

**Master Thesis
Jasper de Jong**

**Cancer Genomics & Developmental Biology
Utrecht University**

Cell Polarity: A comparison of animal and plant systems

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Introduction

All living organisms, from 'simple' unicellular prokaryotes to plants and to mammals, show different degrees of spatial organization. In most multicellular organisms, this spatial organization is easily visible. This organization requires a sense of orientation: what is front and what is back, what is head and what is tail? All organisms, at some point, show a form of polarity: the distinction between two sides of the organism. This polarity can be observed at the level of the whole organism, but individual tissues also show polar organization. Even at the cellular and molecular level this polarity can be observed. The epithelial cells lining our gut are a great example of polar organization within individual cells. The apical side facing the lumen has a completely different membrane organization containing protruding microvilli and has a different function than the basal side of the cell, which has a completely different structural organization. This structural and functional distinction within a cell requires polar organization of cellular components. The polar orientation of cellular components enables a cell to have distinct structural and functional domains. Cell polarity, in turn, relies on the polarized distribution of molecules brought about by molecular polarity. Microtubules, for example, show polarity at the molecular level. Both ends have different characteristics resulting in a specified orientation. Such molecular intrinsic polarity contributes to polarity at the cellular level as will be described in the following chapter.

This form of organization is of great importance during the development of organisms. It is a process that has intrigued researchers for decades. Some aspects of the underlying mechanisms have been identified. Intensive investigation of factors that are involved in the initiation and maintenance of polarity has resulted in some explanatory models, but these are still incomplete. The use of evolutionary distinct model systems allows for a comparison between the basic mechanisms that are employed to obtain polarity. In this thesis I will draw comparisons

between the plant *Arabidopsis thaliana* and the nematode *Caenorhabditis elegans* and to some extent the fly *Drosophila melanogaster*. What do these organisms share in the sense of polarity establishment? What are the main differences and how can this specific knowledge contribute to the overall understanding of the phenomenon of cellular polarity?

Using examples of cellular polarity in these different organisms, I will try to elucidate some of the similarities and differences. This may provide some new ideas of how knowledge of different organisms can lead to new insights of a common phenomenon: cellular polarity.

Building Blocks of Polarity

Polarity at the cellular level can be considered the coordinated orientation of cellular components within a cell and on its plasma membrane, providing different functional domains. This distribution requires strictly regulated transport of different components to the sites where they are needed. The main tool for this intracellular distribution is the cytoskeleton, a structural network consisting of actin filaments and microtubules. The cytoskeleton plays a central role in a number of functions, among which are the establishment of structural properties in different states of the cell (e.g. motility in migrating cells) and the organized distribution of cellular components. This distribution also concerns vesicles that can carry different cargoes. Also, the family of small GTPases should be mentioned as critical players in the polar transport of cellular components. The transport of cellular components, possibly in vesicles aided by small GTPases, via the cytoskeleton forms the fundament of the establishment and maintenance of cellular polarity.

Actin

Actin filaments are polymers composed of globular actin (G-actin) that has the ability to bind and hydrolyse ATP (reviewed by Pollard, 1986). In a dynamic manner, G-actin subunits form polymeric filaments (F-actin) (Fig. 1A). This formation of filaments is preceded by the

formation of small oligomers that have the ability to rapidly elongate, in a process called nucleation (Pollard, 1986). The head-to-tail organization of G-actin subunits provides intrinsic polarity to actin filaments. The two opposite ends of the actin filaments are structurally different. One end contains ADP-bound G-actin (pointed ends) whereas on the other ends consists of ATP-bound G-actin (barbed end) (Fig. 1A) (reviewed by Li and Gundersen, 2008). Both ends display different association and dissociation rates, making the actin filaments dynamic entities.

Although plant cells differ in a number of ways from animal cells, their cytoskeleton shows great structural similarity. Actin filaments possess a similar composition comparable dynamics in plants and animals. After nucleation, ATP-bound actin dimers and trimers are assembled onto the growing barbed ends of the actin filaments. On the opposite pointed end, ADP-actin subunits dissociate (reviewed by Staiger and Blanchoin, 2006).

Several proteins are associated with the regulation of dynamics of actin filaments. One important protein complex is the Arp2/3 complex. This was the first actin assembly factor to be identified and consists of ARPC1-5, Arp2 and Arp3 (actin-related proteins) (reviewed by Goley and Welch, 2006). The ARPC1-5 complex can bind to the side of actin filaments and the Arp2/3 dimer serves as a site of nucleation leading to the formation of branched actin filament structures (Goley and Welch, 2006).

