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Components and targets of the PINOID signaling complex in Arabidopsis thaliana

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Summary

The phytohormone auxin - or indole-3-acetic acid (IAA) - is a major determinant in a wide array of plant developmental processes, such as photo- and gravitropism, apical dominance, embryogenesis and phyllotaxis. Although IAA was the first plant hormone to be isolated and characterized, it was only recently that the mechanisms underlying auxin's action began to be unraveled. Molecular and genetic studies - mostly using the model plant *Arabidopsis thaliana* - have revealed the unique dymanics of auxin and have identified several components that play an essential role in this hormone's functionality. Auxin action was found to depend on dynamic gradients of this hormone generated by PIN protein-facilitated polar auxin transport (PAT). These PIN proteins possess auxin efflux activity and direct PAT through their asymmetric subcellular localization. The polar PIN localization is dynamically maintained and regulated through cyclic trafficking of PIN loaded vesicles along the actin cytoskeleton between endosomal compartments and the plasma membrane (PM).

Previously, the protein kinase PINOID (PID) was identified as regulator of the apico-basal polar localization of PIN proteins, as above-threshold levels of this signaling enzyme direct PIN trafficking to the upper (apical) side of plant cells. A yeast two-hybrid screen identified four PID Binding Proteins (PBPs): the two calcium binding proteins PBP1 and TCH3, PBP2 and the COP9 signalosome subunit CSN8 (Figure 1). This thesis describes a more detailed study of several components and the identification of putative targets of the PID signaling complex.

Chapter 2 focuses on PBP2, a likely scaffold protein that - based on its possible localization at the cytoskeleton in onion cells - was considered as a promising link between PID and PIN vesicle trafficking. The presumed scaffold function of PBP2 was apparent, since the protein consists of two domains that are known to mediate protein-protein interactions: an amino-terminal Bric-a-brac, Tramtrack and Broad Complex/Pox virus and Zinc finger (BTB/POZ) domain, and a carboxy-terminal Transcriptional Adaptor putative Zinc Finger (TAZ) domain. In contrast to previous results, *in vitro* phosphorylation assays showed that PBP2 is not a phosphorylation target of PID, but that instead PID activity is repressed by PBP2. *In vitro* pull down assays suggested that PID interacts with the BTB/POZ domain, and transient expression of both proteins fused to GFP in Arabidopsis protoplasts suggested that this interaction occurs in the cytoplasm.

The likely scaffold function of PBP2 indicated that other PBP2 interacting proteins (PBP2IPs) play a role in the PID signaling pathway. In an yeast two-hybrid screen sixteen putative PBP2IPs were identified that classified as cytoskeletal proteins, transcription factor-like proteins or proteins with an enzymatic function in primary metabolism. Three PBP2IPs, the putative microtubule-associated PBP2 BINDING MYOSIN-LIKE PROTEIN (PBMP), the transcription factor-like PBP2 BINDING MYB PROTEIN (PBMYB), and an uncharacterized AUXIN-INDUCIBLE PBP2 BINDING

PROTEIN (APBP), are analyzed in more detail. *In vitro* pull down assays showed that PBMP and PBMYB interact with the C-terminal TAZ domain containing portion, whereas APBP interacts with the N-terminal part of PBP2. *In vitro* phosphorylation assays did not show any evidence that these PBP2 partners are phosphorylated by PID, implying that PBP2 is not a scaffold for PID substrates. Further analysis did not provide evidence that PBMYB and PBMP are part of the PID signaling complex. In contrast, for APBP we concluded that it may be involved in modulating flowering time, and that it possibly competes with PID for the interaction with PBP2, and as a consequence activates the kinase by relieving it from PBP2-mediated repression. Overall, the data presented in Chapter 2 indicate that PBP2 is a scaffold protein with multiple functions, one of which is to be recruited to the PID signaling complex to regulate PIN polar targeting.

Chapter 3 describes the more detailed analysis of the interaction of PBP2 with two paralogous plant-specific microtubule motor proteins, PBP2 BINDING KINESIN 1 (PBK1) and 2 (PBK2). *In vitro* pull down and phosphorylation assays corroborated the scaffold function of PBP2, since the PBKs bind the C-terminal TAZ domain portion and the PID kinase binds the N-terminal BTB/POZ domain portion of PBP2. The possible existence of such a protein complex at the cytoplasm-plasma membrane boundary was corroborated by the overlapping spatio-temporal expression of *PID, PBP2* and the *PBKs*, and by the fact that the proteins - when fused to GFP – co-localize in the cytoplasm of Arabidopsis protoplasts. Analysis of *pbk1/pbk2* mutant plants and *35S::PBK1* overexpression lines showed phenotypes that were also observed in mutants defective in PAT and in *pid* loss-of-function seedlings, respectively. These observations suggest that the PBKs are involved in the suppression of PID kinase activity. As PBP2 was shown to inhibit PID activity *in vitro*, we propose that PBK1 and PBK2 transport PBP2 to suppress PID and PID-like activity at specific subcellular locations, thereby providing polarity to the signaling of these kinases (Figure 1A).

