Calcium- and BTB domain protein-modulated PINOID protein kinase directs polar auxin transport

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Chapter 1

Calcium and BTB proteins: generic signaling components with specific roles in auxin transport

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Transport driven auxin gradients and maxima orient plant development

As sessile organisms, plants have developed various mechanisms for perceiving and responding to changes in their environment such as light, temperature, gravity and drought. Auxin is a central regulator in many of these adaptive responses. Studies of Darwin on the tropic bending of canary grass and oat coleoptiles towards a unidirectional light source provided the first indications of a transported growth substance that mediates this adaptive response (Darwin, 1880). Later, the plant hormone indole-3-acetic acid (IAA) was identified as the key regulator of tropic responses and was named auxin after the greek word for "to grow" (Went, 1937). The asymmetric lateral distribution of auxin during tropic responses is mediated by its polar cell-to-cell transport. This polar auxin transport (PAT) is crucial for many developmental processes, creating auxin gradients and maxima in specific tissues in the plant, e.g. embryo, root tip, leaves and flowers primordia, that regulate cell division, elongation and differentiation, and in this way are instrumental for plant architecture (Sabatini et al., 1999, Reinhardt et al., 2003, Benková et al., 2003, Weijers et al., 2005).

The chemiosmotic model proposed for PAT in the 1970's predicted that polar auxin flow is driven by specific carriers that due to their localization at one side of the cell mediate polar auxin efflux. Molecular genetic research in Arabidopsis has identified the PIN transmembrane proteins as auxin efflux carriers (Gälweiler et al., 1998, Luschnig et al., 1998, Petrášek et al., 2006), that determine the direction of PAT through their asymmetric subcellular localization (Wisniewska et al., 2006). PIN localization is highly dynamic, regulated by vesicles trafficking, protein degradation and phosphorylation, and is responsive to both internal and external signals, among which auxin itself (Geldner et al., 2001, Friml et al., 2004, Abas et al., 2006, Sauer et al., 2006).

The first, and yet only identified determinant in the polar distribution of PIN proteins is the PINOID (PID) serine/threonine protein kinase. Above threshold levels of PID activity induce movement of PIN proteins from the basal (root facing) to the apical (shoot facing) side of cells, thereby changing the direction of auxin transport (Friml et al., 2004). Recently it has been demonstrated that PID directly phosphorylates PIN proteins in their hydrophilic loop, and that PP2A phosphatases counteract the activity of this kinase (Michniewicz et al., 2007).

PID activity is determined on the one hand by expression of the *PID* gene, which is auxin responsive (Benjamins et al., 2001) and under strict spatio-temporal regulation (Christensen et al., 2000, Benjamins et al., 2001, Michniewicz et al., 2007). On the other hand, several PID binding proteins have been identified that appear to regulate its enzymatic activity. Two of these are the calcium-binding proteins TOUCH3 (TCH3) and PINOID BINDING PROTEIN1 (PBP1) that interact with PID in a calcium-dependent manner and respectively repress or enhance its *in vitro* activity (Benjamins et al., 2003).

Furthermore, PID was found to interact with the Broad-Complex, Tramtrack, Bric-a-Brac (BTB) and Transcriptional Adaptor Zinc finger (TAZ) domain protein1 (BT1) (Benjamins, 2004), an interaction that may recruit PID into a multi-protein regulatory complex for which BT1 functions as scaffold (Kemel Zago, 2006). In other words, the AGCVIII kinase PID, a key regulator of auxin transport, is modulated through its interaction with calcium-binding- and BTB domain proteins. In this chapter we provide a survey on what is known on calcium signaling and BTB proteins in relation to plant development. Since calcium signaling and calcium-binding proteins have been widely reviewed, we will focus on the role of calcium in signaling pathways involving AGC kinases. We will further discuss the role of calcium and BTB proteins in the regulation of auxin transport in tropic responses such gravi-, photo- and thigmotropism.

Calcium: from plant nutrient to signaling molecule

Calcium is an essential nutrient for plants. It functions as structural component in the cell wall and the plasma membrane, and as a counter-cation in the vacuole. In addition, the cytosolic calcium concentration is an important second messenger in signal transduction pathways. In fact, cytosolic calcium excess is toxic for plants. In nature, plants succeed to adapt their requirement to the available calcium in the surrounding environment. Calcium is taken up by the roots and transported via the xylem to all parts of the plants, especially the leaves, where it is stored in the apoplast or in intracellular organelles such as the nucleus, vacuole, endoplasmic reticulum and plastids (White and Broadley, 2003). A set of calcium transporters (such as the Ca^{2+} ATPases) keeps the calcium concentration in the cytosol low by actively transporting calcium to the stores. This allows the cell to respond quickly and specifically to external stimuli by calcium channel-mediated release of calcium from these stores into the cytosol. In order for these transient cytosolic calcium-peaks to activate the appropriate pathway, cells have developed specific tools that will be discussed below.