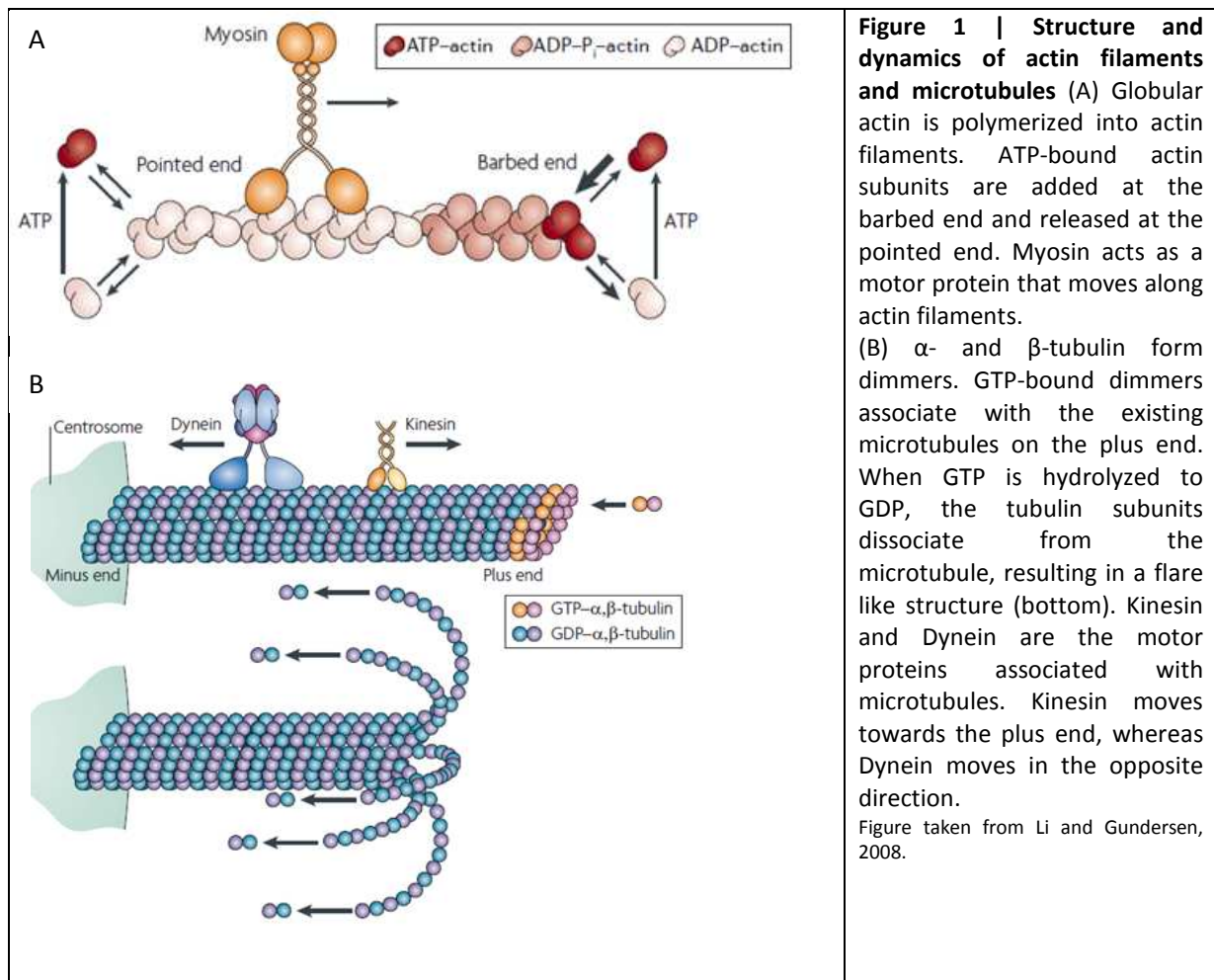
Actin filaments can also be elongated at the growing end. This process is aided by proteins that bind actin and provide nucleation sites (Chhabra and Higgs, 2007). Formin, for example, binds actin filaments and provides a nucleation site at the barbed end. Another nucleation factor called Spir which was first identified in *Drosophila*, can bind actin tetramers that can then able to form a filament (reviewed by Kerkhoff, 2006). Both Formin and Spir creates linear filaments, as opposed to the branched filaments of Arp2/3. In addition to actin polymerization, depolymerization also plays an important role in the maintenance of proper actin dynamics. On the pointed ends actin subunits are

depolymerized and they are added to the barbed end. This phenomenon is called actin treadmilling (reviewed by Cleveland, 1982). Strict regulation of polymerization and depolymerization is of great importance for proper actin turnover dynamics. When turnover needs to be accelerated, depolymerization needs to be enhanced. This is done by a number of protein families (reviewed by Ono, 2007). An increase of depolymerization can be accomplished either by accelerating the rate at which subunits are released, or by severing actin filaments to increase the number of pointed ends available for depolymerization. One family of actin severing proteins is that of Gelsolin. Gelsolin binds to the sides of actin filaments where it can induce a cut and it can bind to the plus ends of filaments blocking the access of nucleation factors (Ono, 2007). In this way an extra minus end is made and the plus end is prohibited from elongating, resulting in an increase of depolymerization. The ADF (actin depolymerizing factor)/cofilin family provides another means of actin severing and depolymerization. ADF/cofilins have the ability to sever actin filaments, but also depolymerize F-actin into G-actin subunits. Additionally, actin interacting protein 1 (AIP1) promotes actin filament disassembly in cooperation with ADF/cofilin and has been shown to be essential in *Arabidopsis* development (Ketelaar *et al.*, 2004). In plants, homologs of actin depolymerizing and severing proteins have been found.

Gelsolin-like proteins have been shown to be present in a number of plant species, but not in *Arabidopsis*. However, twelve ADF-cofilin genes have been predicted in *Arabidopsis*, whereas mammals for example have only one ADF (destrin) and two cofilins (Ono, 2007). These are just some examples of proteins that regulate actin dynamics. In general, the actin dynamics in plants and animals are very much alike. Homologous proteins in plants and animals regulate the process of actin dynamics in similar ways. But does this also account for microtubules?

Microtubules

Microtubules (MTs) are built from α - and β -tubulin heterodimers (Fig. 1B). These subunits



can bind and hydrolyze GTP. The polarity within these subunits results in polar polymeric microtubules with two structurally distinct ends, like actin, due to a head-to-tail association of the subunits. GTP is hydrolyzed by tubulin shortly after assembly into the microtubule. This results in a so called GTP cap on the growing plus end. The shrinking minus end, consisting of GDP-bound tubulin, often is connected to the centrosome or the microtubule organizing center (MTOC). GTP- and GDP-tubulin have different dissociation rates due to their structural differences. This difference provides the dynamic properties of microtubules.

The regulation of microtubule dynamics is somewhat comparable to that of actin. Microtubules in plant and animal cells show similarities as their subunits and microtubule structure are largely conserved. Despite these structural similarities, the organization shows marked differences in plant cells in

comparison to animals. As opposed to animal cells that have centrosomes, most plant cells lack microtubule organizing centers (Wasteneys, 2002). Plants show differently organized microtubule arrays during different stages of the cell cycle (reviewed by Ambrose and Wasteneys, 2008). Crucial in this organization is contact with the cell cortex. For example, microtubules lose their parallel orientation at the cell cortex upon addition of a phospholipase-D antagonist (1-butanol) in *Arabidopsis* seedlings, resulting in disrupted development of roots and cotyledons (Gardiner *et al.*, 2003). This indicates that the connection to the cortex is important for microtubule organization as phospholipase-D associates both with microtubules and the plasma membrane (Gardiner *et al.*, 2003). Another protein that aids in the attachment of microtubules to the cortex is CLASP. Loss of CLASP leads to partial detachment of cortical microtubules resulting in distortion of

organization (Ambrose and Wasteneys, 2008). In addition, interactions between microtubules, aided by microtubule associating proteins (MAPs), are also of great importance for their organization. MAPs function in polymerization (stabilization) and depolymerization (destabilization) of microtubules (Ambrose and Wasteneys, 2008), but also in connecting individual microtubules to form bundles. MAP65 for example, has been shown to homodimerize upon interaction with microtubules and in this way crosslink parallel and antiparallel microtubules (i.e. in opposite direction) (Gaillard *et al.*, 2008; Van Damme *et al.*, 2004). So even without a clearly defined MTOC, plants are still able to properly organize their microtubules in a variety of ways, depending on the location within the cell and the state of the cell (Ambrose and Wasteneys, 2008).

