Calcium- and BTB domain protein-modulated PINOID protein kinase directs polar auxin transport

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Summary

Plant architecture is determined by tightly regulated developmental processes that largely depend on the action of the plant hormone auxin (Tanaka et al., 2006). Auxin has been first discovered as the signaling molecule that directs tropic responses of plants to unidirectional abiotic stimuli such as gravity and light (Darwin, 1880). A major determinant in auxin action, besides its signaling pathway, is its polar cell-to-cell transport (PAT) throughout the plant, which generates dynamic concentration gradients and maxima that are instrumental for organ positioning, meristem maintenance, embryo development and tropic growth responses (Benková et al., 2003, Friml et al., 2002, Friml et al., 2003, Reinhardt et al., 2003, Sabatini et al., 1999). The drivers of PAT are the PIN auxin efflux carriers that control the direction of PAT through their asymmetric subcellular localization (Wisniewska et al., 2006). The polar distribution of PIN proteins is highly dynamic, mediated by actin cytoskeleton-guided vesicle trafficking, and regulated by PIN protein degradation and auxin signaling (Abas et al., 2006, Geldner et al., 2001, Sauer et al., 2006) .

The AGCVIII PINOID (PID) protein serine/threonine kinase is the first, and yet only, identified determinant in the polar subcellular targeting of PIN proteins. Above threshold levels of PID activity induce a polarity switch in PIN localization from the basal (root facing) to the apical (shoot facing) side of the cell (Friml et al., 2004). Recently, it was shown that PID is a membrane-associated kinase (Lee and Cho, 2006) and that PID and PINs partially co-localize at the plasma membrane (Michniewicz et al., 2007). Furthermore, evidence was provided for the PID-dependent phosphorylation of PIN proteins in their large hydrophilic loop, and for the antagonistic action of PID and PP2A phosphatases on the phosphorylation status of PIN proteins (Michniewicz et al., 2007). Although these findings do clarify how PID functions as a regulator of PAT (Benjamins et al. 2001), several important questions about the regulation of the localization and the activity of PID remain unanswered. For example, 1) what are the upstream regulators of the PID kinase, 2) what determines the subcellular PID localization, 3) does PIN phosphorylation occur at the plasma membrane or in the endosomal compartments, and 4) is PID-dependent PIN phosphorylation sufficient to induce a switch in PIN polarity?

Previously, a yeast two-hybrid screen identified two calcium binding proteins PINOID BINDING PROTEIN1 (PBP1) and TOUCH3 (TCH3) and the BTB domain protein PBP2 as PID-interacting proteins. PBP1 is a small protein with a single calcium binding pocket (EF-hand). The corresponding encoding gene is part of a small gene family in Arabidopsis that includes *PBP1H* (for *PBP1 homologue*) and *KIC* (Reddy et al., 2004). TCH3 is an atypical 6 EF-hand calmodulin-like protein that is encoded by a single copy touch-inducible gene in Arabidopsis (Braam and Davis, 1990, Sistrunk et al., 1994). TCH3 and PBP1 bind to PID in a calcium-dependent manner. PBP2 was previously named BTB and TAZ domain protein1 (BT1) (Du and Poovaiah, 2004). BT1 is part of a family of five proteins characterized by the presence an N-terminal BTB domain, a TAZ domain and a C-terminal calmodulin-binding domain, a combination that is land plant specific. BTB

proteins are known to act as scaffold- or linker-proteins that organize protein complexes in various pathways (Albagli et al., 1995), and the specificity of their function may be dependent on the presence of extra domains. Interestingly, none of the PBPs are of targets PID, and instead they negatively (TCH3 and PB2) and positively (PBP1) regulate the activity of this kinase in *in vitro* phosphorylation reactions (Benjamins et al., 2003, Benjamins, 2004). The objective of the study described in this thesis was to identify the *in vivo* significance of the interaction between the PBPs and the PID kinase.

