Calcium-dependent regulation of auxin transport in plant development

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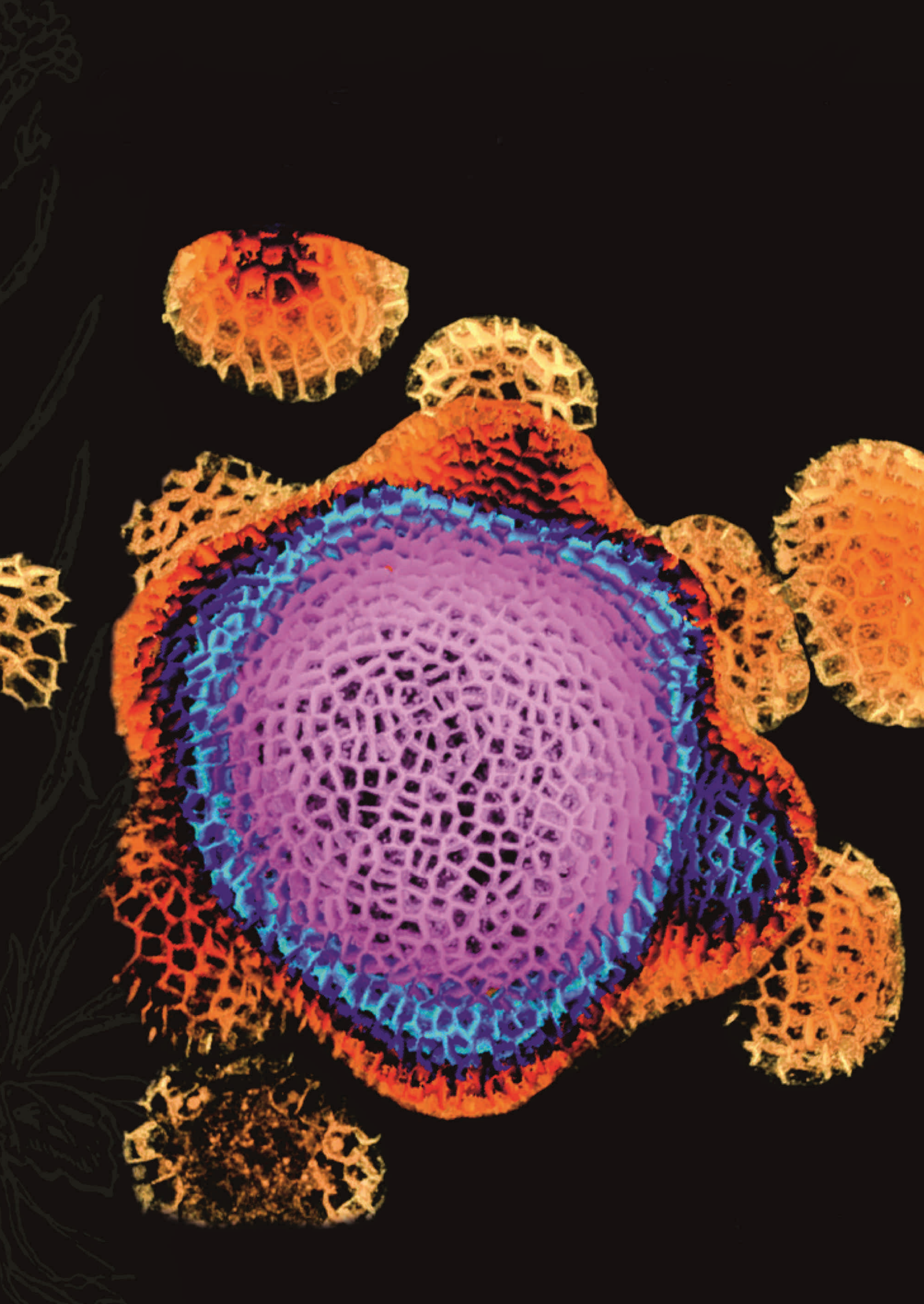

Chapter 1

The role of calcium as second messenger in auxin transport and signaling

(Introduction)

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1. The phytohormone auxin is a pivotal regulator of plant growth, development and adaptation to the environment

Auxin was the first hormone to be discovered. Its existence was already predicted by Charles Darwin and his son in their book *The Power of Movement in Plants* in 1880 (Darwin and Darwin, 1880), and demonstrated later by Went through his coleoptile bending experiments in 1926 (Went, 1926), and its chemical structure, indole-3-acetic acid (IAA), was determined in the 1930s (Kögl et al., 1934). Intensive research has since revealed that auxin is involved in nearly every aspect of plant growth and development.

Auxin plays a crucial role in various developmental processes, including embryogenesis (Winnicki, 2020), organogenesis in the root (Benková et al., 2003; Cavallari et al., 2021) and in the shoot (Benková et al., 2003), which includes phyllotaxis (Bohn-Courseau, 2010; Sassi and Vernoux, 2013), organ patterning (Qi et al., 2014) and morphogenesis (Chitwood et al., 2012; Heisler and Byrne, 2020). It has a broad role in plant growth and development, as it regulates basic cellular processes, such as cell division, cell expansion, and cell differentiation (Perrot-Rechenmann, 2010; Ding and Friml, 2010; Du et al., 2020).

Auxin also is a major determinant in a plant's response to the environment. Due to their sessile nature, plants have evolved a remarkable capacity for morphological plasticity in response to changing and often unfavourable environments. They modify their growth and development through the perception and integration of exogenous signals, utilizing auxin as a coordinative signal. The exogenous signals include light (Halliday et al., 2009; Hohm et al., 2013), gravity (Baster et al., 2013), obstacles (Lee et al., 2020), salinity (Wang et al., 2019), mechanical stress (Nakayama et al., 2012) and osmotic stress (Naser and Shani, 2016), among others. In addition, auxin interplays with other endogenous signals, such as the plant hormones cytokinins, brassinosteroids, ethylene, abscisic acid, gibberellins, jasmonic acid and strigolactones, or important compounds such as nitrate, phosphate, polyamines and sugars

(Saini et al., 2013; Mroue et al., 2018). Through these interactions, auxin specifies organ orientation and positioning by regulating cell growth and division.

Auxin action is primarily achieved through differential concentrations, which are established and maintained by a combination of biosynthesis, homeostasis, degradation and transport, and eventually by its perception and signal transduction. In this chapter, we will focus on the mechanisms of auxin perception and signaling, the differential distribution of this hormone by its polar transport and the involvement of Ca^{2+} signaling in these processes.

2. Auxin perception and signalling

The asymmetric distribution of auxin in tissues is primarily established through local auxin synthesis and polar auxin transport (PAT). This spatial distribution of auxin is then translated into varying intensities of auxin signaling, which in turn determines the rate of cell division, cell expansion and cell differentiation, ultimately influencing the growth and developmental responses of a plant (Chen, 2001; Benková et al., 2003; Paciorek and Friml, 2006; Yu et al., 2022b). Auxin perception and signal transduction occur through different signaling pathways, known as the canonical and non-canonical pathways (Figure 1).

The TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX proteins (TIR1/AFB), the AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressor proteins, and the AUXIN RESPONSE FACTOR (ARF) transcription factors together form the canonical auxin signaling pathway (Reviewed in Yu et al., 2022b). Briefly, intracellular auxin acts like a “molecular glue” and promotes the association of TIR1/AFBs, which are components of the ubiquitin-ligase (E3) complex SKP1-CUL-FBP (SCF), and Aux/IAAs via hydrophobic interaction forces, forming an E2-$SCF^{TIR1/AFB}$-Aux/IAA complex. Aux/IAAs are subsequently polyubiquitinated and degraded by the 26S proteasome, thus releasing their inhibition of ARF transcription factors and leading to activation of auxin-dependent transcription.

Despite the canonical TIR1/AFB-Aux/IAA-ARF pathway explaining the main auxin responses, accumulating evidence challenges it exclusivity. For example, exogenous auxin treatment can trigger an increase in cytosolic Ca^{2+} $[Ca^{2+}]_{cyt}$ within 7-14 seconds (Monshausen et al., 2011) and reduce elongation growth within 30 seconds in the *Arabidopsis thaliana* (Arabidopsis) primary root (Fendrych et al., 2018), which is too fast for the canonical, transcription-mediated pathway of auxin signaling. Moreover, auxin-inhibited clathrin-mediated PIN endocytosis was shown to be TIR1-independent (Paciorek et al., 2005), suggesting the existence of non-canonical pathways that are important in the coordination of plant growth and development. Notably, a recent study showed that TIR1/AFBs possess adenylate cyclase (AC) activity and stimulates cAMP production, which is crucial in regulation of root growth and transcriptional response, indicating that TIR1/AFBs translate auxin signaling in a dual way (Qi et al., 2022; Figure 1).