Motor proteins

As described, the actin and microtubule networks provide different structural functions to cells. The intrinsic polarity of the components provides a means to create polarity on the cellular level. Cellular polarity is the result from the formation of an axis directing internal organization. Along this axis, regulated distribution of structural and functional components defines the orientation of a cell. The cytoskeleton can be considered as the transport route of different types of cellular components. The transportation is carried out by so called motor proteins. These motors have the ability to move along actin filaments or microtubules using the energy of ATP hydrolysis (reviewed by Li and Gundersen, 2008).

The main motor proteins that move along actin filaments are in the family of the Myosins (Fig. 1A) (Li and Gundersen, 2008). Most members move towards the growing barbed ends of the filaments. The different myosin family members display different functionalities. For example, myosin-V is suitable for transport over longer distances as it takes longer before it dissociates from the actin filament than other myosins. Myosin-II plays a role in contractility by facilitating the

sliding of actin filaments (Li and Gundersen, 2008).

The motors that move along microtubules are kinesins and dynein. They move in opposite directions. Most kinesins move towards the growing plus ends and dynein towards the minus end, providing the possibility for two-way trafficking (Fig. 1B) (Li and Gundersen, 2008). The motor proteins have the ability to transport individual proteins, vesicles and even whole organelles along the cytoskeleton, facilitating the internal organization of cells in response to polarity regulating signals. Vesicles are used for transport of molecules within a cell. The interaction of these vesicles with the polarized cytoskeleton plays a key role in the distribution pattern of molecules that play a role in cellular polarization.

Vesicle trafficking

If the cytoskeleton is polarized, vesicles can be directed to specific domains in the plasma membrane. In this way membrane associated proteins can be distributed to specific sites where they are required, providing functional differences within the plasma membrane. A very clear example of the differential organization of membrane domains within one cell can be observed in the earlier mentioned epithelial cells lining the intestinal wall in which the apical and basal domains of the cell have different structure and function and the protein content in these parts of the membrane is very different.

Small GTPases

Also worth to mention in this introduction to polarity factors is the family of small GTPases, also called the Ras superfamily (reviewed by Charest and Firtel, 2007). This family of proteins has been shown to play a role in a great variety of cellular processes, including those underlying cell polarity. They cycle between an active GTP-bound state and an inactive GDP-bound state. Their activity is regulated by two classes of proteins; guanine exchange factors (GEFs) that control the exchange of GTP for GDP and GTPase-activating proteins (GAPs) that mediate GTPase activity resulting in hydrolysis of GTP to GDP. The superfamily consists of five

subfamilies: Ras, Rho, Rab, Ran and Arf GTPases (Charest and Firtel, 2007). The Rho subfamily consists of the Rho, Rac and Cdc42 subgroups. The Ras subfamily can be divided in Rap, Ral and Rheb GTPases. Members of the superfamily are conserved throughout eukaryotes.

Members of the Rab, Ran and Arf families are mostly involved in vesicle trafficking (Nielsen *et al.*, 2008; Charest and Firtel, 2007), whereas Ras and Rho proteins play important roles in signaling cascades transducing extracellular stimuli (Charest and Firtel, 2007). Among the different functions of Rho GTPases is the regulation of the actin cytoskeleton, which explains its involvement in cell polarity (Charest and Firtel, 2007).

Roles for Rho GTPases in polarity have also been observed in plants. Here, ROP GTPases (Rho of plants), are also involved in the modulation of the actin cytoskeleton (Hussey *et al.*, 2006), which, for example, is required for polar outgrowth of cells (Fu *et al.*, 2005).

Altogether, the basic mechanisms underlying polarity and the components involved seem to be well conserved. Structurally, the actin cytoskeleton is very similar in plants and animals as well as the mechanisms regulating actin dynamics. The organization of plant microtubules shows structural differences when compared to animal systems. However, the different organization in plants still allows for a function in cell polarity comparable to animals.

The cellular components described in this chapter are crucial for cell polarity. However, they are also involved in other cellular processes. What makes that they can be employed specifically to function in establishment and maintenance of polarity when it is required? This would require other regulating factors that are specific to the generation of polarity. A great number of factors have been identified that are required for cell polarity. *C. elegans*, *Drosophila* and *Arabidopsis* are used to elucidate these factors and how they function. In the following chapters, I will describe two examples of cell polarity in these systems to compare the processes responsible for creating polarity within cells.

Cellular polarity in asymmetric divisions: From zygotes to stomata

All living multicellular organisms comprise a varying number of different cell types arranged in structurally and functionally different tissues. Originally, they are derived from a single zygote. The development of this single fertilized egg into a complex highly structured multicellular organism is an intriguing process of which many questions have yet been answered but many more are still left unanswered.

In order to create unequal daughters from any apolar cell, polarization is required. This polarization results in molecularly distinct domains that characterize the daughter cells after division. In *C. elegans*, the initial cell divisions have been studied very intensively. The first cell division generates two unequally sized cells with asymmetrically distributed cell fate determinants, each giving rise to different cell lineages (Horvitz and Sternberg, 1982). In *Arabidopsis thaliana*, a comparable situation is observed in which the fertilized zygote divides into two unequal daughter cells. A comparison of this process in the early development of these evolutionary distant organisms may provide insight into the conservation of functional mechanisms underlying the first steps towards the generation of complex multicellularity.

