PIN protein phosphorylation by plant AGC3 kinases and its role in polar auxin transport

Huang, F.

Summary

Summary

As sessile organisms, plants have evolved sophisticated molecular and cellular mechanisms to adapt to changes in their environment, many of which involve the plant hormone auxin. Auxin, or indole-3-acetic acid (IAA), regulates a wide range of plant developmental processes through directional transport generated maxima and minima of this hormone in young developing plant tissues and organs. This so-called polar auxin transport (PAT) is driven by three families of transporter proteins, of which the PIN family of auxin efflux carriers determine the direction of transport through their asymmetric distribution at the plasma membrane (PM). The *Arabidopsis thaliana* genome encodes eight PIN proteins, five of which are actually PM-localized and involved in cell-to-cell PAT. These PM-localized PIN proteins are predicted to consist of two sets of five transmembrane domains that are linked by a large central hydrophilic loop (HL).

The polar localization of PIN proteins is highly dynamic, and is regulated by cellular trafficking mechanisms, ubiquitin-mediated proteolysis, phosphorylation and membrane composition, and is responsive to both internal and external signals (discussed in **Chapter 1**). Following their de novo synthesis, PIN proteins are first placed at the PM in an apolar fashion, and by endocytosis and polar recycling between endosomes and the PM, their polarity is established. The asymmetric distribution of PIN proteins is maintained by constitutive endocytosis and recycling, which is depedent on the actin cytoskeleton and regulated by ADP ribosylation factor guanine exchange factors (ARF-GEFs). One of these ARF-GEFs GNOM is a target of the fungal toxin brefeldin A (BFA), and its treatment induces PIN1 accumulation in endosomal BFA compartments, indicating that GNOM regulates the exocytosis step from endosomes to the PM. Both internal signaling molecules, such as auxin itself, and external signals, such as light or gravity, regulate the polarity and abundance of PIN proteins at the PM, and thereby direct plant development, by interfering with cyclic PIN endo- and exocytosis. Endocytosed PIN proteins are found to be targeted to vacuoles for degradation, which is an important mechanism in the post-translational regulation of PIN proteins.

An important question in auxin research is what determines PIN polarity, or more specifically what directs trafficking of PIN cargoes to one side of the cell. In many biological systems, phosphorylation is an important post-translational modification of proteins that serves as “on-and-off” switch in the regulation of cellular activities and signaling processes. Initial genetic and pharmacological analyses already indicated that PAT is regulated by kinase and phosphatase activities. More recently, the PINOID (PID) serine/threonine protein kinase was identified as key determinant of PIN polarity. High levels of PID activity (*PID* overexpression lines) induce a switch in PIN polarity from

the basal (rootward) to the apical (shootward) side of the root cells, whereas low levels of PID activity (*pid* loss-of-function mutants) result in an apical-to-basal PIN polarity alteration in epidermal cells of the shoot apical meristem. The indicated PIN polarity changes result in changes of the local auxin concentration, consistent with the *PID* gain- or loss-of-function mutant phenotypes. PID was found to act antagonistically with PP2A phosphatases to direct PIN polarity by phosphorylating the PINHL. In order to further investigate the mechanism of PID-mediated PIN polar localization, we set out to identify the phosphorylation sites of PID in the PINHL, and to test the functional significance of PIN phosphorlyation in PIN polarity and intracellular PIN trafficking, and how this directs auxin-regulated plant development in *Arabidopsis thaliana*. Moreover, as PID belongs to the plant-specific AGCVIII protein serine/threonine kinase family where it groups into the AGC3 subclade together with the closely related protein kinases WAG1, WAG2 and AGC3-4, we also tested the functional redundancy among the plant AGC3 kinases in the regulation of PIN polar localization.