Signal identity maintained by signature of transient cytosolic calcium peaks

Since calcium is involved as second messenger in multiple signaling pathways, the downstream components have to be able to distinguish what caused the transient cytosolic calcium peaks. Each signal appears to result in transient peaks of cytosolic calcium with a signal-specific signature that is characterized by its frequency, amplitude, location and duration (Malhó et al., 1998, McAinsh and Hetherington, 1998, Sanders et al., 1999, Rudd and Franklin-Tong, 2001, Ng and McAinsh, 2003). These signatures take the form of calcium spikes, waves and plateaus (McAinsh and Hetherington, 1998, Sanders et al., 1999). The dynamics of calcium signals is the consequence of calcium buffering (by proteins) in the cytoplasm and of the activity of calcium channels and other transporters

present on the membranes of different calcium stores. Each channel type is unique with respect to activation and deactivation or inactivation, localization, gating properties and sensitivity to other cellular parameters, such as membrane potential or pH (Sanders et al., 1999). Opening of calcium channels itself may lead to membrane depolarization and the subsequent activation of other channels. In the end, appropriate calcium "sensor" or "receptor" proteins are needed to interpret the calcium signature and activate the proper transduction pathways (Figure 1) (Sanders et al., 1999, Luan et al., 2002, White and Broadley, 2003, Bouché et al., 2005).

Calcium Receptors: different types for a variety of signaling pathways

Calcium receptors are proteins that monitor the changes in calcium concentration by binding calcium through specific domains called EF hands (Strynadka and James, 1989). Binding of calcium to the EF hands induces conformational changes of the calcium receptor proteins that either activate them or enhance their interaction with other proteins that are in turn activated or repressed (Travé et al., 1995, Sanders et al., 1999, Luan et al., 2002). Several types of calcium receptors are distinguished based on their structural domains (Reddy and Reddy, 2004). The ones called "sensors", the calmodulins (CaM) and calmodulin-like (CaML), are proteins with typically four EF-hands but no effector domain (McCormack and Braam, 2003). They relay a signal through their calcium-dependent interaction with an effector protein, which consecutively leads to its activation or

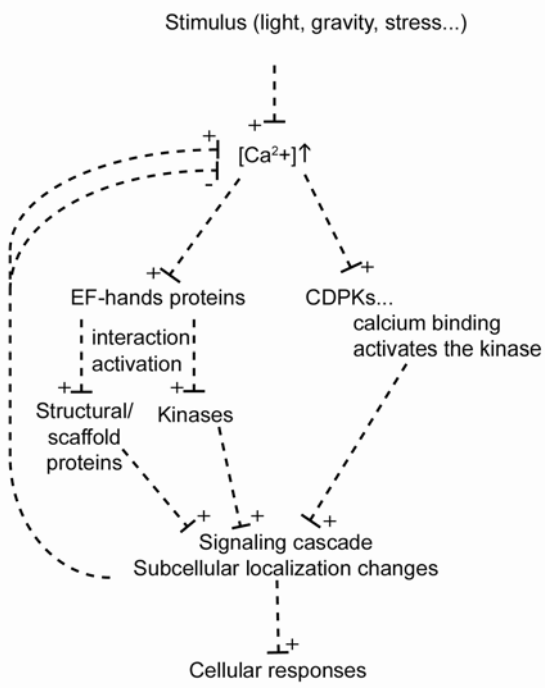

Figure 1. Calcium as second messenger in signal transduction.
Some stimuli (biotic or abiotic, hormone) can induce an increase of the cytoplasmic calcium concentration, which is sensed by at least two types of calcium receptors: the calmodulins and calmodulin-like proteins, or the calcium-dependent protein kinases (CDPKs). In the case of the CDPKs, the calcium signal is directly transmitted to the kinase domain, the activity of which is then enhanced. For the calmodulin(-like) proteins, calcium binding leads to enhanced affinity for other proteins, such as kinases (e.g. the AGCVIII kinases), scaffold proteins (e.g. BT1), or structural proteins (e.g. the kinesin-like calmodulin binding protein KCBP). Activation of the CDPKs, kinases or other calmodulin-binding proteins relays the signal transduction, which gives rise to the appropriate cellular responses, such as bending towards the light source, or activation of defense genes. Additionally, the calcium signal can be enhanced and entertained (spiking, waves) or stopped by a negative feed-back on the calcium channels.

