Protein ubiquitination in auxin signaling and transport

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SUMMARY

Auxin biology is among the oldest fields of experimental plant research, and therefore auxin is one of the most extensively studied plant hormones. The plant hormone auxin (indole-3-acetic acid or IAA) regulates plant development by inducing rapid cellular responses and changes in gene expression. Its regulatory effects on cell division, growth and differentiation are dependent on its transport-driven asymmetric distribution. At the cellular level auxin concentrations are translated into rapid cellular responses and changes in gene expression. Recently, several molecular components involved in auxin-responsive gene expression have been identified. This involves the proteasomal degradation of Aux/IAA transcriptional repressors, thereby allowing auxin response factors (ARFs) to activate the transcription of auxin-responsive genes. Most Aux/IAA proteins are short-lived and degradation of Aux/IAA proteins is essential for auxin signaling. Their half-lives and abundance are dramatically reduced by auxin. Auxin enhances binding of the conserved domain II of Aux/IAA proteins to the receptor TIR1, which is an F-box protein that is part of the E3 ubiquitin ligase complex SCF^{TIR1}. Binding of Aux/IAA proteins to SCF^{TIR1} leads to degradation via the 26S proteasome

Auxin is transported from cell to cell in a polar manner by the asymmetrically distributed PIN auxin efflux carriers. This polar auxin transport (PAT) generates dynamic auxin maxima and gradients. The protein serine/threonine kinase PINOID (PID) is a signaling component in the control of PAT, as it determines the apico-basal polarity of several members of the PIN family of auxin efflux carriers. The PID kinase has been shown to directly phosphorylate the hydrophilic loop of PIN proteins, and to act antagonistically with the PP2A protein phosphatases on the phosphorylation status of PIN proteins. The current model defines that PID regulates polar auxin transport by controlling PIN localization, and thereby determines the direction of auxin flow. PIN proteins are the only functionally characterized PID phosphorylation targets identified to date. In a search for additional targets of PID, yeast-two-hybrid screens identified four interacting partners: the calcium binding proteins TCH3 and PBP1/PBP1H, the BTB/POZ domain protein BT1/PBP2, and subunit 8 of

the COP9 signalosome (CSN8/COP9). None of the PBPs appeared to be phosphorylated by PID, and instead they were found to regulate the activity of this kinase. Moreover, the binding of PID to CSN8 suggested a role for this kinase in regulating protein ubiquitination. The COP9 signalosome (CSN) is a protein complex found in eukaryotic cells that regulates many cellular processes linked to targeted protein degradation. The CSN interacts with, and is essential for the activity of E3 ubiquitin ligases. In Arabidopsis, *cop* mutants display constitutive photomorphogenesis and expression of light-responsive genes in the dark. The interaction network of the CSN is broad and complex, influencing almost every aspect of plant development, among which also auxin response and transport.

The research described in this thesis was directed at unraveling the role of protein ubiquitination in auxin response and transport. As described above, it was well established that the binding of auxin to TIR1 enhances the affinity of this F-box protein for Aux/IAA proteins, and thereby leads to enhanced turn over of these repressor proteins by the 26S proteasome. However, evidence for SCF^{TIR1}-mediated poly-ubiquitination of Aux/IAA proteins was lacking. In **Chapter 2** an *Arabidopsis* cell suspension-based protoplast system was used to find evidence for SCF^{TIR1}-mediated ubiquitination of the Aux/IAA proteins SHY2/IAA3 and BDL/IAA12. Each of these proteins showed a distinct abundance and repressor activity when expressed in this cell system. Moreover, the amount of endogenous TIR1 protein appeared to be rate-limiting for a proper auxin response measured by the co-transfected *DR5::GUS* reporter construct. Co-transfection with *35S::TIR1* led to auxin-dependent degradation, and excess of *35S::TIR1* even led to degradation of Aux/IAAs in the absence of auxin treatment. Expression of the mutant tir1-1 protein or the related F-box protein COI1, which is involved in jasmonate signaling, had no effect on Aux/IAA degradation. The results show that SHY2/IAA3 and BDL/IAA12 are poly-ubiquitinated and degraded in response to increased auxin or TIR1 levels. In conclusion, these data provide experimental support for the model that SCF^{TIR1}-dependent poly-ubiquitination of Aux/IAA proteins marks these proteins for degradation by the 26S proteasome, leading to activation of auxin-

responsive gene expression. It is likely that the differences observed between the two AUX/IAA proteins studied might be linked to differential affinity of these proteins for the TIR1 receptor. For the BDL protein it is hypothesized that affinity might be regulated by phosphorylation (Chapter 4).