The subcellular localization of animal transporters is known to be regulated by direct phosphorylation, often in a large cytoplasmic domain of these membrane proteins. In **Chapter 4** the possibility is addressed that PIN proteins are direct phosphorylation targets of PID. *In silico* analysis of PIN1 revealed twenty-three putative phosphorylation sites, twenty-one of which are localized at the large cytoplasmic loop (CL) of this protein, and five of which are 100% conserved among the CL-containing PINs in Arabidopsis. *In vitro* assays using PID and synthetic PIN1 peptides containing most of the predicted phosphorylation sites identified four highly phosphorylated peptides comprising three of the predicted phosphorylated residues that are 100% conserved in the CL containing PINs. Notably, two of the strongly phosphorylated peptides comprise the T-P-R-X-S-N motif. By testing CLs of different PIN proteins and through site directed mutagenesis we deduced that the

serines 231 and 290, both positioned in the conserved T-P-R-X-S-N motifs, are the major substrates for PID-mediated phosphorylation, and that the serines 377 and 380, that were previously shown to be phosphor-substrates in PIN7 *in vivo*, may also be modified by PID. Our results suggest that the PID kinase affects PIN polarity through direct modification of multiple conserved serine residues in the large cytoplasmic loop of these auxin efflux facilitators.

Polar transport-mediated distribution of auxin results in differential distribution of this hormone, and the cellular auxin concentrations are subsequently translated into a primary gene expression response by the complex and cell-specific interactions between ARF transcription factors and labile Aux/IAA repressors. The abundance of Aux/IAA repressors is controlled by their auxin-induced, SCF^{TIR1} E3 Ligase-dependent proteolysis, a process that is regulated by the COP9 Signalosome (CSN).

Chapter 5 describes the identification of CSN subunit CSN8/COP9 as interacting partner of PID, and that not CSN8, but the linked subunit CSN7/COP15 is phosphorylated by PID *in vitro*. The observation that PID overexpressing plants share constitutive photomorphogenic characteristics with *csn* down-regulated mutant lines suggests that PID may be a repressor of CSN activity. An alternative role for PID as a putative CSN-associated kinase could be to regulate the interaction between E3 ligases and their proteolysis targets. Interestingly, we identified 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 PRXS 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. Analysis of the *pid-bdl* double mutant and transient expression experiments provided important *in vivo* clues for a role of PID as negative regulator of BDL activity during embryogenesis. Considering that BDL is functionally redundant with IAA13, and that IAA13 also contains the PRXS motif, it is plausible that PID affects the activity of both AUX/IAAs. Whether PID controls the stability of BDL and IAA13 or their interaction with ARF5/MP remains to be determined.

Although the mechanisms and roles of PID-mediated regulation of BDL, IAA13 or CSN require further elucidation, our data suggest that the PID protein kinase provides a direct link between auxin transport and -signaling.

Conclusions

The results described in this thesis suggest a duality in the function of the PID kinase, which leads us to propose the following working model (Figure 1). On one hand PINOID functions in the cytosol, where its activity - to mark endosomal PIN proteins for apical targeting (Figure 1A, region 1) or to signal apical-localized PINs to stay in this position (Figure 1A, region 2) - is regulated in sub-cellular manner

through a PBP2-kinesin complex (Chapter 3), and through the calcium binding proteins PBP1 and TCH3 (Figure 1A). On the other hand, PID functions in the nucleus as a CSN-associated kinase that may regulate the activity of the CSN itself, or that regulates the proteolytic degradation of targets of the E3 ligase-CSN complex, such as BDL/IAA12 and IAA13, by phosphorylation (Figure 1B). In this subcellular compartment, it is possible that PID function is again controlled by PBP2. It is likely, however, that PBP2 is also involved in other - PID-unrelated – processes, such as the regulation of gene transcription by the transcription factor PBMYB (Figure 1B).

Interestingly, none of the possible phosphorylation targets of PID identified thus far, namely PIN1 (Chapter 4) BDL or CSN7 (Chapter 5), show a strong interaction with PID. Conversely, none of the PID interactors obtained in a yeast two-hybrid screen are phosphorylation targets of this protein kinase, but instead three of the PBPs seem to regulate PID kinase activity (Chapter 2). All together, these results indicate that PID interacts very transiently with its substrates. This is in contrast to what has been observed for mitogen activated protein kinases that need to stably interact with their substrates for efficient phosphorylation. It could be, however, that PID uses non-substrate proteins in order to be anchored to and efficiently modify its phosphotargets. The use of CSN8 as an adaptor protein for PID-mediated phosphorylation of CSN7 may be an example of such a behavior. However, the true relationship between PID and its putative phosphor-substrates remains to be established.