Chapters 2 and 3 describe the further functional analysis of the calcium-binding proteins. *In vitro* and *on chip* phosphorylation assays confirmed the regulatory role of TCH3 and PBP1 on PID activity, and for TCH3 it could be shown that it interacts with the catalytic domain of PID, whereas PBP1 seems to interact with the N- and C-terminal parts of this kinase. Furthermore, loss- and gain-of-function mutants in *TCH3* and *PBP1* were used to investigate and confirm that both proteins are regulators of the PID kinase activity. *pid* loss-of-function mutant embryos appeared to be sensitized to changes in *TCH3* expression, in that both loss- and gain-of-function of *TCH3* enhanced cotyledon defects. Moreover, overexpression of the *TCH3* coding region reduced the *PID* overexpression phenotype, which is in line with the model that TCH3 negatively modulates PID kinase activity (**Chapter 2**). On the other hand, *pbp1* and *pbp1h* loss-of-function mutations synergistically enhanced *pid* embryo phenotypes, which fits with their stimulatory effect on the *in vitro* PID phosphorylation activity. In addition, PBP1 and PBP1H were found to act as repressors of root growth, possibly though the enhancement of PID function. In contrast, *pbp1-1* loss-of-function partially rescued the *pid-14* inflorescence phenotype. Preliminary observations suggest that this rescue is not observed when two PID-related kinases (*WAG1* and *WAG2*) or *PBP1H* are also knocked out, suggesting that *pbp1* loss-of-function induces feed-back regulation on the activity of the redundantly acting PID-related kinases (**Chapter 3**).

Co-expression of a PID:CFP fusion with TCH3 or PBP1 fused to YFP in Arabidopsis protoplasts and the subsequent detection of Förster Resonance Energy Transfer (FRET) confirmed that PID and the two proteins interact *in vivo*. Interestingly, in both co-transfections the membrane-associated PID:CFP was sequestered from the plasma membrane to the cytosol. This sequestration was auxin-dependent, as it was not observed in auxin-starved protoplasts. Recently, Zegzouti and co-workers provided evidence that association of PID with the plasma membrane is mediated by the amino acid insertion in the PID catalytic domain that is characteristic for the AGCVIII kinases. They suggested that membrane-association was mediated by binding of this insertion domain to phosphatidic acids and phosphorylated inositides (Zegzouti et al., 2006). The sequestration of PID could thus be caused by TCH3 and PBP1 blocking the lipid binding site(s) in PID.

The auxin-dependency of PID sequestration could be related to the well-documented auxin-induced increase in cytoplasmic calcium (Dela Fuente and Leopold,

1973, Felle, 1988, Gehring et al., 1990, Shishova and Lindberg, 2004), which should enhance the binding of PID to the interacting calcium-binding proteins. Indeed, in root epidermis cells, where the expression of *PID* and *TCH3* is known to overlap, auxin treatment provoked a rapid transient release of PID to the cytoplasm, a response that appeared to be dependent on the action of calcium channels and calmodulin proteins such as TCH3 (**Chapter 2** and Figure 1). Preliminary results indicate that *PBP1* overexpression renders PID more resistant to this auxin-induced release from the plasma membrane (**Chapter 3** and Figure 1). It is thus likely that (auxin-induced) elevated levels of cytosolic calcium facilitate the interaction of TCH3 to the PID catalytic domain, preventing a kinase-

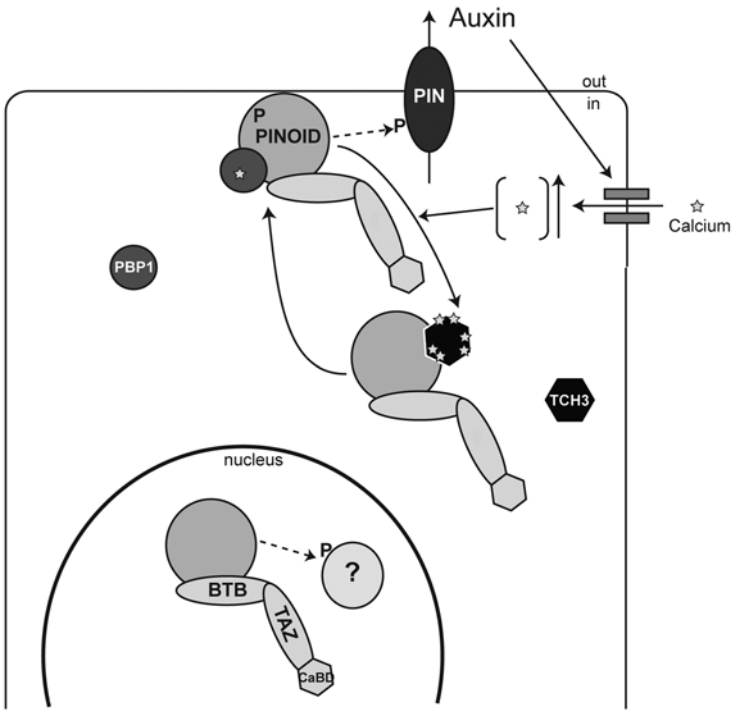

Figure 1. PID activity and subcellular localization is mediated by BT1, calcium and the calcium-binding proteins TCH3 and PBP1.

