We have not only showed that these proteins are the subjects of post-translational modifications but also provided mechanistical explanations by showing at molecular level how their post-translational modification promotes/inhibits their canonical function in light induced signaling.

(A). We have demonstrated that phosphorylation of phyB S86 inhibits light-induced signaling and clarified that phosphorylation negatively regulates the stability of the biologically active phyB Pfr conformer by accelerating thermal relaxation (dark-reversion) of the Pfr conformer into Pr. This observation established a new paradigm by showing that phosphorylation plays an important role in regulating phyB action. Moreover, in collaboration with the laboratory led by Katalin Medzihradszky we determined (i) the precise number and location of all phosphorylated amino acid residues in the N-terminal region of phyB and (ii) clarified to what extent light conditions affect the phosphorylation pattern of phyB. Next to this, in collaboration with Dr. Cornelia Klose from the University of Freiburg, we identified how phosphorylation of the photoreceptor modifies phyB action in shade and at different temperatures by analysing a large set of transgenic plants expressing targetedly mutated versions of phyB in transgenic plants. Collectively we demonstrated that (i) phosphorylation of the phyB N-terminal region does not interfere with phyB Pfr binding to PCHI (a known stabilizator of phyB Pfr form) and (ii) mutation of phyB D453R also inhibits dark-reversion of phyB Pfr without affecting phosphorylation of phyB or its interaction with PCHI. These data suggest that dark-reversion of phyB Pfr is mediated at least partly by the N-terminal region and regulated independently by its phopshorylation, and/or interaction of the full length phyB with PCHI and PIFs. It is evident that integration of these different regulatory mechanisms will be essential to understand how dark-reversion of phyB is controlled under natural growth conditions. The fact that the mutation of D453R (this amino acid residue was shown to be essential for the kinase activity of phyB in vitro) does not affect the phosphorylation pattern of phyB in vivo suggests that (i) phyB does not autophosporylate and that reversible phosphorylation of this photoreceptor is mediated by yet unknown kinases/phosphatases. We have identified two phosphatases which appear, at least indirectly, to be implicated in dephosphorylation of the photoreceptor. Experiments have been undertaken to validate their potential function in mediating reversible phosphorylation of phyB and other proteins known to mediate light induced signaling.

(B) We have provided evidence that beside phosphorylation, SUMOylation of phyB (K996) also negatively regulates phyB action. We demonstrated that reversible SUMOylation of phyB, which is mediated at least partly by the OTS1/OTS2 SUMO proteases, inhibits interaction of the photoreceptor with its downstream signalling partners PIF3 and PIF5. Our data also illustrate that these principally different post-translational modifications inhibit photoreceptor mediated light induced signaling via different molecular mechanisms, phosphorylation decreases life time of the active phpotoreceptor conformer, whereas SUMOylation inhibits its interaction with a component acting downstream of the photoreceptor. The precise mechanism of how these post-translational modifications are integrated and components (kinases/phosphatases and SUMO-ligase/SUMO proteases) mediating reversible modification of these proteins remains to be elucidated.

(C) Independent of the above studies we demonstrated that the PIF3 and PIF5 TFs, important negativ regulators of photomorphogenesis, are also subjects of SUMOylation in vitro. PIFs are active TFs , their transcriptional activity is required at the early phase of photomorphogenesis and regulate expression of a set of selective genes but their transcriptional activity is dismissible in regulating light induced inhibition of hypocotyl elongation and /or cotyledon expansion. In a series of experiments we have demonstrated that PIF3 and PIF5 are indeed SUMOylated in planta. We have also showed that transgenic pif3 null mutants expressing non-SUMOylatable form of PIF3 display hyposensitivity to light (their hypocotyls are longer, cotyledons are smaller and expression of various marker genes is higher as compared to wild type). Since PIF3 negatively regulates R-light-induced phyB-mediated signaling, the phenotype of the transgenic lines suggests that SUMOylation actually modifies both functions of this TF. To validate this somewhat unexpected conclusion we performed (i) in vitro EMSA assays by using SUMOylated or not modified recombinant PIF3 proteins and (ii) in vivo ChIP assays on transgenic lines expressing non-SUMOylatable and native PIF3/YFP fusion proteins. We showed that in vitro SUMOylation of PIF3 inhibits its binding to target promoters. Compromised DNA binding activity of SUMOylated PIF3 explains why expression level of the selected PIF3 dependently expressed genes are lowered in wild type when compared to that found in transgenic lines expressing non-SUMOylatable PIF3. Interaction and binding of PIF3 to the active conformer Pfr has been shown to be involved in regulating the amount of the photoreceptor via light induced co-degradation. Co-degradation of PIF3 and phyB is an essential step in regulating phyB action thus we determined wether SUMOylation could also affect interaction of phyB Pfr with PIF3 and thereby modify co-degradation of these proteins. We have just completed this set of experiments and the data obtained clearly show that SUMOylation of PIF3 compromises its interaction with the photoreceptor. Accordingly we found that levels of accumulated phyB were significantly lower in transgenic lines that express a non-SUMOylatable mutant of PIF3 when compared to native PIF3 expressing lines (in these a large portion of SUMOylated). Taken together the decreased level of phyB and increased activity of PIF3 in the transgenic lines expressing non-SUMOylatable PIF3 satisfactorily explain the pronounced hyposensitive phenotype of these lines (longer hypocotyl, smaller cotyledon). These data also indicate that SUMOylation of PIF3 and that of phyB antagonistically modify photomorphogenesis, by inhibiting action of both the photoreceptor (positiv regulator) and PIF3 (negativ regulator). SUMOylation has been shown to mediate various biotic and abiotic stress responses thereby it is likely that SUMOylation plays a role by integrating light and other stress induced signaling to fine tune growth and developmental responses under changing environmental conditions.