Arabidopsis AGC3 kinases and PIN plasma membrane abundance

Gelderen, K. van

Summary

Plants adapt to their environment through a dynamic control of their development and growth, which relies for an important part on the correct distribution of the plant hormone auxin, or indole-3-acetic acid (IAA). Auxin is produced throughout the plant, but developmental processes such as tissue patterning, the initiation of new organs or the direction of growth (tropisms) are the result of auxin response minima and maxima that generally reflect the cellular auxin concentrations, and are in part determined by the directed cell-to-cell transport of auxin through dedicated transporters. The PIN-FORMED (PIN) auxin efflux carriers transport auxin out of the cell and provide direction to this polar auxin transport (PAT) through their asymmetric localization at the plasma membrane (PM). Two types of PIN proteins can be distinguished: those with a long central hydrophilic loop that extends into the cytosol ('long' PINs: PIN1,2,3,4,6 and 7) and those with a very short hydrophilic loop ('short' PINs: PIN5 and 8). The long PINs are PM localized and display either a polar or non-polar distribution, while the short PINs are predominantly ER-localized, where they regulate the amount of auxin available in the cytosol for efflux by the long PINs. The *Arabidopsis* PM-associated serine/threonine kinase PINOID (PID) and its close homologs WAG1 and WAG2 were shown to phosphorylate serine residues in conserved TPRXS motifs in the central hydrophilic loop of long PINs, causing these PINs to move from respectively the rootward (basal) or outward lateral to the shootward (apical) or inward lateral position at the PM of plant cells. Together with the fourth not yet well-studied AGC3-4 kinase, these kinases group to the AGC3 clade of the plant-specific AGCVIII kinases. Their function is nicely illustrated by phenotypes of the *pid* loss-of-function mutants or plants that ectopically express *PID*. *pid* mutants fail to develop lateral organs such as flowers and shoot branches on their inflorescences due to a failure of PIN1 to localize apically, which is required to generate auxin maxima for organ initiation at the inflorescence meristems. In contrast, *35S::PID* overexpression seedlings show agravitropic root growth and eventually experience root meristem collapse due to constitutively apicalized PINs in the root meristem, leading to mis-distribution of auxin and, as a result, to loss of the organizing auxin maximum in the root tip, and thus to loss of root meristem function.

Newly synthesized PINs first arrive at the PM via vesicle transport from the trans-golgi network/early endosome complex (TGN/EE) in an apolar fashion, and their polarity is subsequently established by a dynamic process of endocytosis from the PM to the TGN/EE and recycling back to the PM. Phosphorylation of the hydrophilic loop by the PM associated AGC3 kinases PID, WAG1 and WAG2 confers a signal that is interpreted at the PM in an as of yet unknown manner, although it is known that it requires the action of specific ARF-GTPases and their corresponding GTP exchange factors (ARF-GEFs). The ARF-GEF GNOM is known to mediate PIN recycling to the basal PM, and by specifically inhibiting

this ARF-GEF by the fungal toxin Brefeldin-A (BFA), one can observe the accumulation of PINs in so-called BFA bodies, or even a basal-to-apical shift in PIN polarity following longer treatment with low BFA concentrations. Currently, it is thought that phosphorylation of the PIN hydrophilic loop is a signal that sorts PINs in the apical/outer lateral recycling pathway, while de-phosphorylation causes them to be sorted in the GNOM-dependent basal/inner lateral recycling pathway. The role of the AGC3-4 kinase in this process has not yet been investigated.

Chapter 1 provides an introduction into the topic of PIN polarity and post-translational modifications and also includes a study into the molecular phylogeny of the AGC3 kinases, and the closely related D6 protein kinases (D6PKs), which belong to the AGC1 clade and are known to enhance PAT by phosphorylating the PIN hydrophilic loop at different positions. Homologs of the AGC3 and D6 kinases and their conserved PIN phosphorylation motifs can already be found in early land plants. The expansion and increased conservation of AGC3 and D6 kinases and PINs in later lineages, such as monocot and dicot flowering plants, is in line with their important role in the formation of reproductive organs and in the tropic growth responses that allow plants to adapt to changes in their environment.

