Summary

As sessile organisms, plants respond to changes in their environment by adjusting their growth and development through a highly dynamic combination of signal perception and transduction systems. The plant hormone auxin is a central regulator in these adaptive responses to environmental cues. Polar cell-to-cell transport of the hormone results in its local accumulation or depletion in tissues and organs, which controls plant growth and development by regulating basic processes such as cell division, -differentiation and -elongation. Polar auxin transport (PAT) is driven by PIN FORMED (PIN) auxin efflux carriers that through their asymmetric localisation at the plasma membrane (PM) determine the direction of transport. PIN subcellular localisation is dynamic and established by clathrin-dependent endocytosis and ARF-GEF-(guanine-nucleotide exchange factors for ADP-ribosylation factor GTPases)-dependent recycling (Tanaka et al., 2006).

The *Arabidopsis* PINOID (PID) serine/threonine protein kinase has been identified as a key determinant in the polar distribution of PIN proteins. The *PID* gene is named after the pin-like phenotype of loss-of-function mutant inflorescences, a phenotype that is shared with the *pin1* mutant. The *pid* mutant defects in embryo bilateral symmetry and inflorescence organogenesis are caused by an apical to basal (shootward to rootward) shift in PIN1 polarity in epidermis cells in embryos and inflorescence meristems. In contrast, *PID* gain-of-function results in an opposite basal to apical PIN polarity shift, leading to auxin depletion from the root meristem, and ultimately resulting in its collapse (Benjamins et al., 2001; Friml et al., 2004). PID is a member of the AGC3 clade of plant-specific *Arabidopsis* AGCVIII protein kinases, together with WAG1, WAG2 and AGC3-4. PID, WAG1 and WAG2 act redundantly in regulating the polarity of the PM-localized ('long') PINs by phosphorylating the serine in three conserved TPRXS motifs present in their large PIN central hydrophilic loop (Michniewicz et al., 2007; Huang et al., 2010). Phosphorylation recruits these PINs for GNOM ARF-GEF-independent (apical-, outer lateral-, indentation-specific) recycling, directing auxin flow and regulating cotyledon development, root meristem size and tropic growth responses (Michniewicz et al., 2007; Kleine-Vehn et al., 2009; Dhonukshe et al., 2010; Huang et al., 2010; Ding et al., 2011).

Previously, a six EF-hand calmodulin-like protein CML12/TOUCH3 (TCH3) has been identified by a yeast two-hybrid screen as a PID-interacting protein (Benjamins et al., 2003). TCH3 appeared not to be a phosphorylation target of PID, but instead to regulate the *in vitro* activity of this kinase through its Ca^{2+}-dependent binding (Benjamins et al., 2003). *TCH3* expression is induced by auxin and mechanical stress (Braam and Davis,

1990), and at the same time auxin and environmental signals such as touch and light can induce a rapid increase in the cytosolic Ca^{2+} levels ($[Ca^{2+}]_{cyt}$) (Monshausen et al., 2011). TCH3 therefore seems an ideal candidate to regulate PID activity and thereby induce changes in PIN polarity in response to both endogenous and environmental signals.

The two key objectives of the research described in this thesis were 1) to demonstrate the role of TCH3 as negative regulator of PID activity *in vivo*, and 2) to investigate in which developmental processes in *Arabidopsis* the kinase-CML interaction plays a role, and whether other AGC3 kinases and CMLs or calmodulins (CaMs) act redundantly in these processes.

Chapter 2 follows up on the initial finding of the PID-TCH3 interaction by confirming that TCH3 negatively regulates PID activity by interacting with the catalytic domain of the protein kinase. Furthermore, protoplast transfection experiments combined with Fluorescence Resonance Energy Transfer (FRET) measurements showed that TCH3 interacts with PID and WAG2 *in vivo*, but not with WAG1 or AGC3-4, and that this interaction is auxin dependent. PID and WAG2 are both PM associated kinases, and previous studies suggested that they phosphorylate PINs at the PM (Michniewicz et al., 2007; Dhonukshe et al., 2010). Interestingly, we observed that TCH3 through its interaction was able to sequester the PID and WAG2 kinases from the PM to the cytoplasm. Moreover, in root epidermis cells expressing the complementing *PID::PID-VENUS* fusion construct, PID-VENUS was released from the PM to the cytosol as early as 5 minutes after auxin treatment, and this internalization could be inhibited by pretreatment with the Ca^{2+} channel inhibitor lanthanum, and was delayed in the *tch3-3* loss-of-function and enhanced in the *TCH3* overexpression background, indicating that this internalization is dependent on Ca^{2+} and at least in part mediated by TCH3. In line with its function as negative regulator of the PID and WAG2 kinases, *TCH3* overexpression delayed root meristem collapse by PID overexpression, and seedlings developed short roots, a phenotype also observed for *pid/wag1/wag2* triple mutant seedlings. Moreover, *TCH3* overexpression seedlings and *tch3-3* loss-of-function mutant lines showed a delay in root gravitropism, suggesting the involvement of the TCH3-kinase interaction in root gravitropism. Root gravitropic growth is mediated by PIN-driven redirection of auxin flow to the lower side of the root tip (Tanaka et al., 2006), coinciding with $[Ca^{2+}]_{cyt}$ peaks in cells at this side of the root tip (Monshausen et al., 2011). We could show that Ca^{2+} acts downstream of auxin during root gravitropism. The auxin-induced $[Ca^{2+}]_{cyt}$- and *TCH3* expression levels promote the kinase-CML interaction, through internalization of the kinase, and

depolarisation (and enhanced degradation) of PIN2. This enhances the asymmetric auxin distribution, and thereby root gravitropic growth.