repression (Snedden and Fromm, 2001, Bouché et al., 2005). Downstream targets of the CaMs have non-conserved CaM-Binding domains (Reddy and Reddy, 2004) that are found in a large range of proteins, from enzymes, such as protein kinases, to scaffold proteins (Reddy and Reddy, 2004). Another calcium receptor family is composed of the calcium-dependent protein kinases (CDPK) (Harper et al., 2004). These proteins, called "responders", combine a CaM-like domain with a kinase domain, and binding of calcium directly activates the protein kinase (Cheng et al., 2002). Another important class of calcium sensors is the calcium-dependent ion channels. CaMs, CDPKs and calcium-dependent ion channels have been extensively reviewed before (Luan et al., 2002, McCormack and Braam, 2003, Harper et al., 2004, Reddy and Reddy, 2004). Next to the EF-hand containing CDPKs, the animal protein kinase C (PKC) is also activated by binding calcium, in this case through a C2 domain. Plant homologues of PKCs have not been identified, but instead plants have the related but plant-specific AGCVIII protein kinases (Bögre et al., 2003, Galván-Ampudia and Offringa, 2007). The AGCVIII kinases do not directly bind calcium, but several of these kinases have been linked to calcium signaling through binding with CaM, CaML or CaM-Binding proteins (Baum et al., 1999, Benjamins et al., 2003, Harada et al., 2003, Reddy et al., 2004), and some of them participate in signal transduction pathways that modulate PAT (Friml et al., 2004, Celaya and Liscum, 2005, Santner and Watson, 2006). Below we describe the current knowledge concerning calcium regulation of AGCVIII kinases, and the role it plays in plant development.

Calcium and plant development: the AGCVIII kinases

The AGC family of serine/threonine kinases is named after the cAMP-dependent protein kinase A (PKA), cGMP-dependent protein kinase G (PKG) and calcium-dependent protein kinase C (PKC), animal kinases that are involved in receptor-mediated growth factor signal transduction (Hanks and Hunter, 1995). In plants, the typical PKA, PKG and PKC are not present, but instead a plant specific subfamily of the AGC kinases, AGCVIII, has been identified (Bögre et al., 2003, Galván-Ampudia and Offringa, 2007). The main common feature between animal and plant AGC kinases is their activation by the 3-phosphoinositide-dependent protein kinase1 (PDK1). PDK1 also belongs to the AGC kinase family, and is well-known as a key regulator of other animal AGC kinases (Toker and Newton, 2000, Newton, 2003). PDK1 recruits the kinases at the plasma membrane by binding to a hydrophobic C-terminal domain, called PDK1 Interacting Fragment (PIF), which promotes PDK1-dependent phosphorylation of the AGC kinases in the activation loop. Phosphorylation leads to conformational changes, and PDK1 is released and an auto-phosphorylation in the PIF domain fully matures the kinases, which are then located in the cytosol, ready for activation upon stimulation (Toker and Newton, 2000, Newton, 2003). The majority of the plant-specific AGCVIII kinases are also *in vitro* substrates of PDK1 (Zegzouti et al., 2006), but the *in planta* significance of the PDK1-dependent activation is

still unclear. For the animal PKC, direct calcium binding through the C2 domain induces conformational changes that activates the kinase and targets the protein to the plasma membrane (Shirai and Saito, 2002, Newton, 2003, Halet et al., 2004). The plant-specific AGCVIII kinases have neither a C2 domains, nor EF-hands. Nevertheless some of them were found to be regulated by calcium (Baum et al., 1999, Benjamins et al., 2003, Harada et al., 2003). For three of the AGCVIII kinases, we will further discuss how EF-hand proteins may modulate their activity to translate the calcium signal into a developmental response.