Chapter 2 presents the functional analysis of the *AGC3-4* gene. Surprisingly, unlike the other three kinases, AGC3-4 did not induce the same basal to apical shift in PIN polarity. Loss-of-function *agc3-4* mutants showed minor defects in root growth and gravitropism, but combined *agc3* loss-of-function mutations did not provide evidence for a redundant function with the other AGC3 kinases. *AGC3-4* overexpression resulted in auxin transport related phenotypes, such as dwarf growth, reduced root gravitropism and delayed lateral root emergence, correlating with an increased basal (rootward) PIN polarity. *AGC3-4* expression was predominantly observed in tissues (vasculature, stomatal lineage) where PINs are either basal or apolar. These findings together with the enhanced sensitivity of PIN1 and PIN2 in the *AGC3-4* overexpression line to the ARF-GEF GNOM inhibitor BFA indicate that AGC3-4 promotes GNOM-dependent recycling of PINs back to the PM and thus acts opposite to its close homologs PID, WAG1 and WAG2.

PIN abundance at the PM is regulated by dynamic endocytosis and recycling to the PM, or alternatively, by targeting PINs to the vacuole for degradation. PIN vacuolar targeting has been shown to be important for the regulation of root gravitropism and negative phototropism. In **Chapter 3** we show that phosphorylation by AGC3 kinases reduces PIN vacuolar targeting in protoplasts, embryos and roots. The effect of phosphorylation is dose-dependent, as complete loss-of-phosphorylation leads to a significantly stronger enhancement of PIN vacuolar targeting compared partial loss-of-phosphorylation. Our data point to a dual function for AGC3 kinase-mediated PIN phosphorylation, which besides PIN polarity also controls the abundance of these auxin efflux carriers at the PM of plant cells.

Chapter 4 concerns the role of PID and WAG2 in regulating PIN3 PM abundance during the formation of the dehiscence zone that is required for opening of the Arabidopsis fruit and the subsequent seed dispersal. The transcription factor INDEHISCENT (IND) has been proposed to specify the separation layer by inducing a local auxin minimum at late stages of fruit development. Our results refine the existing model by showing that IND is required at early stages of *Arabidopsis* fruit development to facilitate the auxin-triggered cell divisions that form the dehiscence zone through repression of PID and WAG2 kinase expression.

In **Chapter 5** we investigated the function of the MACCHI BOU 4 (MAB4) BTB-NPH3 scaffold protein and its homologs MAB4/ENP/NPY1-LIKE1,2,3 and 4 (MEL1,2,3,4) in regulating PIN polarity and PM abundance. MAB4/MELs consist of two domains (NPH3 and BTB) that are thus far only known as protein-protein interacting domains. We show that MAB4 and PID interact with the PIN hydrophilic loop (PIN HL) and that this trimeric complex enhances PIN1 and PIN2 stability at the PM by reducing endocytosis and turnover of these PINs. We show that this complex formation contributes to the immobilization of PIN proteins in microdomains. Through this PIN stabilization, MAB4/MELs indirectly enhance PIN polarity, explaining the PIN polarity defects observed in *mab4 mel* higher order mutants.

In **Chapter 6** we show that AGC3 kinases can enhance PIN PM abundance through a novel nuclear function. These kinases were found to interact with COP9 SIGNALOSOME SUBUNIT8 (CSN8) and COP1 and to phosphorylate COP1 and CNS7. Our findings indicate that AGC3 kinases can function in the nucleus, separately from PIN proteins, and affect photomorphogenesis-enhanced PIN PM abundance, possibly via repression of COP1.

Together the chapters in this thesis contribute to the central thought that beside the polarity cue provided by the AGC3 kinases, these kinases can also promote PIN PM abundance, either in a TPRXS phosphorylation-dependent or -independent manner. Phosphorylation of the PIN hydrophilic loop is linked to a decrease in vacuolar targeting or it can lead to recruitment of MAB4/MELs, which both contribute to auxin transport in a positive manner.