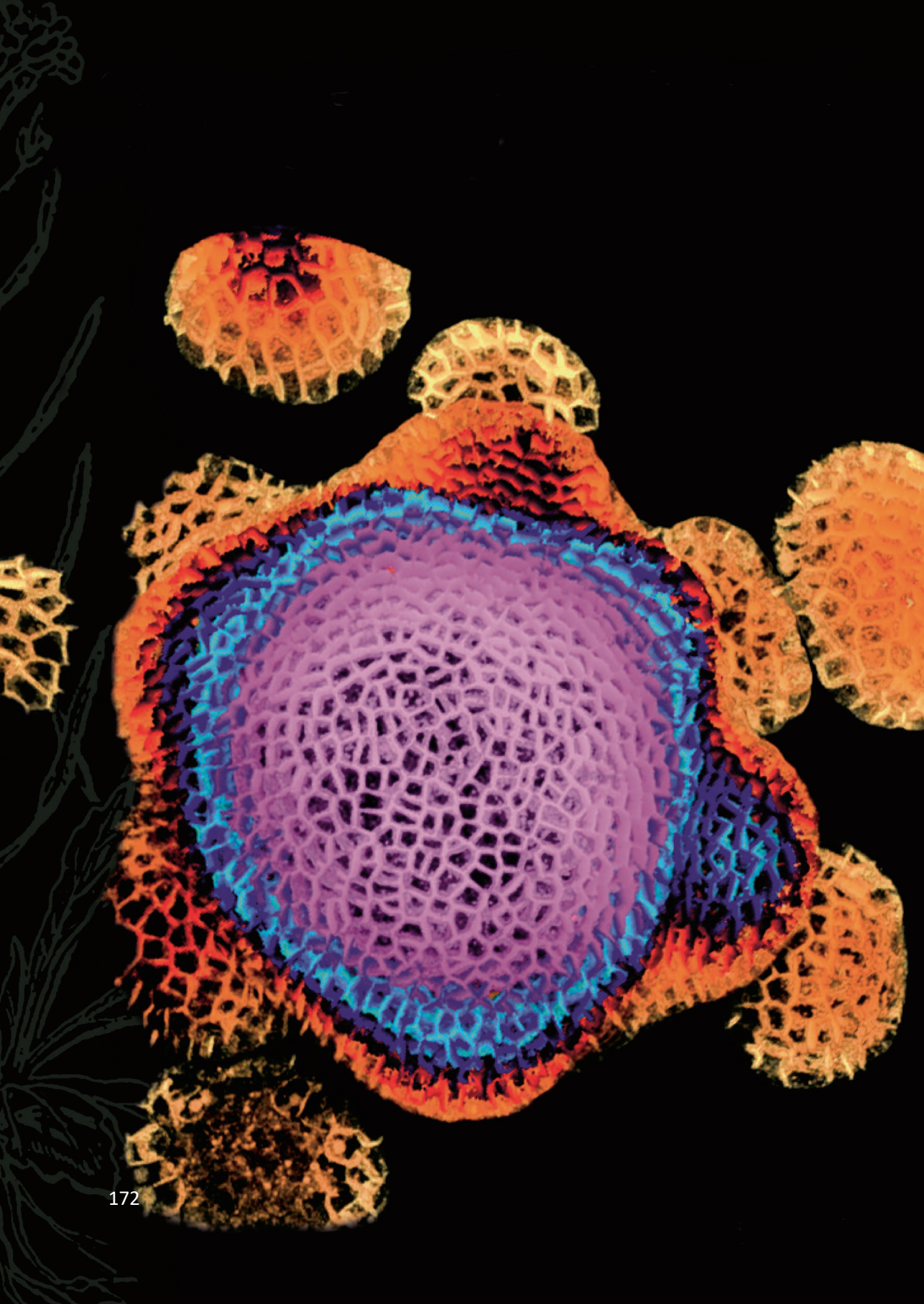
Calcium-dependent regulation of auxin transport in plant development

Wei, X.