The PINOID kinase links calcium and polar auxin transport

The role of calcium as second messenger in modulating auxin responses and PAT is well established. Evidence that calcium is one of the early signals in auxin response came from experiments on wheat protoplasts (Shishova and Lindberg, 2004), maize coleoptile cells (Felle, 1988, Gehring et al., 1990a), parsley cells (Gehring et al., 1990a) and maize and pea roots (Gehring et al., 1990a). A rapid increase in the cytosolic calcium concentration was detected within minutes after auxin application using calcium fluorescent dyes or ion-sensitive electrodes. It was also found that auxin transport is suppressed by application of the calcium chelator EDTA, and restored after application of a calcium solution (Dela Fuente and Leopold, 1973). The finding that the plant AGC kinase PID, involved in the regulation of the PAT, interacts with two calcium-binding proteins, TCH3 and PBP1, in a calcium-dependent manner provided the first molecular link between calcium signaling and PAT (Benjamins et al., 2003). TCH3 is a CaML protein with six EF-hand domains encoded by a touch-responsive gene (Braam and Davis, 1990, Sistrunk et al., 1994). PBP1 is a small protein of the single EF-hand family (Benjamins et al., 2003, Reddy et al., 2004). Neither of the calcium binding proteins is a phospho-target of PID, but instead they regulate PID kinase activity (Benjamins et al., 2003). TCH3 is a negative regulator of PID activity, whereas PBP1 positively regulates the kinase in *in vitro* phosphorylation assays (Benjamins et al., 2003). PID groups to the AGC3 clade of the Arabidopsis AGCVIII kinases, together with three other kinases, WAG1, WAG2 and AGC3-4 (Galván-Ampudia and Offringa, 2007). Functional analysis of WAG1 and WAG2 has indicated that these kinases play a role in root growth. Roots of *wag1 wag2* loss-of-function seedlings show enhanced sensitivity to the PAT inhibitor NPA, and, like PID, the WAG kinases are membrane-associated, suggesting that they may also be involved in the regulation of PIN polar targeting (Galván-Ampudia and Offringa, 2007). Interestingly, similar to *PID* overexpression, overexpression of *WAG2* in Arabidopsis results in agravitropic seedling growth and primary root meristem collapse (C. Galván-Ampudia, unpublished results). It will therefore be interesting to test whether the calcium-dependent regulation by TCH3 and PBP1, as observed for PID, is conserved for the other AGC3 kinases.

Calcium regulation of the trichome development through the KIC-KCBP complex

There is a striking analogy between the components involved in PID signaling and that of another AGCVIII kinase, the kinesin-like calmodulin-binding protein (KCBP) interacting protein kinase (KIPK). KIPK does not classify to the PID clade, but to the largest AGCVIII clade AGC1 (Galván-Ampudia and Offringa, 2007), and interacts with, but does not phosphorylate KBCP (Day et al., 2000). KCBP is a microtubule (MT) motor protein involved in trichome morphology by regulating branching and polar growth (Reddy and Day, 2000, Reddy et al., 2004). In the presence of calcium, KCBP interacts with AtCaM2, and with a small single EF-hand protein, KCBP-interacting calcium binding protein (KIC) (Reddy et al., 2004). Binding of KIC dissociates KCBP from the MT and inhibits its motor activity (Kao et al., 2000, Reddy et al., 2004). *In vivo KIC* overexpression phenocopies *kcbp* loss-of-function mutants and affects trichome morphology, supporting the *in vitro* observation of the negative regulatory function of KIC on KCBP activity (Reddy et al., 2004). The role of KIPK in this pathway is not clear. KIPK may phosphorylate the other KCBP-associated proteins KIC or CaM2. Another option is that KCBP is involved in the subcellular targeting of KIPK. The relationship between KIC, CaM2 and KIPK has not been elucidated yet. Interestingly, KIC belongs to the same protein family as PBP1 (Reddy et al., 2004), and CaM2 is a close homologue of TCH3 (McCormack and Braam, 2003). Taking into account the role of PBP1 and TCH3 on PID kinase activity, KIC and CaM2 may also regulate KIPK kinase activity, suggesting a conserved function of the EF-hand proteins and a conserved regulation of the AGCVIII kinases by calcium.

Light-induced calcium spikes depend on PHOTOTROPIN activity

Light is an important environmental factor required for plant growth and development. To guarantee optimal positioning of the main photosynthetic organs of a plant, the leaves, toward the light source, plants have developed several photoreceptor-dependent mechanisms to orient plant growth, one of which is phototropic growth. Phototropism is a blue light-induced differential growth response resulting in bending of a plant organ in the direction or away of a unidirectional light stimulus (Liscum, 2002). The light receptors involved in phototropism are the phototropins, which are named PHOTOTROPIN1 (PHOT1) and PHOT2 in Arabidopsis (Briggs and Christie, 2002). The PHOT proteins consist of a kinase domain, based on which they classify to the AGCVIII kinase family, and a photoreceptor domain containing two LOV domains that bind the chromophore flavin mononucleotide (FMN) (Liscum and Briggs, 1995, Huala et al., 1997). Based on the current understanding PHOT1 induces phototropic growth in response to unidirectional low intensity light (Briggs and Christie, 2002). In dark conditions, PHOT1 is a membrane-associated protein, and the LOV domains are believed to repress the PHOT kinase activity. Light stimulation alters the redox state of the FMN, which is sensed by the LOV domains and results in a conformational change that releases the LOV-dependent repression on the