Arabidopsis AUXIN BINDING PROTEIN1 (ABP1) has long been reported and is believed to be an auxin receptor responsible for rapid, non-transcriptional signaling events at the cell surface, regulating clathrin-mediated endocytosis to affect PIN distribution, cell expansion, and cytoskeletal rearrangements (Yu et al., 2022b). However, recent findings and studies have raised questions about its importance as an auxin receptor, with some studies claiming that "ABP1 is not required for either auxin signaling or Arabidopsis development" due to the fact that verified *abp1* knockout alleles do not show obvious growth defects or auxin insensitivity (Gao et al., 2015; Dai et al., 2015; Michalko et al., 2015; Gelová et al., 2021). Meanwhile, new evidence supporting ABP1 as auxin receptor has been reported. ER-localized ABP1 can be secreted to the apoplast and can interact with the extracellular domain of the plasma membrane (PM)-localized receptor-like kinase TRANSMEMBRANE KINASE 1 (TMK1) in an auxin-dependent manner. This interaction is required for of a subset of rapid cellular effects, such as an auxin-induced ultrafast global phospho-response, activation of H^+-ATPases and accelerated cytoplasmic streaming, and development mediated by auxin canalization (Friml et al., 2022). Therefore, the role of ABP1 in auxin signaling is much more complicated than previously thought, and further investigation is needed.

Another non-canonical auxin signaling pathway, dependent on the ubiquitin-proteasome system, has been shown by the complex of the Arabidopsis S-Phase Kinase-Associated Protein 2A (SKP2A), E2FC and dimerization partner of E2FB (DPB). Similar to TIR1/AFBs, SKP2A is an F-box protein subunit of the SCF ubiquitin ligase. The binding of auxin promotes the ubiquitin-mediated degradation of the cell cycle repressors E2FC and DPB, thus promoting cell cycle progression (Jurado et al., 2010).

Additionally, another non-canonical pathway dependent on ARFs has been revealed by the variant ARF3, also known as ETTIN (ETT). Unlike the typical Aux/IAA interaction domain, ARF3 harbors a long and intrinsically disordered C-terminal domain – the ETT-specific domain. This domain enables ARF3 to interact with a set of alternative transcriptional regulators that determine plant shape and pattern, such as INDEHISCENT (IND), REPLUMLESS (RPL), BREVIPEDICELLUS (BP), ABERRANT TESTA SHAPE (ATS), PLETHORA5 (PLT5), KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1/3 (KNAT1/3), BABYBOOM (BBM), TEOSINTE BRANCHED1(TB1)/BRANCHED1 (BRC1), CYCLOIDEA AND PCF4/18 (TCP4/18) and TOPLESS (TPL) (Simonini et al., 2016; Simonini et al., 2018; Kuhn et al., 2020). IAA can bind directly to this domain, thus disrupting these interactions and thereby affecting the expression of a group of genes involved in different growth and development processes (Simonini et al., 2016; Kuhn et al., 2020; Figure 1).

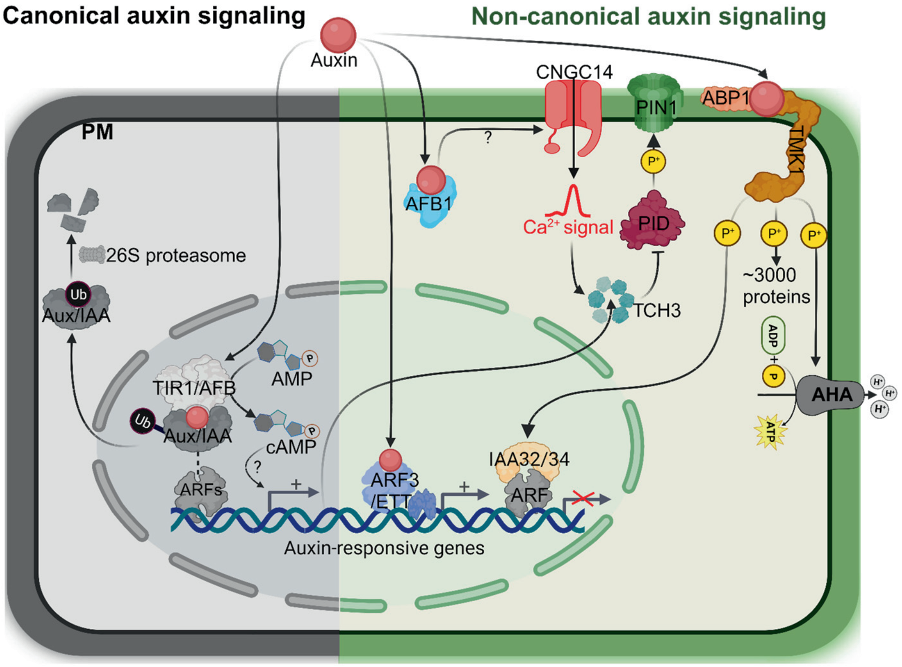

Figure 1. Canonical and non-canonical auxin signaling. Left: canonical nuclear TIR1/AFB-Aux/IAA-ARFs pathway. Right: non-canonical auxin signaling. Details are described in the main text. PM: Plasma membrane.

Similarly to ARF3, there are also nonconformists among the Aux/IAAs. For example, IAA33, lacking the domains I and II that are essential for TIR1/AFB-binding, can be induced by auxin and stabilized by auxin via MITOGEN-ACTIVATED PROTEIN KINASE 14 (MPK14) on the one hand. On the other hand, it can compete with IAA5 to interact and thus release the repression of ARF10/16, helping to maintain the root stem cell (Ding and Friml, 2010; Lv et al., 2020). IAA32 and IAA34, lacking the canonical domain II, are targeted and phosphorylated in the nucleus by the C-terminal kinase domain of TMK1, which is cleaved off from TMK1 after auxin treatment, regulating differential growth of the apical hook (Cao et al., 2019; Figure 1). The other two noncanonical Aux/IAAs also lacking the canonical domain II are IAA20 and IAA30 (Dreher et al., 2006). Overexpression of IAA20 and IAA30 shows similar auxin-related aberrant phenotypes: affected gravitropic growth, malformed vasculature of cotyledons and collapse of the root apical meristem (Sato and Yamamoto, 2008; Muller et al., 2016). Furthermore, their double mutant *iaa20/30* forms ectopic protoxylem (Muller et al., 2016). Hence, it is likely that IAA20 and IAA30 may also have a potential to play some roles in auxin signaling.

Overall, the understanding of auxin signaling has expanded beyond the canonical TIR1/AFB-Aux/IAA-ARFs pathway, with accumulating evidence supporting the existence and importance of non-canonical pathways. Further investigations are needed to fully elucidate the intricate mechanisms underlying auxin signaling and its roles in plant growth and development.

3. Polar auxin transport driven by PIN proteins

3.1 Mechanisms of auxin distribution

Auxin functions through the development of gradients and concentration maxima and minima within developing tissues, providing positional cues for diverse developmental processes (Geisler, 2021). It is primarily synthesized in the shoot and redistributed throughout the plant by two distinct, but interconnected, transport systems: fast passive transport in the phloem and slow cell-to-cell PAT. Phloem transport delivers auxin from it synthesis sites to recipient organs, while PAT distributes auxin in a precise manner to establish and maintain local auxin gradients and maxima/minima mainly in developing tissues or in response to exogenous and endogenous signals (Adamowski and Friml, 2015).

At the cellular level, the chemiosmotic hypothesis explains PAT based on the chemical properties of the principal auxin form, IAA (Rubery and Sheldrake, 1973; Raven, 1975; Goldsmith, 1977; Figure 2). As IAA is a weak acid, a portion exists in a protonated state (IAAH) in the slightly acidic pH of the apoplast (pH 5.5), becoming hydrophobic and able to passively diffuse through the PM. Once inside the neutral cytoplasm (pH 7.0), auxin becomes deprotonated (IAA^-) and unable to pass the PM easily by diffusion, thus getting “trapped” inside the cell. For auxin to be transported out of the cell, the action of efflux carriers is required, and these efflux transporters need to be polarly localized at the PM to control the directionality of auxin flow.

3.2 PIN efflux carriers

According to the chemiosmotic hypothesis, the directionality of intercellular auxin transport depends on the polar subcellular localization of different types of auxin transporters. In Arabidopsis, four main classes of auxin transporters have been identified: the PIN-FORMED (PIN) efflux carriers, the PIN-likes (PILS), the AUXIN RESISTANT 1 (AUX1) and AUX1-LIKE (LAX) influx transporters, and the ATP-binding cassette B (ABCB)-type transporters (reviewed by Grones and Friml, 2015, and Geisler, 2021; Figure 2). Apart from these four major classes, some proteins from other transporter families have been reported to transport auxin, such as NITRATE TRANSPORTER 1.1 (NRT1.1) and WALLS ARE THIN 1 (WAT1) (reviewed in Geisler 2021; Figure 2). Among these transporters, the PIN efflux carriers play a crucial role, as they are rate-limiting for PAT, and their asymmetric localization within cells determines the directionality of intercellular auxin flow and thus the position of the auxin maximum or minimum formed (Petrášek et al., 2006; Wiśniewska et al., 2006; Prasad and Dhonukshe, 2013).