Summary

Due to their sessile nature, plants have evolved an intricate system to survive in their ever-changing and often unfavourable environment. The plant hormone auxin plays a central role in this system, as it regulates very basic cellular processes, including cell division, growth and differentiation. As a result, it is involved in an extraordinarily broad variety of biological mechanisms to promote and influence plant growth and development in response to a wide range of endogenous and environmental signals.

Over the past decades, a wealth of information has been produced on the biosynthesis, metabolism, transport and signalling of this hormone. In **Chapter 1**, we review some of these mechanisms, focussing on auxin perception and signal transduction, its distribution by polar auxin transport (PAT) driven by the PIN-FORMED (PIN) auxin efflux carriers, and how the bivalent kation calcium (Ca^{2+}), a universal second messenger, is involved in translating both endogenous and environmental signals into auxin-mediated developmental and growth responses. Intensive research in the last decades has revealed several signalling routes for auxin. Firstly, there is the canonical nuclear auxin signalling pathway, involving the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN-SIGNALING F-BOX (AFB) F-box proteins that through auxin-mediated recruitment and ubiquitination of Aux/IAA repressors liberate AUXIN RESPONSE FACTORs (ARFs) to activate auxin responsive gene transcription. Secondly, the AUXIN BINDING PROTEIN 1 (ABP1) signalling pathway has been revamped by the finding that coordinated binding of auxin to ABP1 and the receptor-like TRANSMEMBRANE KINASE 1 (TMK1) activates signalling at the PM, leading to e.g. cell wall acidification by activation of H^+-ATPases. Moreover, identification of the role of the S-Phase Kinase-Associated Protein 2A (SKP2A), ARF3, and IAA32/33/34 has provided a better understanding of the complexity of the cellular auxin response machinery. Subsequently, we review the current advances and

understanding of PIN-driven PAT. The directionality and rate of intercellular auxin flow is mainly dependent on the asymmetric localisation and activity of PIN proteins at the PM, which involves Ca^{2+} signalling and is regulated by phosphorylation of their central hydrophilic loop by plant-specific AGC protein kinases.

One of these protein kinases in *Arabidopsis thaliana* (Arabidopsis), the AGC3 kinase PINOID (PID), is a key regulator of PAT, acting as a binary switch in apical-basal polar targeting of PIN auxin efflux carriers, thereby determining the direction of auxin flow. PID was found to interact with CALMODULIN-LIKE 12/TOUCH 3 (CML12/TCH3) and the EF hand containing PID-BINDING PROTEIN 1 (PBP1) in a Ca^{2+} dependent manner, providing one of the first molecular insights into how Ca^{2+} could possibly affect PAT. CML12/TCH3 binding negatively regulates PID kinase activity both *in vitro* and *in vivo*, and its interaction with CML12/TCH3 sequesters PID from the plasma membrane (PM) to the cytosol, in a Ca^{2+}- and auxin-dependent manner. Moreover, increased local auxin levels trigger a transient increase in cytosolic Ca^{2+} levels ($[Ca^{2+}]_{cyt}$) and a rapid and substantial up-regulation of *CML12/TCH3* expression. Therefore, building upon these previous findings, at the end of **Chapter 1** an alternative model for auxin feedback regulation of PIN polarity is proposed, involving Ca^{2+}-CML12/TCH3-PID signalling.

In **Chapter 2**, we conducted further studies on the Ca^{2+}-regulated PID kinase. The *tch3* loss-of-function mutant showed only a mild phenotype, strongly suggesting that CaMs and other CMLs function redundantly with CML12/TCH3. Consequently, our first step was to test the interaction of the closely related homologs of CML12/TCH3 with PID.

Through *in vitro* pull-downs and Bimolecular Fluorescence Complementation (BiFC) assays, we showed that a confined clade comprising seven CaMs and three CMLs (CML8, CML10, CML11) most closely related to CML12/TCH3 also interact with PID. In Arabidopsis protoplasts, co-expression of these CaM/CMLs

and PID in the presence of auxin resulted in dissociation of PID from the plasma membrane (PM). To gain a deeper understanding, we conducted a comparative study on the spatio-temporal expression of the corresponding *CaM/CML* genes and *PID* using *promoter:GUS* reporter fusions. This analysis revealed differential but largely overlapping expression patterns in most tissues throughout all developmental stages.