The results in Chapter 2 are in line with the paradigm that targeted protein degradation occurs through the specific recognition by the proteasome of proteins that have been marked with ubiquitin chains. In **Chapter 3,** evidence is provided that this paradigm does not hold for all plant proteins. The Arabidopsis calcium binding protein PINOID BINDING PROTEIN 1 (PBP1) was originally identified as an interactor of the PINOID (PID) kinase, which was shown to control PID activity in vitro and its subcellular localization in vivo. Using an Arabidopsis cell suspension protoplast system it was found that PBP1 is a highly unstable, poly-ubiquitinated protein that can be stabilized by inhibition of proteasomal degradation. Co-expression of PID did not affect PBP1 poly-ubiquitination. Surprisingly, substitution of all the lysines (K) in PBP1 for arginines (R) blocked poly-ubiquitination, but did not affect its proteasomal degradation. The mutant (K→R) protein retained all tested wild type functions, including its interaction with PID and its subcellular localization. Translational fusions of the lysine-less PBP1 with YELLOW FLUORESCENT PROTEIN (YFP) were ubiquitinated, which indicates that the PBP1 sequence contains a cis-acting motif that is recognized as an ubiquitination signal but not as a degron, since the YFP fusions were not unstable. PBP1 is the first example of a plant protein that is degraded by the proteasome in an ubiquitination-independent pathway. Although the functions of PBP1 ubiquitination remain to be elucidated, our results show that its proteasomal degradation is not dependent on ubiquitination.

Chapter 4 describes the identification of the labile auxin response repressor BODENLOS (BDL/IAA12) as *in vitro* phosphorylation target of PID. The observation that PID-mediated phosphorylation possibly occurs in the PRSS motif close to the SCF^{TIR1}-interacting domain II of BDL/IAA12 suggests that this event plays a role in the stability of this repressor protein. Blockage of the identified phosphorylation site has minor negative effects on the repressor

activity of the BDL protein in protoplasts and *in planta*, but plants carrying a phosphorylation-insensitive version of the gain-of-function bdl protein fail to reproduce the *bodenlos* phenotype. Additionally, the phosphorylation-insensitive bdl protein is much less stable and has a more restricted tissue distribution in the root tip. This indicates that the control of BDL via phosphorylation might be an important mechanism regulating Arabidopsis root development. Although the mechanisms and roles of PID-mediated regulation of BDL require further elucidation, our data suggest that the PID protein kinase regulates both auxin transport and auxin-responsive gene expression.

Chapter 5 describes a further study on the interaction of PID with the CSN subunit CSN8/COP9. *In vitro* phosphorylation assays showed that not CSN8, but the linked subunit CSN7/COP15 is phosphorylated by PID *in vitro*. PID-dependent phosphorylation of CSN7 appeared not to be essential for CSN functions *in planta,* at least not under the growth conditions tested. In protoplasts CSN8 sequesters PID to the cytoplasm and nucleus and enhances the PID ubiquitination that was already described in Chapter 3. The association of PID with the CSN may be related to the control of PID turn over, however another possibility could be that PID regulates the interaction between its phosphorylation targets BODENLOS/IAA12 and PIN proteins and their corresponding E3 ubiquitin ligases. The identification of PID as CSN-associated kinase reveals an unexpected new aspect of PID signaling that links the action of this kinase to control of ubiquitination.

In conclusion, our results show that poly-ubiquitination of proteins plays a central role in the action of the plant hormone auxin, and that the PID protein kinase provides an unexpected link in the communication between auxin transport and auxin response.