The PIN proteins are a plant-specific family of transmembrane (TM) proteins, named after the pin-shaped inflorescence of the Arabidopsis *pin formed 1* (*pin1*) mutant. They consist of two hydrophobic TM regions, interrupted by a short or long hydrophilic loop (HL) (Okada et al., 1991; Viaene et al., 2013). A recent biophysical study showed that of PINs form a homodimer structure, each monomer composed of a transport and scaffold domain with a clearly defined auxin binding site (Ung et al., 2022). And a proline–proline crossover next to the binding site is crucial for structural changes during auxin transport, which is independent of proton and ion gradients. In Arabidopsis, eight PINs have been identified, which are divided into "long" (PIN1-4, 6 and 7) and "short" (PIN 5 and 8) PINs based on the length of their HL (Křeček et al., 2009; Viaene et al., 2013). The long PINs (PIN1-4 and 7) predominantly show polar PM-localization, determining the direction of auxin flow (Petrásek et al., 2006; Zažímalová et al., 2010; Adamowski and Friml, 2015). In contrast, the short PINs (PIN5 and 8) do not localize at the PM but reside at the endoplasmic reticulum (ER), where they mediate auxin flow between the cytoplasm and ER to regulate subcellular auxin homeostasis (Mravec et al., 2009; Bosco et al., 2012a; Dal Bosco et al., 2012b; Ding et al., 2012; Figure 1). PIN6 is considered as a member of the long PIN subfamily due to its high sequence similarity in the TM regions, the presence of an HL that is only partially reduced in size (Křeček et al., 2009), and its phosphorylation state-dependent localization at both the PM and ER (Simon et al., 2016; Ditengou et al., 2018; Sisi and Růžička, 2020).

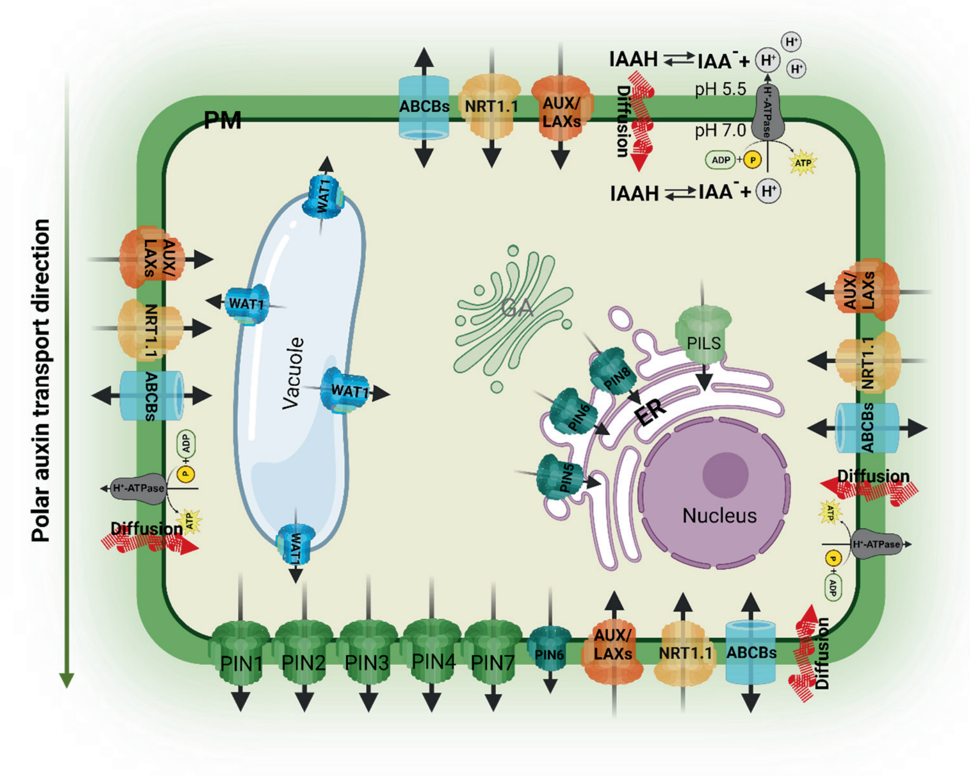

Figure 2. Mechanisms of subcellular auxin distribution and cell-to-cell PAT and the auxin transporters involved. Schematic representation of a cell in a row, indicating passive auxin diffusion and the different auxin transporters involved in subcellular auxin distribution and PAT. The direction auxin diffusion and (polar) auxin transport is indicated by arrows. ER: Endoplasmic reticulum; GA: Golgi apparatus; PM: Plasma membrane.

3.3 Polar auxin transport regulated plant growth and development

PIN-driven PAT (primarily through the long PINs PIN1-4 and 7) plays a crucial role in modulating auxin-dependent plant growth and development and a plant's response to exogenous and endogenous signals.

In Arabidopsis, during embryogenesis, PIN1, 3, 4, and 7 are expressed in different places and at different times, mediating the dynamic and shifting pattern of auxin accumulation. This contributes to the establishment of apical-basal polarity and induces the formation of primordia for aerial and underground organs (Friml et al., 2003). Single mutations in these PINs cause developmental defects in early embryogenesis, with aberrations becoming increasingly severe in multiple mutant combinations (Friml et al., 2002; Friml et al., 2003; Blilou et al., 2005; Vieten et al., 2005; Jenik and Barton, 2005).

During seedling development, at the root tip, PIN1, PIN2, PIN3, PIN4, and PIN7 work together to establish a local "reflux loop" (or "reverse fountain") of auxin, generating a symmetrical auxin gradient with a maximum close to the root tip. This gradient maintains root stem cell activity, root growth and patterning (Friml et al., 2002a; Friml et al., 2003; Blilou et al., 2005; Kleine-Vehn et al., 2010; Wabnik et al., 2011). For apical hook formation, PIN1, 3, 4 and 7 cooperate in regulating the dynamic, asymmetric distribution of auxin, resulting in differential growth at the apical hook (Žádníková et al., 2010). PIN1-driven PAT also plays a major role in lateral roots initiation (Benková et al., 2003), vascular tissue differentiation and regeneration (Galweiler et al., 1998; Mattsson et al., 1999; Scarpella et al., 2010; Marcos and Berleth, 2014; Mazur et al., 2016), leaf and flower positioning (phyllotaxis) and organogenesis (Okada et al., 1991; Reinhardt et al., 2000, 2003; Aloni et al., 2003; Qi et al., 2014; Deb et al., 2015; Xiong and Jiao, 2019).

The dynamic polar subcellular localization of PIN proteins enables them to redirect auxin flow and form auxin gradients, leading to differential cell elongation and organ bending in response to endogenous and external signals. Gravity is a fundamental environmental signal that modulates plant growth through gravitropism. PIN2 is the main player mediating auxin transport in root gravitropism, as indicated by the strong agravitropic phenotype of Arabidopsis *pin2* mutant roots (Luschnig et al., 1998). PIN2 transports auxin from the root tip, where gravity is perceived, to the elongation zone, where growth is regulated (Abas et al., 2006; Sukumar et al., 2009; Lešková et al., 2020). PIN3 and PIN7 are also required for root gravtropism, polarly localizing at lower sides of columella cells to transport auxin towards the lower side of the root tip after gravistimulation (Friml et al., 2002b; Kleine-Vehn et al., 2010; Wang et al., 2015a, Ruiz Rosquete et al., 2018).

Light is another essential environmental signal for plant development. In etiolated hypocotyls, PIN3 apolarly localizes in endodermal cells, but with unilateral blue light stimulation, PIN3 gradually polarizes to and is stabilized at the endodermal cell sides away from the light source. This directs auxin flow to the shaded side of the hypocotyl, resulting in shoot growth toward the light source (positive phototropic response) (Ding et al., 2011). Conversely, Arabidopsis roots exhibit a negative phototropic response. In dark grown roots, PIN1 and PIN2 become less polarized at the PM and show internalized localization in intracellular compartments. PIN2 was proposed to stabilize at the PM at the light side and to be removed from the PM at the shaded side of the root through vacuole-targeted degradation (Wan et al., 2012), while PIN3 was reported to polarize to the lateral membranes towards the light in the root columella cells at the root tip. As a result, auxin was proposed to accumulate at the illuminated root side to promote growth, causing roots to grow away from the light (negative phototropic response) (Zhang et al., 2013). However, this does not fit with the observation that elevated auxin levels at the lower side of the root during the root gravitropic response trigger the opposite (positive gravitropic) growth response. More recently, evidence was provided that the positive phototropic response of the root does not require an asymmetric auxin distribution, and that the elevated auxin levels observed at the illuminated side only appear after the first phototropic root bending, probably caused by the counteracting positive gravitropic response (Kimura et al., 2018). The exact mechanism of root phototropism still remains to be established.

PIN-driven PAT also plays a role in response to abiotic stress. For example, salinity induces internalization of PIN2 from the PM at the side of the root proximal to the area of high salt concentration, and therefore differential PIN2 distribution redirects auxin flow to the root side without salt, resulting in the root growing away from the high salt concentration area (Galvan-Ampudia et al., 2013). In addition to responding to environmental signals, PIN-driven PAT is also involved in responses to endogenous signals, such as auxin (Sauer et al., 2006; Baster et al., 2013; Han et al., 2021), cytokinins (Šimášková et al., 2015; Marhavý et al., 2014), gibberellins (Mäkilä et al., 2023; Willige et al., 2011), strigolactones (Zhang et al., 2020), brassinosteroids (Retzer et al., 2019) and phospholipids (Ischebeck et al., 2013; Tejos et al., 2014).