kinase domain (Christie et al., 1998). Upon autophosphorylation, PHOT1 moves to the cytoplasm where it triggers an unknown signaling cascade that leads to differential growth between the lit and shaded side of the plant (Gallagher et al., 1988, Reymond et al., 1992a, Reymond et al., 1992b, Sakamoto and Briggs, 2002). Unfortunately, up to now, besides the autophosphorylation sites, no other PHOT1 phosphorylation targets are known. An early event in the transduction cascade is a PHOT1-dependent calcium release from the apoplast through plasma membrane channels (Baum et al., 1999, Harada et al., 2003). This light-induced peak in cytosolic calcium has been observed in cells at the shaded side of maize coleoptiles where cells show increased elongation, resulting in the coleoptile bending in response to unidirectional light (Gehring et al., 1990b). This is seemingly in contrast with the action of PHOTs, which, as light receptors, are expected to act at the lit side. This suggests that the light-dependent activation of PHOT leads to a mobile signal that induces calcium peaks in cells at the shaded side. Furthermore, in monocotyledons calcium peaks are not immediate, and only start after 5 minutes and reach their maximal level around 15 minutes, whereas in dicotyledonous plants, increase of calcium is observed within seconds after the light stimulus (Harada and Shimazaki, 2007). The meaning of the involvement of calcium in the phototropic response remains to be elucidated. An interesting possibility is that PHOT1, as initiator of a phototropic signaling cascade, modulates the activity of other AGCVIII kinases through their calcium-induced interaction with TCH3 and PBP1 in cells at the shaded side.

Scaffold proteins in plant development

Scaffold proteins are important for bringing proteins together during signal transduction, and are characterized by the presence of multiple protein-protein interaction domains. The 14-3-3 proteins are well known for their role of multi-functional scaffold proteins. Dimerized 14-3-3 proteins interact with phosphorylated proteins and allow transduction of the signals (Sehnke et al., 2002, Ferl, 2004, Bridges and Moorhead, 2005). BTB domain proteins are also typical scaffold proteins. BTB domain proteins have been first described in *Drosophila melanogaster* as scaffolds in transcription factor complexes, are found in many eukaryotes including mammals and plants and are involved in various cellular processes (Bardwell and Treisman, 1994, Albagli et al., 1995, Stogios et al., 2005, Gingerich et al., 2005, Gingerich et al., 2007). In Arabidopsis, eighty BTB proteins have been classified in ten subfamilies depending on other domains present (Gingerich et al., 2005). Up to now, only few of these proteins have been functionally characterized.

Studies in both plants and in animals indicated that several families of BTB proteins serve as a substrate adapter protein in CULLIN3 (CUL3)-containing E3 ligase complexes (Krek, 2003, Xu et al., 2003, Pintard et al., 2004, Moon et al., 2004, Wang et al.,

2004, Gingerich et al., 2005, Dieterle et al., 2005, Weber et al., 2005, Figueroa et al., 2005). Their BTB domain interacts with CUL3 whereas a second protein-protein interaction motif, such as MATH or NPH3, is used to specifically recruit target proteins for ubiquitination and subsequent degradation by the 26S proteasome. Both in animals and in plants, the BTB-MATH protein family has been well characterized for the identification of the target proteins (Xu et al., 2003, Pintard et al., 2004, Dieterle et al., 2005, Figueroa et al., 2005, Gingerich et al., 2005, Weber et al., 2005).