The PAR proteins

In the 1980's, genes that are involved in the first embryonic division of *C. elegans* were identified in a screen for mutants defective in embryonic development. Initially four *par* genes were identified (partitioning defective) (Kemphues *et al.*, 1988). In wild type *C. elegans*, the first zygotic division results in a larger anterior blastomere (AB) and a smaller posterior blastomere (P_1) (Sulston *et al.*, 1983). In *par-1*, *par-2* and *par-3* mutants these two blastomeres are of equal size (Kemphues *et al.*, 1988). In these three mutants and in the *par-4* mutants, the second cleavage of the P_1 blastomere differs from wild type as the division plane is transversely rather than longitudinally. The cleavage plane in wild type P_1 blastomeres is perpendicular to that of the

AB blastomere resulting from a rotation of the spindle complex, which does not occur in the four *par* mutants (Kemphues *et al.*, 1988). From other similar screens two more *par* genes were identified (*par-5* and *par-6*) (Morton *et al.*, 2002; Watts *et al.*, 1996). Mutations in those two *par* genes also result in equally sized AB and P₁ blastomeres. In addition to the *par* genes, the screen also identified the atypical protein kinase C (PKC-3) to be involved in the first asymmetric division (Tabuse *et al.*, 1998).

Characterization of the PAR proteins has led to a model that explains how they contribute to the generation of polarity of the zygote and its asymmetric division. Localization studies of the PAR proteins and PKC-3 showed a clear asymmetric distribution in the one-cell zygote. PAR-3, PAR-6 and PKC-3 become localized in the anterior periphery (Etemad-Moghadam *et al.*, 1995; Hung and Kemphues, 1999; Tabuse *et al.*, 1998), whereas PAR-1 and PAR-2 are localized in the posterior periphery of the one-cell embryo (Fig. 2A) (Boyd *et al.*, 1996; Guo and Kemphues, 1995). PAR-4 and PAR-5 are localized uniformly throughout the one-cell embryo to the cortex and in the cytoplasm (Morton *et al.*, 2002; Watts *et al.*, 2000). To understand the role of the PAR proteins in the generation of zygotic polarity, their regulators

had to be identified. How do the PAR proteins acquire their polar localization? Localization studies in *par* mutants showed that PAR-3 and PAR-6 require each other for their anterior localization (Etemad-Moghadam *et al.*, 1995; Hung and Kemphues, 1999). PKC-3 colocalizes with PAR-3 and PAR-6 (Tabuse *et al.*, 1998). Another study showed that PAR-1 localization is dependent on *par2* and *par-3* and that PAR-3 localization depends on *par-2* as it restricts PAR-3 from the posterior side (Boyd *et al.*, 1996). PAR-2 does not depend on *par-1* for proper localization (Boyd *et al.*, 1996). Additionally, PAR-5 is required for mutual exclusion of the cortical PAR domains. *par-5* RNAi results in overlap of the PAR proteins that are separated in wild type (Morton *et al.*, 2002). It was also found that reciprocal inhibition plays an important role in establishing two separate PAR-domains (Fig. 2A). PKC-3 phosphorylates PAR-2, limiting its localization to the posterior domain (Hao *et al.*, 2006). PAR-2 on the other hand prohibits posterior localization of PAR-3-PAR-6-PKC-3 through PAR-1 and PAR-5 (Hao *et al.*, 2006). These findings imply that the PAR proteins regulate each other's localization. But what is upstream of the self-regulation of the PAR proteins? What polarizes the polarizers?

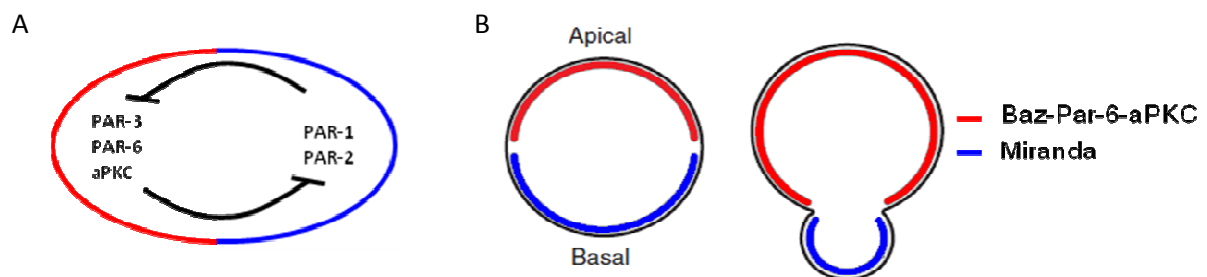


Figure 2 | Polarization of the PAR proteins in the *C. elegans* zygote and *Drosophila* neuroblasts

In both the *C. elegans* zygote (A) and *Drosophila* neuroblasts (B), PAR proteins show polar localization. The anterior and the posterior PAR proteins mutually exclude each other from their domains. In *Drosophila* neuroblasts, the PAR proteins that localize anteriorly in the *C. elegans* zygote are observed apically (Baz is the PAR-3 homolog). Miranda is localized basally and this localization specifies the cell fate of the basal ganglion mother cell. Figure adapted from Prehoda, 2009

CDC-42

An important discovery in linking PAR proteins to polarity was the direct interaction of the mammalian PAR-6 with CDC-42 (Joberty *et al.*, 2000). It was later shown that this interaction also occurs in *C. elegans* (Gotta *et al.*, 2001). Gotta *et al.* also showed that CDC-42 is involved in the establishment of polarity in the one-cell *C. elegans* embryo. *cdc-42* RNAi resulted in the loss of asymmetric localization of the PAR proteins (Gotta *et al.*, 2001). CDC-42 is a Rho GTPase that was first discovered in yeast and is known to play a role in establishing polarity (Adams *et al.*, 1990). Cdc42 has numerous downstream targets and participates in a variety of cellular processes (reviewed in Erickson and Cerione, 2001). But how does it function in the establishment of polarity in the *C. elegans* embryo? Due to their physical interaction, CDC-42 may act through PAR-6 in the establishment of polarity. This idea is supported by work in mammalian cells showing disruption of cell-cell junctions by ectopic expression of PAR-6 or a dominant-negative form of CDC-42 (Gao *et al.*, 2002; Joberty *et al.*, 2000). Additionally, the PAR-PKC-3 complex was shown to be involved in the directed migration of astrocytes, which is triggered by an integrin-CDC-42 signaling pathway (Etienne-Manneville and Hall, 2001). In the *C. elegans* embryo, CDC-42 localizes to the anterior cortex, but does not require the anterior PARs (PAR-3 and PAR-6) to do so (Schonegg and Hyman, 2006). However, Schonegg and Hymann showed that CDC-42