3.4 The establishment of PIN polarity

Mounting evidence suggests that subcellular trafficking of PIN proteins is the primary mechanism for the establishment and maintenance of PIN polarity (Adamowski and Friml, 2015). PIN proteins undergo continuous and dynamic cycling between their PM polar domain and endosomal compartments (Kleine-Vehn and Friml, 2008; Grunewald and Friml, 2010; Marhava, 2022). The newly synthesized PINs enter the endomembrane system through the endoplasmic reticulum (ER), where they are folded and glycosylated before being transported to the Golgi apparatus. They undergo further modifications after moving to the trans-Golgi network (TGN) and are then sorted to the PM (Grunewald and Friml, 2010). As the secretion of newly synthesized PIN1 from the TGN to the PM has been suggested to be non-polar (Dhonukshe et al., 2008), we will mainly focus on the post-secretion regulation of PIN proteins. During developmental processes or in response to endogenous and exogenous signals, PIN proteins can be endocytosed, trafficked to the trans-Golgi network (TGN)/early endosome (EE), and then transported to different polar domains at the PM by exocytosis or directed to the vacuole for degradation by multivesicular bodies (MVB). These processes determine the abundance and polarity of PINs at the PM (Cheng and Wang, 2022).

3.5 PIN phosphorylation and auxin transport polarity and activity

The polarity and activity of PIN proteins at the PM have been reported to be regulated by post-translationally modifications through phosphorylation at various residues in the central PIN hydrophilic loop (PIN HL) (reviewed in Bassukas et al., 2022). This strongly indicates that not only the trafficking of PIN per se, but also the specific polarity signals within the protein, determine the localization and activity of the protein at distinct polar domains (Grunewald and Friml, 2010). Consistently, an increasing number of protein kinases have been shown to be capable of phosphorylating multiple sites within the PIN sequences, suggesting that the PIN polarity and activity signals are related to the phosphorylation status of these proteins.

3.5.1 PID/WAG AGC3 kinases

Among the known PIN phosphorylating protein kinases, PINOID (PID) is one of the most well-characterized regulators of PIN polarity. PID, together with its homologs WAG1, WAG2 and AGC3-4 compose the AGC3 subfamily of the plant-specific AGCVIII group of kinases. These kinases are related to and thus are named after the protein kinase A, protein kinase G and the Ca^{2+}-activated protein kinase C group of kinases found in other eukaryotic organisms (Rademacher and Offringa, 2012). PID, WAG1 and WAG2 instruct apical asymmetric PIN localisation by phosphorylating the serine (S) residue in three conserved TPRXS motifs (S1-S3: S231, 252, and 290 in PIN1) within the PIN-HL (Michniewicz et al., 2007; Dhonukshe et al., 2010; Huang et al., 2010). Arabidopsis *pid* single or *pid*, *wag1*, *wag2* multiple mutants exhibit an increasing preference for basal PIN localization, resulting in either defects in cotyledon initiation during embryogenesis (Bennett et al., 1995; Cheng et al., 2008), the development of a pin-shaped inflorescence phenocopying that of the *pin-formed 1* (*pin1*) mutant (Bennett et al., 1995; Reinhardt et al., 2003; Friml et al., 2004), or agravitropic root growth (Dhonukshe et al., 2010). By contrast, overexpression of these kinases leads to a basal-to-apical switch of PIN1, PIN4 and PIN2 in the root meristem, causing depletion of the auxin maximum and leading to agravitropic root growth and eventually collapse of the root meristem (Benjamins et al., 2001; Friml et al., 2004). The remarkable change of PIN polarity in loss- or gain-of-function mutants of the *PID* and *WAG* genes, is mirrored by PIN polarity changes observed in plants expressing the engineered phosphomimic (resulting in apical/shootward localization) and phospho-dead (resulting in basal/rootward localization) versions of PIN proteins (Huang et al., 2010; Dhonukshe et al., 2010). Moreover, the mutants defective in subunits of the antagonistically acting trimeric phosphatases, which includes the protein phosphatase 2A (PP2A) (Michniewicz et al., 2007), PP1 (Guo et al., 2015), and PP6 (Dai et al., 2012), exhibit similar apical/rootward localization of PIN proteins as *PID/WAG* overexpression lines. These findings show a direct correlation between PID/WAG-mediated phosphorylation and apical localization of PINs. Notably, in addition to their role in directing a PIN basal-to-apical shift, phosphorylation conducted by PID or WAG2 also enhanced the transport activity of PINs in the Xenopus oocytes system (Zourelidou et al., 2014).

However, phosphorylated PIN1 at S1-S3 sites have also been detected with phosphosite-specific antibodies at the basal as well as the apical side of the PM in different root cell types, in embryos, and shoot apical meristems, or even in the *pid* mutant (Weller et al., 2017), indicating that a more complex model is required to explain the effects of PIN phosphorylation on their polar localization and activity.

3.5.2 AGC1 kinases

Apart from the AGC3 kinases, the AGC1 clade kinase D6 PROTEIN KINASE (D6PK) and three related D6PK-LIKE1-3 were found to phosphorylate S1-S5 of PINs, but preferably S4-S5 (Zourelidou et al., 2014). D6PK acts redundantly with PID/WAGs in phosphorylating S1-S3, suggesting that these kinases share some functional redundancy (Zourelidou et al., 2009; Zourelidou et al., 2014). However, in D6PK loss-of-function and overexpression plants, unlike PID or WAG loss-of-function and overexpression plants, the PIN polarity does not change (Zourelidou et al., 2009). D6PK colocalizes with PIN1, PIN2 and PIN4 at the basal (rootward) PM of Arabidopsis root cells, where it rather regulates the rate of PAT (Zourelidou et al., 2009; Barbosa et al., 2014). D6PK and D6PKL1-3 play a crucial role in hypocotyl phototropism and gravitropism, shade-avoidance, as well as lateral root and shoot differentiation (Zourelidou et al., 2009; Willige et al., 2013; Kohnen et al., 2016). An additional AGC1 kinase that was found to phosphorylate and activate PIN proteins to regulate PAT is PROTEIN KINASE ASSOCIATED WITH BREVIS RADIX (PAX) (Marhava et al., 2018). PAX is specifically expressed in the protophloem and activates PIN-mediated auxin efflux, whereas its auxin-sensing repressor BREVIS RADIX (BRX), which is a PM-associated, polarly localized protein that is specifically expressed in the protophloem, strongly dampens this stimulation by inhibiting PAX activity. PAX and BRX act together as a molecular rheostat to regulate protophloem development and root patterning (Marhava et al., 2018). Notably, the PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PDK1) was found to phosphorylate the activation loops of PAX and D6PK to activate these protein kinases (Zegzouti et al., 2006; Xiao and Offringa, 2020; Tan et al., 2020).

3.5.3 MAPKs and RLKs

Besides the AGC kinases, PIN proteins are also regulated through phosphorylation by MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) and RECEPTOR-LIKE PROTEIN KINASES (RLKs).

MAPKs are a conserved family of protein kinases found in all eukaryotes. They function in a signal transduction cascade that is designed to transduce and amplify signals through sequential phosphorylation: MAPKs are activated through phosphorylation by MAPK KINASES (MAPKKs), which are again activated through phosphorylation by MAPKK KINASES (MAPKKKs) (Enders et al., 2017; Lin et al., 2021). In plants, their signaling has been mostly linked to responses to high salinity, drought, extreme temperature and insect and pathogen infections (Lin et al., 2021). More recently, the MAPKs MPK4 and MPK6 were found to phosphorylate the threonines (T) within the three TPRXS motifs that were also targets of the AGC kinases (Dory et al., 2018). MAP KINASE KINASE 7 (MKK7) acts upstream of MPK6. Induction of expression of their upstream activator MAPK KINASE 7 (MKK7) or activation of the MAPK pathway by application of the pathogen-derived flg22 peptide was found to alter the trafficking of PIN1, resulting in MPK6-dependent intracellular accumulation of PIN1 in root cells (Dory et al., 2018). Furthermore, the MKK7-MPK6 cascade was reported to lead to phosphorylation of Ser 337 in the PIN1-HL, to regulate the basal localization of PIN1 in xylem parenchyma cells and PAT in the primary stem, thereby controlling shoot branching (Zhang et al., 2010; Jia et al., 2016).

RLKs belong to a large family of PM localized kinases. They consist of a ligand-binding extracellular domain, a single transmembrane domain, and a cytoplasmic kinase domain. The auxin-regulated RLKs CANALIZATION-RELATED AUXIN-REGULATED MALECTIN-TYPE (CAMEL) together with CANALIZATION-RELATED RLK (CANAR) interact with and phosphorylate PIN1, possibly at T129, T234, S240, T257, and S408, which are not shared by any previously reported protein kinases (Hajny et al., 2020). PIN1 subcellular trafficking and basal polarization are impaired in Arabidopsis *camel* or *canar* loss-of-function mutants causing defects in leaf venation and vasculature regeneration after wounding (Hajny et al., 2020).

4. Calcium signalling and its involvement in auxin action

4.1 Ca^{2+} is a ubiquitous second messenger for both plant development and early stress responses

The divalent cation calcium (Ca^{2+}) is a crucial component of the plant growth system, playing a vital role in the formation of cell walls, cell membranes and various cellular processes (Hepler and Wayne, 1985; Eklund and Eliasson, 1990; Xu and Heath, 1998). Additionally, Ca^{2+} acts as a ubiquitous second messenger, translating environmental challenges and developmental cues into signal-specific changes in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) , referred to as the 'calcium signature' (Verret et al., 2010).