The BTB domain proteins in PHOT signaling

BTB proteins have been reported to be involved in the PID and the PHOT pathways as scaffold and regulator proteins. Two BTB domain proteins of the same family, NON PHOTOTROPIC HYPOCOTYL3 (NPH3) and ROOT PHOTOTROPISM2 (RPT2), interact with PHOT1 (Motchoulski and Liscum, 1999, Sakai et al., 2000). The LOV domains of PHOT1 bind to the coiled-coil domain of NPH3 and with the BTB domain of RPT2, whereas the two BTB proteins homo- and heterodimerize with their respective BTB domain (Motchoulski and Liscum, 1999, Inada et al., 2004). Genetic analyses suggest that both BTB proteins are required for the phototropic response, with *nph3* resembling *phot1* and *rpt2* phenocopying *phot2*, indicating that they are downstream components of the PHOT1 and the PHOT2 signaling pathways, respectively. They are both associated with the plasma membrane where they may be part of a PHOT complex in darkness (Motchoulski and Liscum, 1999, Inada et al., 2004, Celaya and Liscum, 2005). Recent data indicate that NPH3 is phosphorylated in the dark, and that its dephosphorylation in the light is dependent on PHOT1 (Pedmale and Liscum, 2007). The two BTB proteins are proposed to act as modular scaffold proteins bringing together in a dynamic signaling complex comprising PHOT1, or PHOT2, and the early actors of the phototropic response, such as phosphatases and kinases (Liscum, 2002). The fact that the BTB domain of NPH3 may interact with CUL3 suggests that NPH3-dependent recruitment of proteins for degradation by the 26S proteasome is a crucial part of the phototropic response (Pedmale and Liscum, 2007).

BTB proteins in PID signaling

Two BTB proteins have been reported to act in or in parallel to the PID signaling pathway. Firstly, the PINOID BINDING PROTEIN2 (PBP2), initially identified as CaM binding protein BT1, for BTB and TAZ domain1 (Du and Poovaiah, 2004), was identified as interactor of PID (Benjamins, 2004). PBP2/BT1 is part of the land plant specific *Arabidopsis thaliana* BT protein family which includes four other members (Du and Poovaiah, 2004). Interaction studies and functional analyses of the BT protein family suggest that these proteins may be scaffold proteins that provide cross-talk between various signaling pathways (Du and Poovaiah, 2004, Kemel Zago, 2006, Ren et al., 2007).

Interestingly, BT2 is involved in the auxin-induced enhancement of telomerase activity, and *BT2* overexpression lines show reduced auxin sensitivity (Ren et al., 2007).

A second BTB protein of the NPH3-like family, MACCHI-BOU4/ENHANCER OF PINOID/NAKED PINS IN YUC MUTANTS1 (MAB4/ENP/NPY1), has recently been implied in or parallel to the PID signaling pathway. *mab4/npy1* loss-of-function mutants show auxin transport-related phenotypes, such as aberrant cotyledon numbers and deviations in floral organ patterning (Treml et al., 2005, Cheng et al., 2007, Furutani et al., 2007). Genetic analysis of *mab4* indicates that MAB4 is involved in organogenesis synergistically with PID by controlling PIN1 localization in embryo and inflorescence meristems (Furutani et al., 2007).

Calcium and BTB proteins regulate development through the AGC kinases

From the previous part we conclude that the PHOTs and the AGC3 kinases are key regulators of auxin-dependent growth and patterning, and that the signal transduction by both types of AGCVIII kinases involves BTB proteins and calcium. Below we discuss the possible role of these two components based on different examples of auxin-controlled plant development.

Organogenesis

Auxin transport is an important regulator for the positioning and the emergence of a newly formed organ. It generates auxin maxima that precede the occurence of the primordial of future lateral organs, such as lateral roots, leaves and flowers (Benková et al., 2003, Reinhardt et al., 2003, Heisler et al., 2005). Mutants impaired in auxin transport and signaling, such as *pin1*, *pid* and *monopteros* are defective in the formation and positioning of lateral organs at the inflorescence meristem (Okada et al., 1991, Bennett et al., 1996, Hardtke and Berleth, 1998). PID regulates auxin transport by directing the subcellular localization of the PIN auxin-efflux transporters proteins (Friml et al., 2004). And the PID kinase activity during lateral organ formation is crucial for the formation of proper inflorescence in Arabidopsis (Reinhardt et al., 2003). The scaffold protein MAB4/NPY1 undoubtedly acts synergistically with PID-dependent signal transduction. But its role in PID signaling is unclear. Also for BT1 and cytosolic calcium, a role in organogenesis is still elusive (Kemel Zago, 2006, Cheng et al., 2007, Furutani et al., 2007). Interestingly, organ formation is responsive to touch (Jaffe, 1973, Braam, 2005), and we could speculate on the involvement of TCH3 in regulating polar auxin transport by repressing PID-dependent phospho-signal to the PIN proteins (Benjamins et al., 2003). In addition, BT1 has a CaM-binding domain, but it is not known whether TCH3 or PBP1 can interact with it (Du and Poovaiah, 2004). The exact function of this CaM-binding to BT proteins is not known, but

a study on the regulation of telomerase by BT2 suggests that BT proteins become activated by increase cytosolic calcium (Ren et al., 2007).