Chapter 3 describes a study to identify the function of the PID-CML interaction throughout plant development. The mild effects of *tch3-3* loss-of-function suggested that some of the 7 CaMs and 50 CMLs in *Arabidopsis* act redundantly with TCH3. Indeed we could show that PID interacts with, and is sequestered by the closely related CML10, but not by the more distantly related CML24/TCH2. This result confirmed that there is redundancy among the CMLs, but it also showed some level of specificity, especially with respect to the ability of CMLs to recruit PID from the PM. In view of this redundancy, and the likely pleiotropic function of CMLs, we decided to unravel this process from the kinase side, by generating 'untouchable PID': a kinase that does not interact with TCH3, but is still fully functional as a PM associated, PIN phosphorylating kinase. AGCVIII kinases are characterised by an insertion of 36 to 90 amino acids in the kinase catalytic domain (Galván-Ampudia and Offringa, 2007), and it has been shown that the PM association function of PID localises to this insertion domain (ID) (Zegzouti et al., 2006). By generating mutant versions of PID having deletions or specific amino acid substitutions in this ID we could show that PM association and TCH3 binding functions overlapped to a region in the middle of this ID. Careful amino acid sequence and 3D structure analysis revealed that this part contains a partially overlapping amphipathic alpha-helix and IQ-motif. Both structures are known to interact with CaMs in animal cells (Snedden and Fromm, 2001; Bahler and Rhoads, 2002; Lu and Taghbalout, 2013), and through *in vitro* pull downs we could show that this specific region does not only interact with TCH3 and CML10, but also with an *Arabidopsis* CaM. Moreover, the amphipathic alpha helix is also known as a high affinity PM association domain (Lu and Taghbalout, 2013). Fusion of the amphipathic alpha helix/IQ-motif to GFP resulted in a PM associated fusion protein that could be sequestered to the cytosol by TCH3 binding. Amino acid substitution of residues that were part of the IQ-motif but outside the alpha helix still resulted in loss of PM association, indicating that the two functions tightly overlap. Although this has prevented us from generating an 'untouchable' PID version, our studies have provided valuable information on the function of the PID kinase. Its tight association with the PM, possibly enhanced through the interaction with phospholipids, takes care that it stays close to the PIN phosphorylation targets. However, the two overlapping CaM/CML binding domains that are located in the heart of the kinase catalytic domain will allow TCH3 to recruit and inactivate PID when both the $[Ca^{2+}]_{cyt}$ and TCH3 levels are

sufficient. This may explain the relatively late occurrence of PID internalization during root gravitropism, which was observed only a few hours after gravistimulation (Chapter 2).

The *pid* loss-of-function mutant phenotypes suggest that the kinase is important for the initiation and positioning of new aerial organs. In fact, preliminary results by Reinhardt and coworkers (Reinhardt et al., 2003) suggested that PID is involved in maintaining the regular spiral phyllotaxis in *Arabidopsis* rosettes and inflorescences. In addition, mechanical stress in the shoot apical and inflorescence meristems was reported as a key determinant in generating phyllotactic patterns (Heisler et al., 2010). As *TCH3* transcription is induced by auxin and mechanical stress (Braam and Davis, 1990), and the same signals also elevate $[Ca^{2+}]_{cyt}$, the TCH3-PID interaction could be an important signalling event in phyllotactic patterning.

Chapter 4 describes studies that further investigate the role of the AGC3 kinases and their interaction with CaMs and CMLs in phyllotaxis. By using AGC3 kinase loss-of-function mutants or *pin1* mutants plants expressing non-phosphorylatable or phosphomimic versions of PIN1-GFP we could show that reversable PIN1 phosphorylation by these kinases is important to maintain the spiral phyllotaxis in *Arabidospsis* with a 'golden' divergence angle of 137.5 °. In general, mutants showed an increased variation in the divergence angle. Especially PID and WAG2 appeared important, as their loss-of-function regularly led to a shift from spiral to decussate (alternating 90 °and 180 °divergence angles) phyllotaxis. In some cases we found angles around 270 °, suggesting that the organ initiation event in between had been unsuccessful, and implying that kinase activity is important for focussing the auxin maximum required for organ initiation. Plants overexpressing TCH3 or *tch3* loss-of-function mutants only showed weak irregularities in phyllotactic patterns, and the expression of TCH3 in shoot and inflorescence meristems was weak. However, by using the *PID::PID-VENUS* reporter line we could show that both auxin and mechanical stress induce Ca^{2+}-dependent internalization of PID-VENUS in inflorescence meristems. A role for TCH3 in this process is unlikely, however, based on results in Chapter 3 possibly CML10 or one of the CaMs are involved in modulating the phyllotactic pattern by recruiting PID and WAG2 from the PM in response to auxin and mechanical stress signals.

In conclusion, based on the functional analysis of PID, and its interaction with TCH3 *in vivo* described in this thesis, we propose a new model in which the PID-CaM/CML module translates both endogenous and external signals into changes in development

and growth by modulating the subcellular localisation of PINs. But there are still some unanswered questions that need further investigation. For example, it would still be desirable to generate an untouchable PID to study the function of the PID-CaM/CML interaction throughout development. This would require further fine mapping of the PM association and CaM/CML binding domains in PID, possibly through high throughput amino acid substitution analysis. Such studies will provide important new insights into the capacity of plants to change their growth and development in response to both internal and environmental signals.

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