To decipher the diverse stimuli-induced Ca^{2+} signatures, plants have evolved an extensive array of Ca^{2+} sensors (Roberts and Harmon, 1992; Vogel, 1994). The majority of the Ca^{2+} sensors are sensor relay proteins, which include Calmodulins (CaMs), Calmodulin-like proteins (CMLs) and Calcineurin B-like proteins (CBLs) (Sanders et al., 2002; Hashimoto and Kudla, 2011). The Arabidopsis CaM/CMLs family harbours seven *CaM* genes and 50 *CMLs* (McCormack and Braam, 2003). While lacking other identifiable functional domains, the CaM/CMLs possess a varying number of Ca^{2+} binding domains called "EF hands". They function by altering downstream target activities through Ca^{2+}-dependent protein–protein interactions (Hashimoto and Kudla, 2011). Similarly, CBLs act as sensor relay proteins due to their lack of enzymatic activity. They can interact with a specific family of proteins known as CBL-interacting protein kinases (CIPKs), playing a crucial role in response to wounding, cold, drought, salt stress, regulation of ABA sensitivity and biosynthesis, and nutrient sensing (Luan et al., 2002). In contrast, calcium-dependent protein kinases (CDPKs) are sensor-responders. They contain both a Ca^{2+} sensing domain with EF-hand motifs and a protein kinase domain. Binding of Ca^{2+} results in activation of the kinase domain leading to the subsequent phosphorylation of target proteins (Hashimoto and Kudla, 2011).

In conclusion, Ca^{2+} serves as a pivotal messenger in plant development and early stress responses, with specialized sensors, such as CaM/CMLs, CBLs, and CDPKs, enabling plants to interpret and respond to various environmental and developmental cues.

4.2 Calcium as second messenger in auxin signalling

Over the past decade, the growth in genetic studies and related technology development provide an abundant amount of evidence showing that certain rapid auxin responses cannot be fully explained by the nuclear canonical TIR1/AFB-Aux/IAA-ARFs pathway. Instead, they suggest the involvement of new pathways that rely on TIR1/AFBs receptors localized outside the nucleus and/or TMK receptors localized to the PM (reviewed in McLaughlin et al., 2021, Pérez-Henríquez and Yang, 2023). For instance, auxin rapidly inhibits root growth within 30 seconds, which is too rapid to depend on transcriptional regulation. However, its dependence on TIR1/AFB signalling suggests the existence of an extranuclear TIR1/AFB-dependent signalling pathway (Fendrych et al., 2018). Indeed, recent studies have shown that all six TIR1/AFBs exhibit both nuclear and cytoplasmic subcellular localization in Arabidopsis root cells, with specifically AFB1 being predominantly located in the cytoplasm (Prigge et al., 2020). Consistent with these finding, AFB1 was reported to mediate the rapid root growth inhibition, as the single *afb1* loss-of-function mutant shows a significant defect in rapid root growth inhibition (Fendrych et al., 2018; Prigge et al., 2020; Serre et al., 2021).

Ca^{2+} signalling is one of the earliest responses to auxin (Hasenstein and Evans, 1986; Gehring et al., 1990; Shishova and Lindberg, 2004; Monshausen et al., 2011). The auxin induced rapid inhibition on root growth is accompanied by a CYCLIC

NUCLEOTIDE-GATED CHANNEL 14 (CNGC14)-dependent transient increase in $[Ca^{2+}]_{cyt}$. Inhibition of root growth is significantly delayed in the *cngc14* loss-of-function mutant, indicating the involvement of Ca^{2+} signalling in rapid auxin response (Dindas et al., 2018; Shih et al., 2015). Moreover, auxin triggered transient increase in $[Ca^{2+}]_{cyt}$ requires TIR1/AFB-based auxin signalling (Dindas et al., 2018). A recent study has shown that the cytoplasmic AFB1 induces this non-genomic rapid auxin response, and that AFB1 is dependent on and acts upstream of CNGC14-mediated Ca^{2+} signalling, mediating the rapid root growth inhibition (Dubey et al., 2023).

4.3 Calcium signalling involved in polar auxin transport

The first reports linking Ca^{2+} signalling to PAT date back several decades. In 1973, Dela Fuente and Leopold (1973) found that PAT in sunflower stem sections was significantly suppressed by washing with the Ca^{2+} chelator ethylenediaminetetraacetate (EDTA), but could be restored by subsequent application of $CaCl_2$. Additionally, PAT was observed to be dampened under low Ca^{2+} conditions (Dela Fuente, 1984). Further studies by Lee et al. (1984) reported that gravistimulation induces polar movement of calcium across the root tip of maize and pea from the upper side to the lower side, coinciding with the basipetal movement of auxin. Subsequent research confirmed these findings and suggested that gravity-induced increase in $[Ca^{2+}]_{cyt}$ constitutes an upstream event of PAT (Plieth and Trewavas, 2002; Toyota et al., 2008).

Ca^{2+} signalling plays a regulatory role in various processes involving PAT, including phototropism (Baum et al., 1999; Harada et al., 2003; Harada and Shimazaki, 2007; Zhao et al., 2013), response to inositol trisphosphate (Zhang et al., 2011), coping with toxic heavy metals (Li et al., 2016), shoot apical meristem (SAM) development (Li et al., 2019), and maintenance of the quiescent center (QC) in the root meristem (Goh et al., 2012). These findings highlight the crucial interplay between auxin and Ca^{2+} in plant physiological processes.

As previously mentioned, the asymmetrically distributed PIN proteins provide directionality in and perform the rate-limiting step in PAT (Petrášek et al., 2006; Wiśniewska et al., 2006). PIN-driven PAT can be regulated at multiple levels, including transcription, protein degradation, subcellular trafficking, polarity, and activity (Cheng and Wang, 2022). Since the direct involvement of Ca^{2+} signalling in PIN transcription and protein degradation is scarcely reported, this discussion will mainly focus on how Ca^{2+} signalling translates environmental challenges and developmental cues into alterations in PIN polarity, activity, and subcellular trafficking, subsequently leading to changes in plant growth and development.

4.4 Ca^{2+} signalling in the regulation of PIN subcellular trafficking

Ca^{2+} has been reported to play a role in regulating both protein endocytosis and exocytosis, including PIN proteins, thereby affecting protein abundance and polarity at the PM (Figure 3).

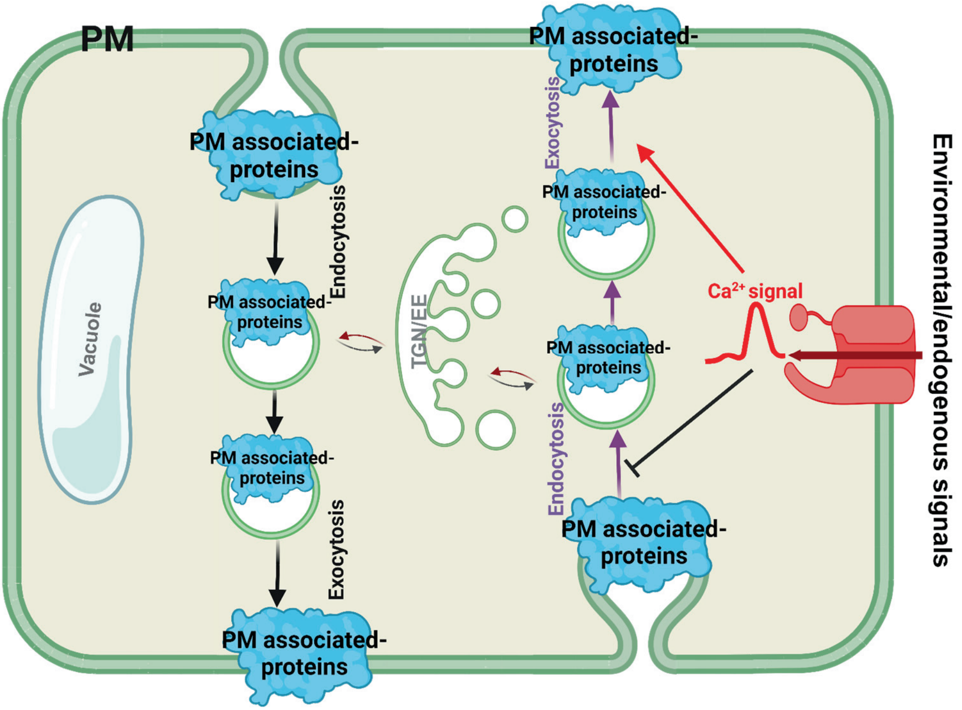

Figure 3. Schematic depiction of the effect of endogenous and environmental signal-induced elevated $[Ca^{2+}]_{cyt}$ on protein exocytosis and endocytosis. Elevation in the $[Ca^{2+}]_{cyt}$ has an inhibitory effect on endocytosis and a stimulating effect on exocytosis. TGN/EE: Trans-golgi network /early endosome; PM: Plasma membrane.