Phototropism

Phototropic response induces a differential cellular expansion between the lit side and the shaded side, causing a phototropic stem bending towards the unidirectional light source. The unequal growth response appeared to be triggered by an auxin gradient across the stem. This differential auxin distribution between the lit and shaded sides was initially vizualized using the auxin responsive reporters in etiolated seedlings (Li et al., 1991, Friml et al., 2002), and was recently demonstrated by auxin measurements in etiolated Brassica seedlings (Esmon et al., 2006). This auxin gradient requires lateral movement of active auxin, as observed in maize, Arabidopsis and Brassica seedlings (Briggs et al., 1957, Briggs, 1963, Baskin et al., 1986, Friml et al., 2002, Esmon et al., 2006). As discussed above, the direction of auxin transport is determined by the asymmetric placement of the PIN auxin efflux carriers at the plasma membrane (Wisniewska et al., 2006). *pin3* loss-of-function mutants are partially defective in phototropic responses and PIN3 proteins show lateral localization in the hypocotyl (Friml et al., 2002), making PIN3 an excellent candidate for driving a lateral auxin distribution to the shaded hypocotyl side. The current hypothesis is that the blue light-induced PHOT1 signal transduction leads to a change in PIN3 localization, which induces lateral auxin transport to the dark side of the hypocotyl. This implicates that a PHOT1-activated signaling is directly involved in the regulation of the PIN localization. Since PID and possibly the other AGC3 kinases are determinants of PIN polar targeting, it may be interesting to test their involvement in the phototropic growth response. One interesting possibility is that the PHOT1-induced calcium peak that regulate the activity of the kinases via their interaction with TCH3 and PBP1 (Galván-Ampudia and Offringa, 2007). On the other hand, the BTB protein NPH3 is crucial for the lateral redistribution of auxin, since in mutants of the *NPH3* rice homologue *COLEOPTILE PHOTOTROPISM1*, auxin is not found in the shaded side after blue-light stimulation and coleoptile bending is not observed (Haga et al., 2005). It is interesting to note that NPH3 is a phospho-protein, and that blue light induces PHOT1-dependent dephosphorylation (Pedmale and Liscum, 2007). Again, the AGC3 kinases may play a role in keeping NPH3 in the phosphorylated state that interacts with PHOT1, while repression of kinase activity followed by dephosphorylation of NPH3 may lead to its release to the cytosol, where it will be able to initiate changes in auxin transport.

Root growth: gravi- and thigmotropism

Another tropic response is the root response to gravity reorientation, called gravitropism. Gravity is sensed by specialized cellular organs named statoliths, derived from plastids and filled with starch granules. Evidence of their involvement in the gravitropism comes from

starchless mutants. The columella cells, located at the root tip, are the sensors of the gravity vector in the root and responsible for the reorientation of the root growth upon alterations in this gravity vector (Blancaflor et al., 1998). In the shoot the statoliths are located in the endodermis, and some shoot agravitropic mutants identified indeed lack the endodermis. Dynamic auxin movement was observed using auxin responsive *DR5* promoter-reporter constructs in gravi-stimulated roots (Friml et al., 2002, Ottenschlager et al., 2003, Paciorek et al., 2005, Swarup et al., 2005). Moreover, several loss-of-function mutants with altered in auxin transport in roots display agravitropic phenotypes, confirming a role for auxin transport in the root growth response to gravity changes (Bennett et al., 1996, Benjamins et al., 2001, Friml et al., 2002). The PIN3 auxin efflux transporter randomly localizes at the plasma membrane in the root columella initials, but rapidly relocates to the new bottom side of the cells upon changes in the gravity vector, thereby creating a lateral auxin gradient between the lower and the upper side of the root (Friml et al., 2002). Consequently, the auxin-induced differential cell elongation between the two sides results in the root reorientation toward the gravity vector (Ottenschlager et al., 2003). Calcium was found to be involved in and to enhance the auxin transport in gravi-stimulated roots (Lee and Evans, 1985, Plieth and Trewavas, 2002). Several studies suggest a correlated polar mouvement of calcium and of auxin in gravi-stimulated roots (Lee and Evans, 1985 and references within). Moreover gravitropic stimulation of Arabidopsis seedlings induces a specific calcium signature in cells of the root (Plieth and Trewavas, 2002). However up to now, no BTB proteins or kinases have been identified that are clearly involved in PIN3 re-localization, which is the primary cue for gravitropic root growth. In fact, the polarity of PIN3 does not seem to be sensitive to *PID* overexpression (Friml et al., 2004). And the complete agravitropy of *PID* overexpression seedlings can be attributed to the apicalization of the other three PIN proteins expressed in the root tip.