The results presented in this thesis provide interesting new insights into putative molecular mechanisms of PID action, the most important of which is the direct PID-mediated phosphorylation of PIN proteins in their cytoplasmic loop. Acknowledged PID-sensitive PINs direct auxin transport in roots (PIN1, PIN2 and PIN4), hypocotyls (PIN1), inflorescence meristems (PIN1) and embryos (PIN1, PIN4), whereas PID function is limited to the latter two tissues. Assuming that PID-like signaling is essential for PIN polarity throughout the whole plant, it is logical to assume that other PID-related kinases regulate PINs in other tissues than in inflorescence meristems or embryos. Our previous analysis of the Arabidopsis genome identified twenty-two other members of the plant specific family of protein kinases to which PINOID belongs. Most likely, some of these members are also putative PINs regulators, and the comparative study of their function and activity will help to clarify their role in regulating the direction of PAT.

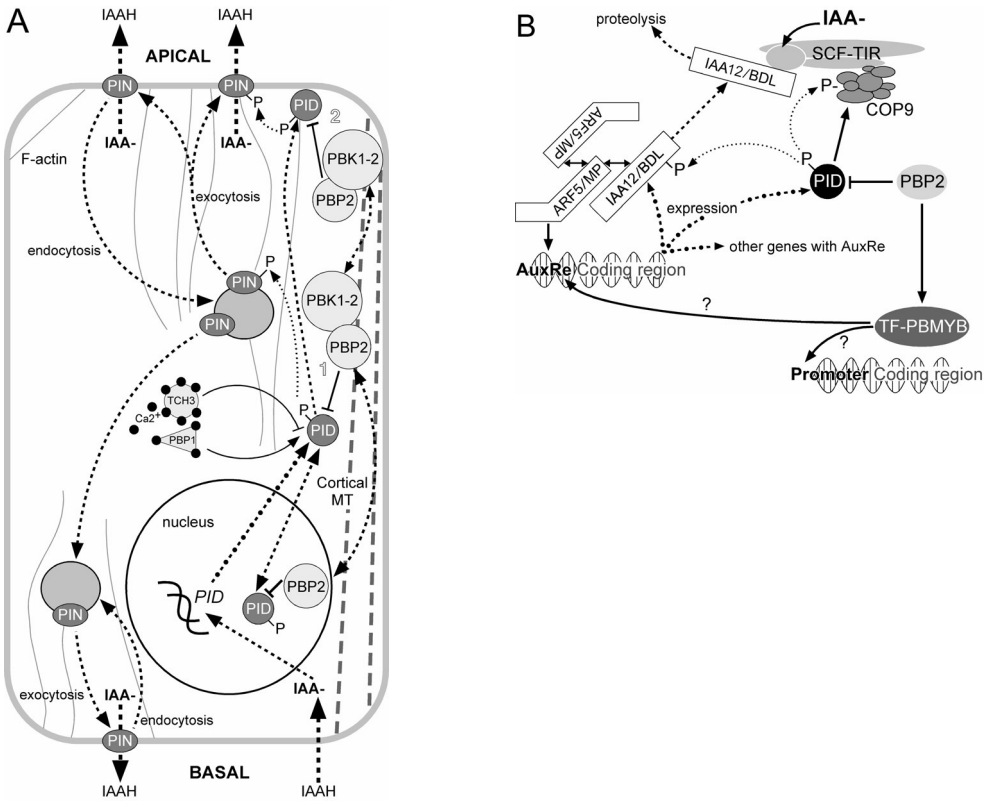

Figure 1. Model for the mechanisms of PID-mediated regulation of PAT. (A) Protonated (non-charged) auxin enters the cell by passive diffusion or by active carrier-mediated uptake and induces expression of auxin responsive genes, such as *PID*. In response to cytosolic calcium levels, PID activity is enhanced by the calcium binding protein PBP1 or repressed by the calmodulin TCH3. The PBK-PBP2 complex moves along cortical microtubuli to repress PID activity in different cellular locations: 1) at the endosomes were PID-mediated phosphorylation of PINs marks them for exocytosis to the apical cell pole, or 2) at the plasma membrane where PID-mediated phosphorylation prevents endocytosis, thereby signaling PINs to stay at their current apical position. Controlled PID-mediated PIN phosphorylation ultimately results in modulation of cellular auxin efflux. (B) In the nucleus, auxin induces the expression of genes containing auxin responsive elements (AuxRe) such as *PID* and *IAA12/BDL*. This induction is enabled by initial auxin binding to TIR1, which enhances SCF^{TIR1}-Aux/IAA interaction, leading to enhanced proteolysis of Aux/IAA proteins. The degradation of the Aux/IAA protein BDL releases its repression upon ARF5/MP, therefore enabling ARF5 activation of auxin responsive genes. BDL phosphorylation by CSN-associated kinase PID could stimulate BDL binding to TIR1, and enhance BDL degradation. Alternatively, PID phosphorylation could weaken the BDL-ARF5 interaction. PID-CSN interaction could also result in CSN repression upon phosphorylation. Continuous lines indicate protein-protein or protein-DNA interactions; thick punctuated lines indicate mobility; thin punctuated lines indicate phosphorylation; thick punctuated lines with dots indicate gene expression.