4.4.1 Effect of Ca^{2+} signalling on endocytosis

The PM localized PIN proteins are constitutively internalized via clathrin-mediated endocytosis. The involvement of Ca^{2+} signals in the regulation of endocytosis has been reported both in animals and plants. In synaptic terminals of retinal bipolar neurons, elevated $[Ca^{2+}]_{cyt}$ strongly inhibits endocytosis, as indicated by Ca^{2+}-induced increases in membrane capacitance (Von Gersdorff and Matthews, 1994). In muscle cells, rapid influx of Ca^{2+} accelerates exocytosis of the Glucose Transporter type 4 protein (GLUT4), but slows down its endocytosis (Li et al., 2014). Moreover, caffeine-induced Ca^{2+} release on sarcoplasmic reticulum was shown to inhibit endocytosis (Page et al., 1994).

TPLATE is a plant-specific interactor of clathrin (Van Damme et al., 2011). It is part of a unique multi-subunit protein complex (the TPLATE complex, TPC) that acts in concert with the adaptor protein complex 2 (AP2 complex), dynamin-related proteins and clathrin, to regulate plant growth via its role as a major adaptor module for clathrin-mediated endocytosis (Gadeyne et al., 2014). Several TPC subunits contain Ca^{2+} binding EF hand motifs, suggesting a role for Ca^{2+}-dependent regulation; however, further validation is need (Day et al., 2002; Bar et al., 2008; Gadeyne et al., 2014). Treatment with caffeine, which increases the $[Ca^{2+}]_{cyt}$ by inducing intracellular Ca^{2+} store leaks, was found to inhibit endocytosis by dislodging TPLATE and CLATHRIN LIGHT CHAIN2 from the cell plate during cytokinesis, which is consistent with findings in animals that high $[Ca^{2+}]_{cyt}$ inhibits endocytosis (Page et al., 1994; Van Damme et al., 2011).

4.4.2 Effect of Ca^{2+} signalling on exocytosis

Following endocytosis, vesicles containing PIN proteins can be directed to recycling endosomes (RE) and from there PIN cargo is delivered back to the PM. Ca^{2+} is well known for its role in activation of exocytosis in both animal and plant cells. The paradigm for the role of Ca^{2+} in exocytosis is in neurotransmission, where a local elevation in the $[Ca^{2+}]_{cyt}$ in the axon termini triggers the exocytosis of neurotransmitter-filled vesicles to activate downstream neurons (Benarroch, 2013). Ca^{2+} also triggers granule exocytosis in mast cells, and hormone exocytosis in endocrine cells (reviewed in Pang and Südhof, 2010). The direct activation effect of elevation of $[Ca^{2+}]_{cyt}$ on exocytosis in plants was demonstrated by Ca^{2+}-induced increases of membrane capacitance in protoplasts from barley aleurone (Homann and Tester, 1997), maize coleoptiles or root caps (Sutter et al., 2000; Carroll et al., 1998) or tobacco leaves (Sutter et al., 2012).

The stimulating effect of Ca^{2+} on exocytosis is mediated by specific Ca^{2+} sensing proteins, named SYNAPTOTAGMINs (SYTs). SYTs function as the primary Ca^{2+} sensors to stimulate exocytosis in mammalian cells. SYT1, SYT2, and SYT9 function as Ca^{2+}-sensors in synaptic exocytosis, SYT1 and SYT7 are major Ca^{2+} sensors in endocrine large dense-core vesicle exocytosis, SYT2 functions as the major Ca^{2+} sensor for exocytosis in mast cells, SYT4 functions in postsynaptic exocytosis in Drosophila, and SYT7 mediates Ca^{2+}-triggered lysosome exocytosis in fibroblasts (Pang and Südhof, 2010). SYTs contain a N-terminal transmembrane region, and two C-terminal Ca^{2+}-binding C2-domains that act via Ca^{2+}-dependent interactions with both the fusing phospholipid membranes and the membrane fusion machinery (Pang and Südhof, 2010). In plants, calcium-dependent candidate regulators of exocytosis include CaMs, CDPKs, NADPH oxidases (NOX), actin-binding proteins, ANNEXINS (ANNs) and SYNAPTOTAGMINS (SYTs) (Žárský et al., 2009). The SYNAPTOTAGMIN family in Arabidopsis has five members (Craxton, 2004). SYT1 is indispensable for maintaining PM integrity under salt or freezing stress, likely by facilitating the process of exocytotic membrane resealing (Schapire et al., 2008). SYT2 participates in pollen germination and pollen tube growth (Wang et al., 2015b). SYT3 and SYT1 act redundantly in preventing PM damage and promoting abiotic stress resistance (Ruiz-Lopez et al., 2021). SYT4/5 contribute to Arabidopsis immunity to *Pseudomonas syringae* DC3000 (Kim et al., 2022). However, involvement of SYTs in exocytosis in plants needs further study.

ANNEXINs were initially identified in animal cells and named for their ability to "annex" or bring together membranes. They were initially proposed to be involved in membrane fusion processes (Clark et al., 2012). ANNEXINS are composed of two principal domains: the divergent NH2-terminal “head” and the conserved COOH-terminal protein core, which contains a conserved Ca^{2+}- and membrane-binding motif (Gerke and Moss, 2002). Root cells of Arabidopsis seedlings ectopically expressing ZmANN33 or ZmANN35 show a more intact PM after chilling stress, which is likely due to the accelerated exocytotic process (He et al., 2019). Arabidopsis ANN5 plays a role in the maintenance of membrane integrity in pollen grains exposed to osmotic or ionic imbalances (Lichocka et al., 2022). Overexpression of ANN5 leads to increased resistance of pollen germination and pollen tube growth to the exocytosis-inhibiting fungal toxin Brefeldin A (BFA), and this effect is modulated by Ca^{2+} fluctuations that occur within pollen cells (Zhu et al., 2014).

4.5 Calcium signalling in the regulation of PIN polarity/activity

The shift in PIN polarity might result from other regulators that interact with PID/WAGs, enhancing the efficiency of this polarity shift. Several regulators of PID or WAGs have been identified. The PHOSPHOLIPASE D (PLD)-derived phosphatidic acid (PA) has been reported to bind to the PID insertion domain (ID) in the catalytic domain to promote PID-mediated PIN2 phosphorylation and subsequent root growth under salt stress (Wang et al., 2019). The 3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE-1 (PDK1) was initially reported to interact with PID through the C-terminal PDK1-interacting-fragment (PIF) domain and promote PID kinase activity by phosphorylating the activation segment of PID (Zegzouti et al., 2006). However, Xiao and Offringa (2020) have shown that both PID functionality and its subcellular localization do not depend on PDK1 function. Instead, PDK1 plays a more important role in regulation of PAT by phosphorylating and activating the AGC1 kinases PAX and D6PK (Xiao and Offringa, 2020; Tan et al., 2020; Figure 3). Some other regulators, like BTB and TAZ domain scaffold protein 1 (BT1/PBP2) (Benjamins et al., 2003; Robert et al., 2009) and 14-3-3 (Xiao, 2019), were also reported to interact with PID, however these interactions have mainly been shown by *in vitro* biochemical assays and their biological function is yet to be determined.

The first molecular clue that Ca^{2+} signaling is involved in regulating PIN-driven PAT was provided by the finding that PID interacts with the CALMODULIN-LIKE 12/TOUCH 3 (CML12/TCH3) and the EF hand containing PID-BINDING PROTEIN 1 (PBP1) in a Ca^{2+} -dependent manner (Benjamins et al., 2003). Later, CML12/TCH3 was also found to interact with WAG2 (Fan, 2014). CML12/TCH3 is a negative regulator of PID kinase activity both *in vitro*, as shown by phosphorylation assays, and *in planta* where *TCH3* overexpression reduces *PID* overexpression induced root meristem collapse (Robert, 2008; Galván-Ampudia, 2009). Consistently, Zhang et al. (2011) showed that modulation of PIN polarity by the inositol trisphosphate (InsP3)-mediated increase of $[Ca^{2+}]_{cyt}$ is PID/WAG-dependent. Elevating InsP3 or $[Ca^{2+}]_{cyt}$ levels interfered with apical localization of PIN2 in young cortex cells of *35S:PID* seedlings, thereby delaying the *35S:PID*-mediated root apical meristem collapse, whereas treatments decreasing $[Ca^{2+}]_{cyt}$ enhanced the root collapse of *35S:PID* plants and affected the basal PIN2 polarity in young epidermal cells of *pid wag1 wag2* roots (Zhang et al., 2011).

Apart from PID and WAG2, no involvement in Ca^{2+}-dependent regulation has been reported for the other kinases phosphorylating PIN proteins, as discussed above. Below a few other kinases are presented that do seem to play a role in Ca^{2+}-dependent regulation of PAT.

4.5.1 CRKs

The CDPK-RELATED KINASE5 (CRK5), a member of the Arabidopsis Ca^{2+}/calmodulin-dependent kinase-related kinase family (CRKs) (Harper et al., 2004), was reported to phosphorylate PINs to regulate plant development (Rigo et al., 2013). In Arabidopsis, the CRK family consist of eight highly conserved members. They share a conserved C-terminal CaM-binding domain, but have sequence divergence in their N-terminus except for two conserved domains that are responsible for membrane-association (Harper et al., 2004; Leclercq et al., 2005;. Rigo et al., 2013).