Chapter 2 describes the identification of three evolutionarily conserved TPRXS(N/S) motifs within the PIN1HL, and provides evidence that the central serine residues located in the motifs are phosphorylated by PID. Loss-of-phosphorylation PIN1:GFP protein (serine to alanine) induced inflorescence defects, correlating with their basal localization in the shoot apex, and induced internalization of PIN1:GFP during embryogenesis, leading to strong embryo defects. Conversely, phosphomimic PIN1:GFP (serine to glutamic acid) showed apical localization in the shoot apex but did not rescue *pin1* inflorescence defects. Both loss-of-phosphorylation and phosphomimic PIN1:GFP proteins were insensitive to *PID* overexpression. The basal localization of loss-of-phosphorylation PIN1:GFP increased auxin accumulation in the root tips, partially rescuing *PID* overexpression-induced root collapse. Collectively, our data indicate that reversible phosphorylation of the conserved serines in the PIN1HL by PID (and possibly by other AGC kinases) is required and sufficient for proper PIN1 PM localization, and is thus essential for generating the differential auxin distribution that directs plant development.

In **Chapter 3**, the functional conservation of the identified phosphoserines in three TPRXS(N/S) motifs (**Chapter 2)** was tested in the PIN2 protein, and the functional redundancy among AGC3 kinases was investigated. We identified the PID homologs WAG1 and WAG2 as new PIN polarity regulators, and showed that the AGC3 kinases PID, WAG1 and WAG2, but not other plant AGC kinases instruct recruitment of PIN proteins into the apical recycling pathway by phosphorylating the central serine residues in three TPRXS(N/S) motifs within the PINHL. This phosphorylation-triggered apical PIN recycling competes with the ARF-GEF GNOM-dependent basal recycling pathway to promote apical PIN localization. Moreover, AGC3 kinase-mediated phosphorlyation of PIN proteins enhances their PM localization. Our data show that by directing polar PIN

localization and PIN-mediated PAT, the AGC3 kinases redundantly regulate cotyledon development, root growth and root gravitropic response, indicating their involvement in both programmed and adaptive plant development.

In **Chapter 4,** we further investigated the role of PID-mediated phosphorylation of PIN1 in its intracellular trafficking. By inducible expression of PID in tobacco BY-2 cells, we confirmed the role of PID as positive regulator of auxin efflux. Expression of wild type or non-phosphorylatable PIN1:GFP versions in *Arabidopsis* protoplasts showed that phosphorylation at the TPRXS(N/S) motifs reduced the rate of PIN1 targeting to the vacuoles. Also in seedling roots, non-phosphorylatable or phosphomimic PIN1:GFP proteins showed respectively enhanced or reduced vacuolar targeting compared to wild type PIN1:GFP. Collectively, our data indicate that besides the function as PIN polarity determinant, PID promotes auxin efflux by phosphorylating PIN1 at the TPRXS(N/S) motifs to enhance their PM localization, and as a result to reduce PIN1 targeting to and degradation in the vacuoles.

In conclusion, the identification and functional analyses of the evolutionarily conserved TPRXS(N/S) motifs in the HL of PIN proteins described in this thesis advance our understanding of molecular mechanisms underlying AGC3 kinase-directed PIN polar localization, and provide new insights into redundant kinase action on polar cargo targeting, and thus mediated organogenesis and plant development, that may be operational in all land plants. The strong embryo defects induced by loss-of-phosphorylation PIN1:GFP proteins have never been reported in *pin1* or *pid* mutants, indicating a central role for PIN1 during embryogenesis that is masked in the *pin1* loss-of-function mutant by functional redundancy with other PIN family members, and also supporting the functional redundancy among the three AGC3 kinases during embryogenesis. These results lead to intriguing new questions, such as: i) what cellular mechanism distinguishes phosphorylated over non-phosphorylated PINs and recruits them to the apical recycling pathway, ii) whether the serines in the TPRXS(N/S) motifs are functionally conserved in other PINs in *Arabidopsis* or in PIN homologs in other plant species, and iii) what upstream regulators of the PID kinase are involved in mediating the effect of external signals on PIN polar localization? These research questions form important future challenges in the auxin transport field.