During growth, roots are constitutively submitted to environmental cues such as gravity and contact with the soil. As a consequence, root growth follows a wavy pattern, as a result of concomitant circumnutation (natural rotating movement of the root during growth) and gravitropic and thigmotropic (touch) stimulation. As it is unlikely that PID is involved in triggering gravitropic responses, the other two kinases of the AGC3 clade, WAG1 and WAG2, which have been implied in the regulation of root waving (Santner and Watson, 2006), are probably involved in re-directing PIN3 localization during gravitropic and thigmotropic root growth. It will be interesting to test whether the interaction of TCH3 and PBP1 with the WAG kinases is conserved, since they could provide the missing link between gravitropism-induced increases in cytosolic calcium and re-orientation of auxin transport.

Thesis outline

The function of the PID protein kinase in auxin transport has been largely clarified by the demonstration of its activity on the polar targeting of PIN auxin efflux carriers (Friml et al., 2004), and by the identification of the large central hydrophilic loop of PIN proteins as its likely phosphorylation substrates (Michniewicz et al., 2007). However, importamt questions that remain are how PID activity and subcellular localization are regulated and what could be the *in planta* significance of the PID-dependent PIN phosphorylation. Some of the answers come from the isolation of calcium-binding proteins and BTB domain proteins as PID-binding partners (Benjamins et al., 2003, Benjamins, 2004). PID BINDING PROTEIN1 (PBP1) and TOUCH3 (TCH3) are calcium-dependent regulators of PID kinase activity *in vitro*, and the fact that the *PID* overexpression phenotype is enhanced by calcium channel and calmodulin inhibitors suggests that these PBPs link calcium signaling and auxin transport regulation by the PID kinase (Benjamins et al., 2003).

Chapter 2 and **Chapter 3** describe the further study on the role of TCH3 and PBP1 in Arabidopsis. Alterations in the expression of these genes in the *pid-14* loss-of-function mutant confirmed the function of a negative regulator, for TCH3, and positive regulators, for PBP1 and its close homologue PBP1H, of the PID kinase activity (Chapters 2 and 3). Further analysis in Chapter 3 indicated that PBP1 and PBP1H function redundantly.

Besides regulating the enzymatic PID activity, calcium was also implied in determining the subcellular localization of PID (Chapter 2). In epidermal cells of the root tip PID is mainly plasma membrane-associated (Michniewicz et al., 2007). Upon exogenous auxin application, however, PID was found to be rapidly and transiently released into the cytoplasm (Chapter 2). Inhibitor studies indicated the involvement of calcium channels and CaMs in PID sequestration, and suggested that this was mediated by binding to CaM or CaMLs, such as TCH3, due to auxin-increased cytoplasmic calcium concentrations (Dela Fuente and Leopold, 1973, Felle, 1988, Gehring et al., 1990a, Shishova and Lindberg, 2004) (Chapter 2). On the other hand, *PBP1* overexpression partially stabilized PID at the plasma membrane of root epidermis cells (Chapter 3). In conclusion, auxin seems to regulate both the subcellular localization and the enzymatic activity of the PID kinase through the action of calcium and calcium binding proteins.

The BTB domain protein PBP2/BT1 has been previously identified as a third PID-binding partner (Benjamins, 2004). Here we further analyzed the function of this protein. **Chapter 4** first describes a detailed functional analysis of the Arabidopsis family of land plant-specific BT proteins, which comprises five members (Du and Poovaiah, 2004). Interestingly, BT1 is an unstable protein and a target for degradation by the 26S proteasome. However, reports concerning its interaction with CUL3 are contradictory (Gingerich et al., 2005, Dieterle et al., 2005, Weber et al., 2005, Figueroa et al., 2005).

Genetic and expression analyses showed that the five BT proteins are functionally redundant and essential for female gametophyte development.

In **Chapter 5** we analyze in more detail the function of BT1 and its family members in the PID pathway. The results indicate that the BT proteins bind to PID through their BTB domain, and have a negative regulatory function on PID kinase activity *in vitro*. The instability of the protein may be necessary to fine tune this effect of BT1 on PID activity *in vivo*. For at least four of the five BT proteins we were able to show that they interact with PID, suggesting that all BT act in PID signaling. Interestingly, the absence of BT function rescued the *PID* overexpression seedlings phenotypes, suggesting that BT proteins are crucial regulatory components for PID function. Co-expression studies in Arabidopsis protoplasts indicate a yet unrevealed function for PID in the nucleus, and suggest that the BT proteins function by regulating the subcellular localization of the kinase.

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