CRKs are Ca^{2+} responsive kinases. In *Nicotiana tabacum,* the biochemical activity of NtCPK5 is calcium-dependent (Wang et al., 2005). In Arabidopsis, biochemical studies have shown that the autophosphorylation activity of CRK1 and CRK3 is remarkably increased (up to 10-fold) in the presence of CaM and Ca^{2+}, whereas their substrate phosphorylation activity is only two- to threefold increased (Wang et al., 2004; Du et al., 2005). Regarding CRK5, its autophosphorylation and trans-phosphorylation activity were found to be similar in the presence or absence of CaM and Ca^{2+} (Rigo et al., 2013). This might be because CRK5 requires some yet undefined Ca^{2+} binding partners, as suggested previously (Harmon, 2003; Du et al., 2005). CRK5 was found to phosphorylate PIN1, 2, 3, 4, and 7 *in vitro*, but the phosphorylation sites have not yet been identified (Rigo et al., 2013; Baba et al., 2019a; Baba et al., 2019b). CRK5 has been reported to mediate phosphorylation of PIN proteins in various developmental processes. For instance, CRK5 was found to phosphorylate PIN2 to regulate root gravitropism and lateral root formation (Rigo et al., 2013), PIN3 to mediate apical hook development (Baba et al., 2019b) and PIN1 to coordinate embryonic development (Baba et al., 2019a). In *crk5* loss-of-function mutants, apical (shootward) localization of PIN2 in root epidermis cells is reduced and the protein shows mixed apolar and apical localization in the cortex of the root transition zone. CRK5 phosphorylation may thus control PIN2 polarity, stability and/or degradation (Rigo et al., 2013; Figure 3).

4.5.2 CDPKs

Recently, the Arabidopsis CALCIUM-DEPENDENT PROTEIN KINASE 29 (CPK29) has been reported to directly interpret Ca^{2+} signals from internal and external triggers to modulate PIN trafficking and polarity by targeting a specific Serine residue in the PIN HL not phosphorylated by other kinases (Lee et al., 2021). The *cpk29* loss-of-function mutant exhibits a decrease in polar PM localization and enhanced internal accumulation of PIN proteins, suggesting its involvement in Ca^{2+}-enhanced PIN exocytosis (Lee et al., 2021; Figure 3).

In tomato, StCDPK1 was reported to phosphorylate StPIN4 *in vitro*, suggesting a possible role in regulating the polarity of StPIN4 (Santin et al., 2017).

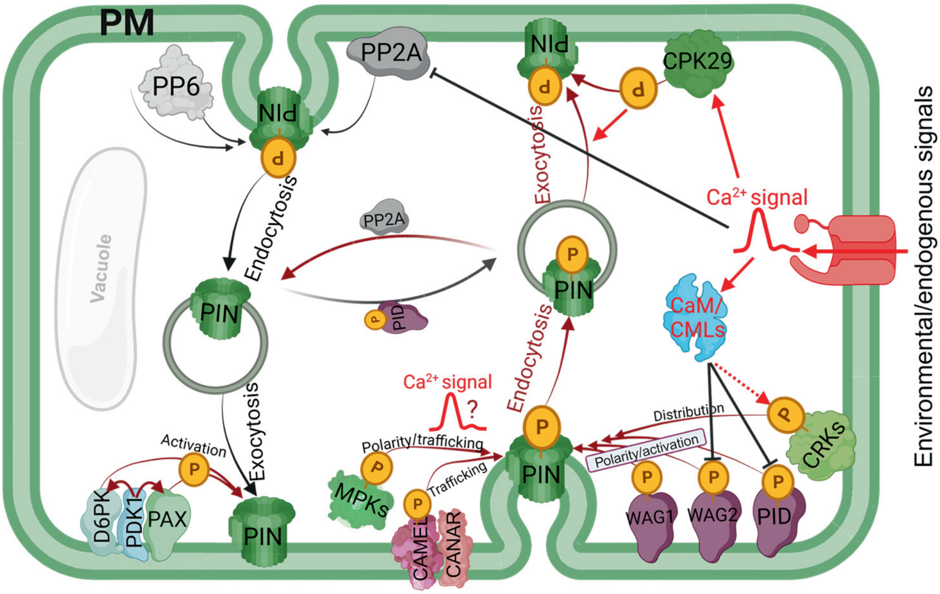

Figure 4. Model showing the role of Ca^{2+} signalling in protein kinase-mediated regulation of PIN polarity, activity or trafficking. Details are described in the main text. P: Phosphorylation. PM: Plasma membrane.

5. Feedback of auxin on its own transport: a role for Ca^{2+} signalling

In 1969, Tsvi Sachs proposed the canalization hypothesis, describing the feedback of auxin on its own transport (reviewed in Smith and Bayer, 2009). The subsequent identification of PIN proteins as determinants of the rate and direction of PAT in later years (reviewed in Marhava, 2022) has provided scientists with the molecular tools to understand the mechanism behind this feedback and its importance in auxin-dependent development.

Firstly, auxin was shown to feedback on PIN polarity through auxin signaling-mediated transcriptional changes in gene expression. Sauer et al. (2006) showed that local auxin application or auxin accumulation during de novo formation of vasculature and lateral roots leads to relocalization of PIN1 and PIN2 at the PM, which is cell-specific, *PIN* transcription-independent, but still involves the TIR1/AFB-Aux/IAA signaling pathway leading to ARF-dependent transcription. Prát et al. (2018) reported that the auxin responsive gene encoding the transcriptional activator WRKY23 acts downstream of IAA17-ARF7/19 to mediate this process, as *WRKY23* gain- and loss-of-function mutants showed altered auxin-mediated lateralization of PIN1 and PIN2. Hajný et al. (2020) reported that the CAMEL-CANAR RLKs, which phosphorylate PINs and regulates their polarity, act downstream of WRKY23. These findings represent an elegant mechanism of auxin feedback on its own transport machinery though the auxin signaling pathway.

Another mechanism of feedback of auxin on PIN polarity occurs through PIN membrane cycling dynamics. Paciorek et al. (2005) found that auxin acts as a general inhibitor of endocytosis, including PIN endocytosis, implying feedback of extracellular, apoplastic auxin on PIN trafficking and polarization. The signaling was recently found to be mediated by the controversial auxin receptor ABP1. Auxin facilitates the interaction of ABP1 and TMKs, which activates the ROP6/RIC1 pathway in root cells and the ROP2/RIC4 pathway in leaf pavement cells, leading to inhibition of clathrin-dependent PIN1 endocytosis (Xu et al., 2010; Xu et al., 2014; Pan et al., 2020). During gravistimulation, auxin-promoted interaction with ABP1 leads to activation and stabilization of TMK1, which in turn enhances its interaction with and phosphorylation of PIN2, at the lower side of the root to reinforce asymmetry in PIN2-mediated auxin fluxes for gravitropic root bending (Rodriguez et al., 2022). ABP1-TMK mediated auxin perception is also required for a subset of auxin responses, such as the activation of PM H^+-ATPases, global protein phosphorylation, regeneration of vasculature around the wound and formation of auxin-transporting channels for the development of vasculature originating from a local source of auxin (Friml et al., 2022).

Besides rapid auxin signaling via the ABP1-TMK receptors, calcium has been proposed to be involved in a swift auxin signaling system mediating rapid growth responses (Shishova and Lindberg, 2010; Plieth and Trewavas, 2002). It has been shown that exogenous auxin, via the AFB1-based auxin signalling pathway, triggers a rapid, transient increase in $[Ca^{2+}]_{cyt}$ in many plant species conducted by Ca^{2+} channels, in particular CNGC2 and CNGC14 (Verret et al., 2010; Vanneste and Friml, 2013;Wang et al., 2017; Dindas et al., 2018; Chakraborty et al., 2021; Yu et al., 2022a). In this way, local increases in auxin concentrations are translated into Ca^{2+} signals to regulate downstream targets.

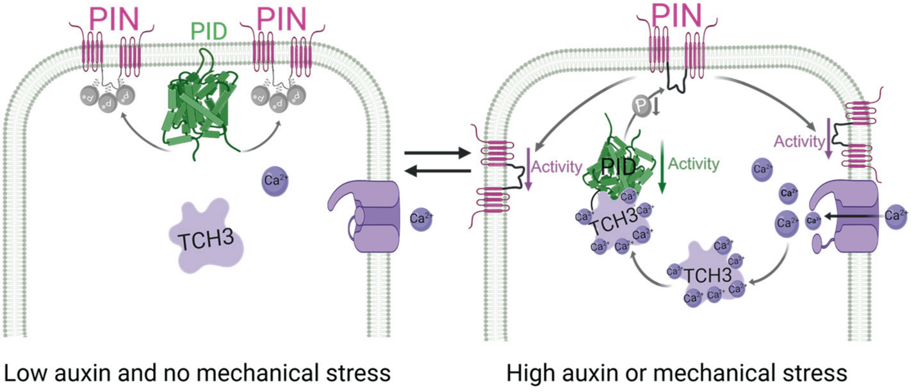

Figure 5. Model for feedback regulation of auxin or mechanical stress on PIN polarity and activity through Ca^{2+}-dependent binding of TCH3 to PID. Mechanical stress and auxin lead to elevated expression of *CML12/TCH3* and trigger elevation of $[Ca^{2+}]_{cyt}$, which activates the

CML/TCH3, leading to sequestration of PID from the PM to the cytosol and a decreased PID kinase activity. As a result, the attenuated PIN phosphorylation leads to reduced PIN polarity and activity.