These results suggested functional redundancy among these CaM/CMLs in regulating PID kinase. To overcome the redundancy and identify the biological function of the CaM/CML-PID interaction, in **Chapter 3** we mapped the CaM/CMLs-binding domain in PID in order to identify the key amino acids responsible for CaM/CMLs-binding, with the final aim to generate an 'untouchable' but still fully functional version of PID. Complementation of the *pid* loss-of-function mutant should then reveal the role of the calcium-dependent CaM/CML binding to PID. First, we confirmed that PID associates to the PM by the insertion domain (ID) in the catalytic kinase core. Subsequent fine mapping of the CaM/CML binding domain in PID showed that both CaM/CML binding and PM association converge at an amphipathic alpha helix in the PID ID. Disruption of this amphipathic alpha helix by substitution of several positively charged arginines by alanines (RtoA) interfered with both CaM/CML binding and PM association. Surprisingly, the PID(RtoA) versions showed the same overexpression phenotypes as wild-type PID and complemented the pin-like inflorescence phenotype of the *pid* mutant, when expressed under the *PID* promoter. These results indicate that dominant PM association is not essential for PID function and that the 'untouchable' PID(RtoA) versions enable us to finally unravel the role of the calcium-dependent PID-CaM/CML interaction in plant development.

In **Chapter 4**, we utilized the *pid-14* mutant lines expressing 'untouchable' PID(RtoA) versions to study the role of the calcium-dependent PID-CaM/CML interaction in plant development. Initial phenotypic analyses of these plants suggested that the mutant PID versions were fully functional, as they did not alter

seedling development or flowering time and complemented the pin-like inflorescence phenotype of the *pid* mutant. However, closer inspection of the inflorescences of these plants showed clear defects in the spiral phyllotaxis, ranging from deviating divergence angles between subsequent flowers and fruits to the simultaneous initiation of flower primordia. These phenotypes were reflected in the increased number and randomized position of PIN convergence points and resulting auxin maxima in the inflorescence meristems (IMs) of the 'untouchable' PID expressing plants.

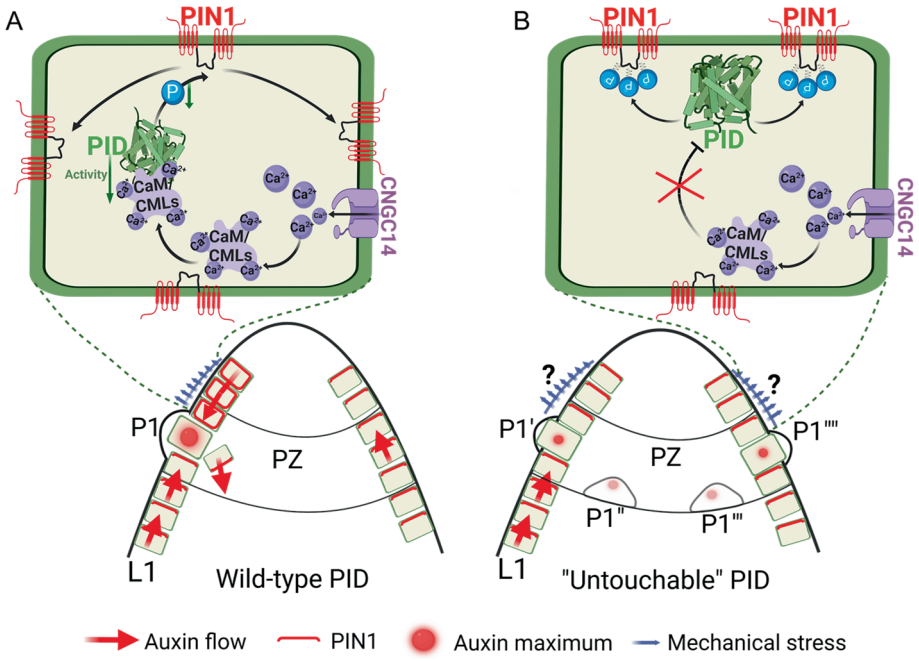


Figure 1. Model for the involvement of the CaM/CMLs-PID interaction in positioning primordia in the Arabidopsis IM. A) A single strong, perfectly positioned auxin maximum appearing in a wild-type Arabidopsis IM. **B)** The stochastic, simultaneous appearance of weaker auxin maxima in Arabidopsis lines expressing "untouchable" PID. L1 indicates the epidermis; PZ = peripheral zone; P1 = newly initiated floral primordia. ? = indicates that due to irregular phyllotaxis the position of mechanical stress will be unclear.