does not properly localize to the anterior cortex upon depletion of myosin II (NMY-2), indicating that actomyosin activity is required for asymmetric distribution of CDC-42. Analysis of the actomyosin network reveals rigorous dynamic changes during the establishment of polarity. After fertilization, cortical cytoplasm moves towards the anterior of the zygote (i.e. away from the sperm entry point), a process called cortical flow (Hird and White, 1993). The flow of the cytoplasm in the interior (cytoplasmic flow) moves in the opposite direction (Hird and White, 1993). The contractile actomyosin network appears to be destabilized near the point of sperm entry, resulting in a flow of cortical NMY-2 and

actin filaments, and of PAR-3, PAR-6 and PKC-3, towards the opposite side of the embryo (Munro *et al.*, 2004). The question remains how exactly the actomyosin network orchestrates this anterior migration of PAR-3, PAR-6 and PKC-3. The actomyosin network could possibly actively anchor those PAR proteins to specific anterior cortical domains or actively exclude them from the posterior cortical domain. Another possibility is that the actomyosin network actively transports PAR-3, PAR-6 and PKC-3 in the anterior direction. Another observation was PAR-2 prohibits recruitment of NMY-2 to the posterior cortex, inhibiting a flow of the anterior PAR proteins back to the posterior cortex (Munro *et al.*, 2004). The anterior PAR proteins are thought to prevent association of PAR-2 with the cortex (Boyd *et al.*, 1996; Etemad-Moghadam *et al.*, 1995). Hao *et al.* (2006) show that aPKC phosphorylates PAR-2, inhibiting its localization to the cortex. This would explain the posterior localization of PAR-2. On the other hand, PAR-2 also excludes the anterior PAR proteins from the posterior cortex (Cuenca *et al.*, 2003). Cuenca *et al.* saw that in embryos depleted of PAR-2 via RNAi, PAR-6 initially shows normal localization to the anterior cortex, but later moves back to the posterior side. This observation contributes to the model in which PAR-3, PAR-6 and aPKC move anteriorly resulting in posterior localization of PAR-2 which prohibits the PAR-3-PAR-6-PKC-3 complex from returning to the posterior side of the embryo. In this way the anterior PAR-complex and the posterior PAR-2 can stay separated. This separation therefore starts with the induction of asymmetric contractions of the actomyosin network, but what causes this asymmetry?

Breaking symmetry

A study in which the entry site of the sperm was altered indicated that the site of sperm entry becomes the posterior side of the embryo (and eventually the adult worm) (Goldstein and Hird, 1996). They also show that a component of the sperm initiates rearrangement of the zygotic cytoplasm. Later experiments showed that the paternal centrosome, or something closely related, is a key component as laser ablation of the

centrosome prevents the establishment of polarity (Cowan and Hyman, 2004). The posterior site of sperm entry is also the site where the acto-myosin network disassembles ((Munro *et al.*, 2004)). How centrosomes might act to generate asymmetry in the actomyosin network is not clear.

Another factor that may act in the generation of the initial asymmetry is the small GTPase RHO-1. RHO-1 is required for the organization of the cortical actomyosin network (Schonegg and Hyman, 2006). Regulation of RHO-1 is carried out by its guanine nucleotide-exchange factor (RhoGEF) ECT-2 (Morita *et al.*, 2005) and the GTPase activating protein (RhoGAP) CYK-4 (Jantsch-Plunger *et al.*, 2000). ECT-2 mediates exchange of the GDP by GTP to activate RHO-1. CYK-4 inactivates RHO-1 signaling by catalyzing GTP-hydrolysis. Before fertilization, both RHO-1 and ECT-2 are located throughout the cortex of the embryo (Motegi and Sugimoto, 2006). After fertilization, both proteins are excluded from the posterior and become enriched anteriorly (Motegi and Sugimoto, 2006). CYK-4 shows a different localization pattern. CYK-4 was found to be enriched in *C. elegans* sperm ((Jenkins *et al.*, 2006)). In embryos depleted from CYK-4 by RNAi, PAR-6 and PAR-3 showed no anterior localization as in wild type, but occupied a much larger area of the cortex, indicating that CYK-4 is required for the proper anterior localization of PAR-3 and PAR-6 (Jenkins *et al.*, 2006).

Analysis of NMY-2 behavior in *cyk-4(RNAi)* embryos suggested that CYK-4 is required to downregulate the actomyosin network posteriorly, inducing asymmetric pulling forces (Jenkins *et al.*, 2006). Thus, the paternally contributed CYK-4 may provide a cue for the initiation of asymmetry after fertilization of the symmetric egg.

But how does the polar organization of the PAR proteins result in asymmetric division into two different daughter cells? To answer this question, it is important to know how the PAR proteins function and what their effectors are. Analysis of the molecular properties of the PAR proteins shows that PAR-1 and PAR-4 are protein kinases (Guo and Kemphues, 1995; Watts *et al.*, 2000). PAR-3 and PAR-6 contain PDZ domains (Etemad-Moghadam *et al.*, 1995;

Hung and Kemphues, 1999) that are involved in protein-protein interactions, providing the possibility for the assembly of protein complexes (reviewed in Hung and Sheng, 2002). PAR-5 is a 14-3-3 protein (Morton *et al.*, 2002). 14-3-3 proteins can bind a number of functionally diverse signaling proteins (reviewed in Fu *et al.*, 2000). PAR-2 contains a zinc-finger domain, implying that it could bind DNA, RNA or other proteins (Leviton *et al.*, 1994). Thus, the PAR proteins are likely to regulate polarity via signaling mechanisms with other proteins. Then what are the downstream targets of the PAR proteins and how are they regulated?

Downstream of the PARs

Regulating cell fate determinants

We now have a model explaining how the initially symmetrically localized PAR proteins can be reorganized and adopt asymmetric positions. But what does this asymmetry of the PAR proteins mean for development? To answer that question downstream effects of the PAR proteins have to be examined. What are the interactors of the PAR proteins, how are they regulated and what are their functions?