The finding that the calcium sensors CML12/TCH3 and PBP1 interact with PID (Benjamins et al., 2003) provided an additional molecular link between Ca^{2+} signaling and PIN activity and polarity. CML12/TCH3 interacts with PID in a Ca^{2+} -dependent manner (Benjamins et al., 2003) and negatively regulates PID activity *in vivo* and *in vitro* (Robert, 2008; Galván-Ampudia, 2009). Moreover, *CML12/TCH3* expression was found to be rapidly up-regulated in response to mechanical stimuli, such as touch, as well as by other stimuli including darkness, temperature shocks or brassinosteroid or auxin signalling (reviewed in Braam et al., 1997). In Arabidopsis protoplasts and epidermis cells at the root tip, the interaction with CML12/TCH3 sequesters PID from the PM to the cytosol in the presence of Ca^{2+} and auxin (Fan, 2014). Based on these results, we propose an alternative model for the feedback of auxin on PIN activity and polarity (Figure 5): local increases in auxin levels or mechanical stress trigger a rapid and substantial up-regulated expression of *CML12/TCH3* and a transient increase in $[Ca^{2+}]_{cyt}$, leading to the activation of CML12/TCH3, which in turn sequesters PID, a crucial regulator of PIN protein polarity/activity, to the cytosol and inhibits its kinase activity. Consequently, PIN protein polarity and activity are decreased, leading to local changes in auxin transport and distribution.

5.1 The role of the Ca^{2+}/TCH3-PID pathway in plant growth and development

The Ca^{2+}-dependent TCH3-PID interaction provides an elegant mechanism how auxin or mechanical stress can modulate and fine tune PAT. The question remains in which plant growth or developmental process this mechanism plays a significant role. In view of the *PID* expression pattern and the phenotypes of *pid* loss-of-function mutants, it seems likely that this mechanism is involved in regulation of root growth and organ initiation in the embryo and inflorescence.

Previous studies in our group have indicated that the Ca^{2+}-dependent TCH3-PID interaction plays a role in the root gravitropic response (Fan, 2014). The gravitropic response of the root is driven by asymmetric redistribution of auxin in the root tip, which is coordinated by PIN proteins (reviewed in Han et al., 2021). Upon gravistimulation, PIN3 and PIN7 polarize to the lower sides of columella cells, thus driving the auxin flow from the auxin maximum towards the lower side of the root tip (Han et al., 2021). This additional auxin is subsequently transported by the apical/shootward localized PIN2 from the root tip, where gravity is perceived, to the elongation zone, where growth is regulated (Han et al., 2021). Simultaneously, the increased accumulation of auxin at the lower side of the root tip induces a transient increase in $[Ca^{2+}]_{cyt}$ (Monshausen et al., 2011) and a rapid and substantial up-regulated expression of *CML12/TCH3* (Antosiewicz et al., 1995; Benjamins et al., 2003). It is known that Ca^{2+} plays an essential role in gravitropism, as gravistimulation induces transient Ca^{2+} signals in many tissues and species (Vanneste and Friml, 2013) and blocking the Ca^{2+} channels inhibits the root gravitropic response, but not the asymmetric distribution of auxin, indicating that Ca^{2+} acts downstream of auxin (Fan, 2014). *PID* is co-expressed with *TCH3* in epidermis cells of the elongation zone of the root tip (Benjamins et al., 2001; Benjamins et al., 2003). Based on these data we proposed that the enhanced $[Ca^{2+}]_{cyt}$ and increased *TCH3* expression lead to TCH3-PID binding, which sequesters PID from the PM to the cytosol and decreases its kinase activity. This results in attenuated PIN2 phosphorylation and thus its apolar localization and reduced auxin efflux activity at the lower side of root tip, enhancing the asymmetric auxin distribution and thus the downward bending of the root tip. The importance of the asymmetric induction of *TCH3* expression during this process was confirmed by the delayed gravitropic response of *TCH3* overexpression seedlings. Not in line with this model is the finding that, *tch3* loss-of-function mutants did not show a significantly altered gravitropic root response, this may be caused by redundancy among the *CAM/CML* genes (Fan, 2014).

Auxin distribution and its polar transport mediated by the dynamic changes in PIN polarity patterns also underlies the initiation and patterning of new organs, for example, phyllotaxis (Yin, 2021). Aerial organs, such as leaves and flowers, are initiated at the shoot apical meristem (SAM) of a plant, and this process is triggered by local PIN1-driven accumulation of auxin (Yin, 2021). The continuous initiation and expansion of aerial organs through cell division and growth induces mechanical stress in the SAM, which has been identified as additional driver of phyllotaxis by regulating auxin transport (Heisler et al., 2010). Li et al. 2019 have shown that mechanical stimulation using laser ablation or drug treatment of the SAM cause transient increases in $[Ca^{2+}]_{cyt}$, leading to changes in PIN1 polarity (Li et al., 2019). By studying *SHOOT MERISTEMLESS* (*STM*), a mechanical stress-responsive gene encoding a master regulator and marker of meristematic identity in Arabidopsis, Landrein et al. (2015) observed that mechanical stress acts in parallel to auxin signalling, providing robustness to the regulation of gene activity in the shoot meristem (Landrein et al., 2015). So far, however, it remains unclear how exactly PIN1 polarity and activity are regulated to determine the next PIN1 convergence point where an organ primordium is initiated. Interestingly, *CML12/TCH3* has been shown to be highly upregulated in response to both mechanical stress and auxin (Braam and Davis, 1990; Benjamins et al., 2001; Benjamins et al., 2003; Fan, 2014). PID is a crucial regulator of PIN polarity, accumulating in the peripheral zone of the SAM and in newly formed leaf primordia, and known for its role in floral meristem outgrowth and floral organ development (Bennett et al., 1995; Friml et al., 2004; Wang et al., 2014). Moreover, providing mechanical stress to the SAM led to Ca^{2+}-dependent sequestration of PID from the PM to the cytosol (Fan, 2014). Based on this, we hypothesize that the Ca^{2+}-dependent TCH3-PID interaction presumably integrates hormonal and abiotic signals triggering elevations in $[Ca^{2+}]_{cyt}$, such as auxin and mechanical stress, to mediate the dynamic changes in PIN polarity and activity in the SAM, thus providing robustness to the phyllotactic pattern. However, this hypothesis needs further validation.

6. Outline of the thesis

In **Chapter 1** of this thesis we review the state of the art of auxin signalling and transport and the involvement of Ca^{2+} as second messenger in these processes. We finalize by proposing a new model how, through the Ca^{2+}-dependent CML12/TCH3-PID interaction, auxin can feedback on PIN polarity and activity. However, the role of the Ca^{2+}-CML12/TCH3-PID module in plant growth and development is still unclear, as single *tch3* loss-of-function mutants do not show a clear phenotype, suggesting that other CMLs, and possibly CaMs, might act redundantly with CML12/TCH3.

To further confirm our hypothesis and identify whether other CaM/CMLs interact with PID, we selected the most closely related homologs of CML12/TCH3, including seven CaMs, CML8, 9, 10, 11, 13 and CML14 and the more distantly related CML24/TCH2 as candidates. In **Chapter 2**, we show that a confined clade comprising seven CaMs and four closely-related CMLs, including CML12/TCH3, interact with PID. In Arabidopsis protoplasts, co-transfection of these CaM/CMLs with PID in the presence of auxin resulted in sequestration of PID from the PM. A comparative study on the spatio-temporal expression of the corresponding *CaM/CML* genes and *PID* using *promoter:GUS* reporter fusions displayed differential but also largely overlapping expression patterns in most tissues throughout all the developmental stages. Interestingly, we observed that the *CaM2*, *CaM7*, *CML10* and *CML12/TCH3* genes are responsive to auxin treatment and gravistimulation. These results confirm our previous suggestion that CML12/TCH3 may acts redundantly with other CAM/CMLs in regulating PID activity, and explain why the *tch3* loss-of-function mutant does not show clear phenotypes.

In **Chapter 3**, to overcome the redundancy of the CaM/CMLs in regulating PID kinase and identify the biological function of the CaM/CML-PID interaction, we fine-mapped the CaM/CMLs binding domain in PID. 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. Moreover, the PID(RtoA) versions showed the same overexpression phenotypes as wild-type PID and complemented the pin-like inflorescence phenotype of the *pid* loss-of-function mutant, when expressed under the *PID* promoter. This indicated that 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 analysis of these plants suggested that the mutant PID versions were fully functional, as they complemented the pin-like inflorescence phenotype of the *pid* mutant and did not alter seedling development or flowering time. 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 auxin maxima in the inflorescence meristems of the ‘untouchable’ PID expressing plants. Our data indicate that Ca^{2+}-dependent regulation of PID activity by CaM/CML binding is required for the accurate spatio-temporal positioning in the SAM of a single auxin maximum at a time.

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