Previous research has indicated that the spiral phyllotaxis observed in Arabidopsis inflorescence meristems (IMs) is the result of an interplay between PIN1-generated auxin maxima and mechanical stress imposed on (cells in) the IM by the

continuous initiation and outgrowth of new floral primordia. Ca^{2+} signalling has been proposed as mediator connecting the auxin and mechanical stress pathways, however, the signalling components involved have remained elusive. Based on the results presented in this thesis, we now propose a model in which the previously discovered Ca^{2+}-dependent regulation of PID activity by CaM/CML binding provides the missing link (Figure 1). In this model, PID phosphorylation-directed apical PIN1 causes cell-to-cell transport of auxin through the shoot epidermis to the peripheral zone of the IM. The growth of the regularly positioned young floral primordia impose mechanical stress on the IM, resulting in a stress maximum in cells just above the position where the next primordium will be initiated. This stress maximum triggers PM-localized Ca^{2+} channels, resulting in elevated cytosolic Ca^{2+} levels and the subsequent activation of CaM/CMLs and their binding to the PID kinase. Due to the inhibited kinase activity, PIN1 proteins become dephosphorylated and their reduced apical polarity results in auxin accumulation in the cells below. The enhanced auxin levels in these cells again lead to elevated cytosolic Ca^{2+} levels and CaM/CML-mediated depolarization of PIN1, finally resulting in a single strong auxin maximum and thus in the initiation of a perfectly positioned floral organ (Figure 1A). In the lines expressing "untouchable" PID, the mechanical stress-induced depolarization of PIN1 does not occur, causing auxin maxima to appear, regularly simultaneous, in a stochastic manner, resulting in the observed defects in spiral phyllotaxis (Figure 1B). The irregular phyllotaxis is likely to have an effect on the mechanical stress in the IM, and thus positions of stress maxima will also be stochastic.

The results described in thesis provide new insights into how organ initiation at the Arabidopsis IM is coordinated and how the spiral phyllotaxis pattern is maintained. Still, there are some unanswered questions. Firstly, in **Chapter 2** we show that a confined clade of the CaM/CMLs gene family interact with and regulate the PID kinase. However, the precise expression pattern of the CaM/CMLs in the IM has to be determined to know which of the PID interacting CaM/CMLs are responsible

for translating mechanical stress and auxin induced Ca^{2+} peaks into changes in PIN1 polarity. Secondly, an intriguing finding in **Chapter 3** of this thesis is that, despite the predominant cytosolic localization of the "untouchable" PID versions, they were able to complement most defects of the *pid* mutant, showing that these mutants version are functional kinase *in vivo*. A more detailed study on how this happened would gain a much better understanding on the significance of the PM-association of PID as one of the key regulators of polar auxin transport. Thirdly, our model in Figure 1 relies on a few assumptions, an important one being the occurrence of a mechanical stress maximum above the epidermal cells in the peripheral zone of the IM where the new primordium will be initiated. Such a stress maximum could possibly be detected by temporary PIN1 depolarization event. Another option would be to use recently designed molecular probes to detect mechanical stress in cell walls. We have tried both options, but they appeared technically challenging. Currently we are trying to use the sequestration of PID to the cytosol to localize the mechanical stress maxima. An alternative option would be to generate a fluorescent probe based on the interaction between PID and CaM/CML. Hopefully, one of the approaches mentioned above will allow us to obtain a better understanding of how the Ca^{2+}-dependent CaM/CML-PID interaction integrates hormonal and abiotic signals that trigger elevations in $[Ca^{2+}]_{cyt}$, such as auxin and mechanical stress, to generate robustness in the spiral phyllotaxis that is typical for the Arabidopsis inflorescence.