One study provides evidence that several PAR proteins are involved in the polarized movement of the cell cortex ((Cheeks *et al.*, 2004)). No or little cortical movement is observed in *par-2*, *par-3*, *par-4* and *par-6* mutants (Cheeks *et al.*, 2004). This cortical flow may provide a means to distribute cellular components unevenly throughout the zygote.

Several proteins have been identified that show asymmetric distribution depending on PAR proteins. Examples of such PAR-dependent proteins are MEX-5 and MEX-6 (Schubert *et al.*, 2000). MEX-5 shows a clear anterior localization in wild type one-cell embryos, but is uniformly distributed in *par-1* and *par-3* mutants (Schubert *et al.*, 2000). Proteins downstream of MEX-5, PIE-1 and MEX-1, appear to be upregulated in the absence of MEX-5 and downregulated upon ectopic MEX-5 expression (Schubert *et al.*, 2000), suggesting downregulation of these

proteins by MEX-5 on the anterior side of the embryo. PIE-1 and MEX-1 are required for the regulation of germ cell fate, segregating with the germ lineage in early embryos that derives from the posterior blastomere to which PIE-1 and MEX-1 are localized (Mello *et al.*, 1992; Mello *et al.*, 1996). In addition, MEX-5 and MEX-6 were shown to stabilize MEX-3 in the anterior (Huang *et al.*, 2002). MEX-3 inhibits the translation of *pal-1* mRNA which is required for specification of the posterior blastomeres (Waring and Kenyon, 1990). *Pal-1* is translated in the 4-cell stage (Hunter and Kenyon, 1996). This 'MEX-system' provides an example of how PAR polarity can be translated into functional polarity (i.e. polar distribution of fate determinants).

PARs and the mitotic spindle

Another important step in generating asymmetry lies in the spindle orientation. Visualization of the mitotic spindle shows asymmetric positioning of the mitotic spindle prior to the first cell division (Albertson, 1984). The forces that act on the posterior spindle pole are stronger than those acting on the anterior pole (Grill *et al.*, 2001). This unequal distribution of pulling forces leads to the generation of a larger anterior daughter (the AB blastomere) and a smaller posterior P₁ blastomere. A role for *par-2* and *par-3* in the regulation of pulling forces was shown ((Grill *et al.*, 2001)) and it had earlier been proposed that PAR-3 may anchor or stabilize microtubules (Etemad-Moghadam *et al.*, 1995). One effector in this function of the PAR proteins is LET-99 (Wu and Rose, 2007). The anterior PAR-3 and the posterior PAR-1 both inhibit localization of LET-99, creating a central band of LET-99 (Wu and Rose, 2007). LET-99 has been shown to play a role in the organization of the mitotic spindle (Rose and Kemphues, 1998). This role of the PAR proteins in spindle positioning shows how the asymmetric localization of the PAR proteins can contribute to development.

The PARs in Drosophila

The role that the PAR proteins play in polarity is observed in more organisms than just *C. elegans*. The PAR system is also observed and

investigated in mammals, *Xenopus* and *Drosophila*. In *Drosophila*, its functioning has been studied mainly in epithelial cells and asymmetrically dividing neuroblasts (reviewed by Prehoda, 2009). *Drosophila* embryonic neuroblasts delaminate from the neuroectoderm in which they already display an apical-basal polarity (Prehoda, 2009). They undergo an asymmetric division resulting in an apical neuroblast and a smaller ganglion mother cell (GMC) (Fig. 2B) (Prehoda, 2009). These two cell types are characterized by (the presence or absence of) a subset of proteins that have been distributed asymmetrically (Prehoda, 2009). The protein Miranda (Mira) plays a central role in this unequal distribution. It is located on the basal side (Fig. 2B) where it binds three asymmetrically distributed proteins (Pros, Staufen and Brat) (reviewed in Gonczy, 2008), resulting in the segregation of these factors specifically with the basal GMC. The basal localization of Mira depends on the apically localized Par-3-Par-6-aPKC complex (Gonczy, 2008). Association of the Lethal giant larvae (Lgl) protein with Par-6 and aPKC results in its inhibition via phosphorylation that promotes dissociation of Lgl from the apical cortex (Betschinger *et al.*, 2005). Active Lgl is required to recruit Mira to the basal cortex, explaining that Mira is restricted to the basal side of neuroblasts (Betschinger *et al.*, 2003). However, Lgl was later found to be a potent inhibitor of aPKC activity (Lee *et al.*, 2006). This finding, combined with later experiments showing that aPKC directly phosphorylates Mira (Atwood and Prehoda, 2009), implies that the phenotype (ubiquitous Mira expression) observed in the presence of a non-phosphorylatable Lgl (Betschinger *et al.*, 2003) may result from the loss of aPKC activity. Hence, aPKC seems to directly prohibit Mira from localizing apically. The following question then is how is aPKC restricted to the apical side?

In *C. elegans* zygotes, we see that PAR-3 and PAR-6 are interdependent for their anterior localization (Etemad-Moghadam *et al.*, 1995; Hung and Kemphues, 1999) and that PKC-3 (aPKC in *Drosophila*) and PAR-3 are also mutually dependent (Tabuse *et al.*, 1998). In *Drosophila*, Bazooka (Baz, the homolog of

PAR-3) does not depend on Par-6 or aPKC (Rolls *et al.*, 2003). In contrast, Par-6 localization does depend on aPKC (Rolls *et al.*, 2003). This implies that Par-3 acts to localize aPKC. Atwood *et al.* (2007) propose that Baz acts via Cdc42 to recruit Par-6-aPKC. Although these results give some insights into how polarization of these proteins may result in asymmetry that eventually defines two different cells, the initial step in creating polarity in *Drosophila* neuroblasts remains elusive. In *C. elegans*, a role has been shown for factors deposited by sperm (Cowan and Hyman, 2004; Jenkins *et al.*, 2006). A comparable initial step that explains polarity of the neuroblasts has yet to be identified in *Drosophila*.

But how is the Par-3-Par-6-aPKC complex prevented from localizing to the basal cortex in *Drosophila* neuroblasts? Is there a mechanism comparable to the reciprocal inhibition of the anterior and posterior PAR proteins in the *C. elegans* zygote?