PID is a plasma membrane-associated protein kinase in proximity of its phospho-targets, the PIN auxin efflux carriers. Through its BTB domain, BT1 might regulate PID localization, notably in the nucleus, where yet unknown PID phosphorylation targets are localized. Low calcium levels stabilize membrane association and potentiate PID activity by interaction with PBP1. Increases in calcium concentrations, via calcium channels in the plasma membrane, for example in response to elevated auxin levels, stimulate the interaction with the calmodulin-like TCH3, and this inhibits PID activity and triggers the dissociation of PID from the plasma membrane. P: phosphate group from a phosphorylation event, stripped line: phosphorylation reaction, plain line: signaling event, stars: calcium.

lipid interaction and resulting in sequestration of the kinase away from its phospho-targets to the cytoplasm (Figure 1). Changes in subcellular localization are a commonly used cellular mechanism to regulate protein activity by sequestering proteins away from their targets. To our knowledge, however, the calcium- and calmodulin-dependent release of the PID kinase represents a novel mechanism of how the activity of kinases that steer the polar subcellular targeting of transporter proteins is regulated.

Chapter 4 describes the functional analysis of the Arabidopsis *BT* gene family to which *PBP2/BT1* belongs. A comparison of gene structure and amino acid sequence analysis of the encoded proteins indicated that the five genes group into three clades, that the genes have a clade-specific expression pattern, and that the corresponding proteins have a clade-specific subcellular localization. Furthermore, BT1 is shown to be a short-lived protein targeted for degradation by the 26S proteasome. Genetic analysis of the *BT* family indicated that the genes are functionally redundant and that *BT2* and *BT3* are essential for both male and female gametophyte development.

Chapter 5 describes a study on the link between BT proteins and PID. BT1 was found to interact with PID through its BTB domain, and to repress PID kinase activity *in vitro*. Moreover, *BT1* and *PID* expression patterns in Arabidopsis plants overlap, and PID:CFP and BT1:YFP fusion proteins co-localize in the cytoplasm of Arabidopsis protoplasts, indicating that *in vivo* interaction is possible. In fact, the proteins were found to alter each other subcellular localization. In protoplasts expressing both fusion proteins, BT1:YFP is also found at the plasma membrane, whereas PID:CFP becomes localized to the nucleus. Not only does this provide *in vivo* evidence for the interaction between BT1 and PID, but it also uncovers that PID possibly functions in the nucleus (Figure 1). *In vitro* pull-down assays indicate that at least four of the five BT proteins interact with PID, which corroborates the observed functional redundancy among the *BT* genes (**Chapter 4**).

The negative regulatory function of BT1 was confirmed *in vivo* by the fact that *BT1* overexpression enhanced *pid* loss-of-function mutant phenotypes and reduced *PID* overexpression phenotypes. Interestingly, *PID* overexpression, which normally does not lead to strong inflorescence phenotypes, significantly enhanced the *bt* loss-of-function inflorescence and silique phenotype. This indicates that the effect of *PID* overexpression on the inflorescence is normally suppressed by BT proteins, and only becomes visible in plants carrying multiple *bt* mutations. In contrast, the multiple *bt* mutations rescued the *PID* overexpression seedling phenotypes, indicating that BT scaffold proteins do not only repress PID activity, but are also essential components of PID signaling (**Chapter 5)**.

In conclusion, the functional analyses of the PBPs described in this thesis uncover a new mechanism of protein kinase activity regulation via calcium signaling, and present novel roles for the BT proteins, not only in PID signaling, but also more in general in plant development. Interesting questions about the biological relevance of the fine tuning of PID activity by these upstream regulators remain unanswered. For example, how does the

subcellular sequestration of PID by calcium signaling affect the PIN function and localization? And what is the exact function of the BT proteins in the PID signaling pathway, and which other proteins are parts of the BT-PID complex? The first steps and components in PID signaling have now been established, but clearly much more details need to be uncovered before the molecular mechanisms behind the dynamic kinase-dependent steering of polar auxin transport in response to endogenous developmental programs and external (abiotic) signals will be completely understood.

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