In *lgl* mutants, aPKC is observed at the basal cortex, implying a role for Lgl in aPKC localization (Atwood and Prehoda, 2009). In contrast, regulation in the opposite direction has also been observed (Betschinger *et al.*, 2003). However, the mechanism through which Lgl restricts aPKC is yet unknown. A possible mechanism might be through Par-6 as it was found to bind to Lgl, but this is still unclear (Betschinger *et al.*, 2003).

Altogether, the interaction with Lgl provides an additional role of the Par proteins to establish distinct cortical domains to what has been observed in *C. elegans*. However, it looks very similar to the reciprocal inhibition of the anterior and posterior PAR proteins observed in the *C. elegans* one-cell embryo.

From these two different organisms, and from data observed in for example mammalian cells, we can clearly see a conserved role for the PAR proteins in the establishment and maintenance of polarity.

Can we also see similar mechanism in plant development? The cytoskeleton is largely

conserved, but does this also account for the regulators of polarity? A comparison of plant and animal systems may provide further insights into this complex mechanism.

Asymmetric divisions in *Arabidopsis*

Like in *C. elegans*, the one-cell embryo of *Arabidopsis* divides asymmetrically to produce two cells that differ in size. In plants, the number of genes identified in polarity of the zygote is far less extensive than in *C. elegans*. However, some mechanisms have been explored and these principles show similarity to what is seen in *C. elegans*. Additional information on asymmetric cell division in plants comes from certain cell types in the plant epidermis. Combining the knowledge of the plant asymmetric divisions with those observed in animal systems may ultimately give a more complete view on this crucial process

Asymmetric zygotic division in Arabidopsis

As described for *C. elegans*, *Arabidopsis*' first embryonic cell division too results in the formation of two cells of unequal size (Fig. 3). The smaller apical cell will give rise to the embryo, whereas the larger basal cell forms the extra-embryonic suspensor structure. The following embryonic development is strictly controlled and virtually invariant (reviewed by Jenik *et al.*, 2007). In *C. elegans* a clear central role can be attributed to the family of PAR proteins in establishing and generating polarity. However, no homologs of the PAR proteins have been identified in plant genomes. It is therefore very interesting to see what kind of molecular mechanisms are employed by plants to generate polarity. Which proteins and what kind of processes are required? Is the plant system comparable to that observed in animals? The current literature on the first embryonic division in *Arabidopsis* is less extensive than that for *C. elegans* but some

interesting research has been conducted to provide insights into this important process.

In a mutation screen in *Arabidopsis*, a MAPKK kinase was identified to be involved in the distinction of the apical (embryonic) and basal (extra-embryonic) cell fates (Lukowitz *et al.*, 2004). The first division in loss-of-function mutants results in two cells of similar size. Before the zygotic division, wild type zygote elongates about 3-fold (Mansfield and Briarty, 1991). *yda* mutants showed suppression of this initial zygotic elongation and the cells derived from the basal cell eventually adopted an embryonic fate instead of forming the extra-embryonic suspensor structure. In contrast, gain-of-function mutants show exaggerated development of the suspensor and reduced embryonic development, suggesting that this gene suppresses the embryonic fate (Lukowitz *et al.*, 2004). This gene, called *YODA* (*YDA*), encodes a mitogen-activated protein kinase kinase (MAPKK) kinase. The effects on later embryonic divisions and differentiation imply that the initial zygotic polarity is of importance for embryonic development (Lukowitz *et al.*, 2004). The fact that this gene encodes a MAPKK kinase suggests that it is part of a MAP kinase signaling pathway. Indeed, the downstream MAPK kinases, *MKK4* and *MKK5*, and the MAP kinases *MPK3* and *MPK6* were identified to act downstream in asymmetric cell divisions (Wang *et al.*, 2007).

The zygotic division in *mpk3* and *mpk6* mutants resembled *yda* mutants in that it resulted in two equally sized daughters (Wang *et al.*, 2007).

To understand how such a MAPK cascade can influence polar organization to create an asymmetric division, downstream targets have to be elucidated. Another question addresses the other side of the signaling cascade: how is the activity of *YDA* regulated? Experimental evidence addressing these questions is still limited in the *Arabidopsis* zygote. However, a recent finding by Bayer *et al.* (2009) gives an indication of a possible regulating mechanism upstream of *YDA*. They report that *SHORT SUSPENSOR* (*SSP*) participates in the activation of the *YDA* MAP kinase cascade. Paternal

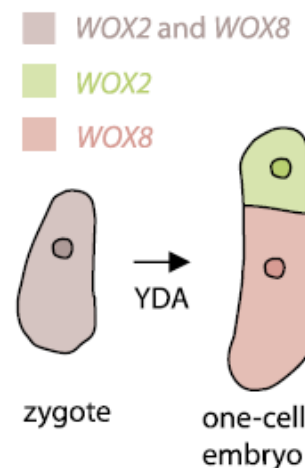


Figure 3 | Division of the *Arabidopsis* zygote

Before the zygote divides, it increases up to three times in length. Then it divides into a small apical daughter cell that will give rise to the embryo, and a larger basal cell that will form the extraembryonic suspensor. *WOX2* and *WOX8* were shown to segregate with the apical and basal daughter cells, respectively. Figure taken from Abrash and Bergmann, 2009

transcripts of *SSP* are delivered to the egg cell via the sperm cell during fertilization and they are translated after this delivery (Bayer *et al.*, 2009). *SSP* is part of a family of interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinases. The mechanism of action has yet to be proven, but it is proposed that the kinase domain of *SSP* may function in protein binding, rather than having catalytic activity (Bayer *et al.*, 2009). For a substantial number of plant IRAK/Pelle kinases it is observed that they don't require kinase activity for their function, suggestive of signaling through a phosphorylation-independent mechanism (reviewed by Castells and Casacuberta, 2007). Mammalian IRAKs mediate activation of MAPK pathways. For example, binding of IRAK-1 to a complex containing the MAPKKK TAK1 induces phosphorylation of TAK-1, resulting in translocation of the TAK-1 complex to the cytosol which is required for its activity (reviewed in Janssens and Beyaert, 2003). The finding that *SPP* acts upstream of the MAPKKK *YDA*, suggests that a similar event may occur in *Arabidopsis*. How *SSP* becomes activated and via what mechanism it results in activation of *YDA* has yet to be determined.

Additionally, it is still uncertain whether a MAP kinase signaling cascade initiates asymmetry or that the cascade itself is activated in an asymmetric manner.

Although the egg cell already shows a polarized organization as the nucleus is located apically and the vacuole on the basal side (Mansfield and Briarty, 1991), *SSP* gives a strong indication that the sperm contributes to the establishment of polarity in the *Arabidopsis* zygote (Bayer *et al.*, 2009). Further experimental data on the contribution of sperm to the polarity of the *Arabidopsis* zygote are scarce. However, experiments in fucoid algae (which are only distantly related to plants) imply that the point of sperm entry is an important cue for polarity, determining the orientation of the growth axis (Hable and Kropf, 2000). Actin is uniformly distributed in unfertilized eggs, but a so called actin patch is formed at the sperm entry site (Hable and Kropf, 2000). Actin dynamics were shown to be important for polarization of the zygote (Hable *et al.*, 2003). An important factor controlling the actin dynamics in the fucoid zygotes is the Actin-related protein (Arp2/3) complex, which is known to serve as a nucleation site for actin filaments (Hable and Kropf, 2005). It would be interesting to see if comparable actin dynamics and the role of ARP2/3 herein, can be observed in *Arabidopsis*. It is very likely that actin dynamics play an important role in the initial *Arabidopsis* cell division, given the central role of actin in polarity (e.g. the asymmetric contractions of the actomyosin network in the *C. elegans* zygote). It would be possible that actin serves to transport and asymmetrically distribute cellular components (e.g. cell fate determinants or polarity factors such as the PARs in the *C. elegans* zygote). What these factors are has yet to be determined.

WOX genes

To stay with the zygote, I first describe the role of a family of homeodomain transcription factors. These *WOX* (*WUSCHEL related HOMEODOMAIN*) genes were identified to play a role in cell fate determination during early embryonic development (Haecker *et al.*, 2004). *WOX2* and *WOX8* mRNAs are found

together in the *Arabidopsis* egg cell and zygote and upon division they segregate to the apical and basal daughter, respectively (Fig. 3) (Haecker *et al.*, 2004). An intriguing question is whether the *WOX2/WOX8* asymmetry is established before or after cell division. Haecker *et al.* propose, based on their observations in two-cell embryos (they saw no coexpression in the two-cell stage) that *WOX2* and *WOX8* are separated before cytokinesis or directly after, before the time point at which they analyzed the two-cell embryos. In case of the latter scenario, mRNA degrading factors or translational regulators would be required and they would have to be distributed asymmetrically. Thus, both scenarios require polar distribution of components in the zygote (Haecker *et al.*, 2004), just as observed in the *C. elegans* zygote. Other *WOX*-family members become expressed later in development (Haecker *et al.*, 2004). So from these observations, the *WOX2* and *WOX8* proteins appear to be effectors of the first asymmetric division rather than inducers. However, the *WOX* genes do show to be important players in the establishment of the apical-basal polarity of the developing embryo (Breuninger *et al.*, 2008; Haecker *et al.*, 2004). It would now be interesting to see what regulates the specific expression domains of *WOX2* and *WOX8*. As the *YDA*-signaling cascade is known to influence zygotic division and basal cell fate, this might be an intracellular signaling pathway involved in the regulation of *WOX* expression (Lukowitz *et al.*, 2004; Wang *et al.*, 2007). However, no direct evidence for this interaction has yet been found. It would be interesting to analyze *WOX* behaviour in the absence of *YDA* signalling. This could provide information on the possible interaction of *YDA* signaling with the *WOX* genes.

The asymmetric distribution of *WOX2* and *WOX8* transcripts resembles that of the anterior and posterior separation of PAR proteins in the *C. elegans* embryo. However, the proposed polarized distribution of transcripts in the *Arabidopsis* zygote is even more resembling of the *Drosophila* egg, in which maternal factors are laid out in a polarized fashion before fertilization

(reviewed in Riechmann and Ephrussi, 2001). As it seems, the rearrangements leading to the polar distribution of several cellular components is initiated at the time of fertilization.

Paternal contribution of *SSP* makes it likely that fertilization initiates molecular polarity as observed in *C. elegans*.

Combining information derived from different systems may help in understanding any process investigated. In the case of asymmetric cell division, another asymmetric division in *Arabidopsis* has been studied intensively. This shows, for instance, a role for *YDA* in different types of asymmetric divisions.

Asymmetry in stomatal development

In the epidermis of plant leaves specialized cell complexes are formed that are required for gas exchange. The so-called stomata arise from a series of symmetric and asymmetric divisions (reviewed by Bergmann and Sack, 2007). Multipotent stem cells, the meristemoid mother cells (MMCs), divide asymmetrically to create a small meristemoid and a larger stomatal lineage ground cell (SLGC) (Fig. 4). The meristemoid undergoes a number of asymmetric divisions, creating more meristemoids before differentiating into a guard mother cell (GMC) (Fig. 4). This GMC divides symmetrically to create two guard cells that surround a pore, forming a stoma (Fig. 4). Another asymmetric division occurs when a SLGC produces a new meristemoid (Fig. 4) (Bergmann and Sack, 2007). This division seems to be regulated via cell-cell signaling as the SLGC almost always produces a meristemoid daughter away from the side where it contacts a stomate. In this way, stomata are separated by one cell (known as the one-cell spacing rule) (Geisler *et al.*, 2000). This observation suggests a role for external cues regulating asymmetry rather than intrinsic cues. A gene that is involved in the regulation of this orientated division is *TOO MANY MOUTHS (TMM)* (Geisler *et al.*, 2000; Nadeau and Sack, 2002). In *tmm* mutants, an excess number of stomata displays random positioning because the asymmetric divisions are not oriented properly (Geisler *et al.*, 2000; Nadeau and Sack, 2002). *TMM* encodes a leucine-rich repeat-containing receptor-like

protein (LRR-RLP) with a transmembrane domain, but without an intracellular kinase domain. It was proposed that *TMM* transduces an extracellular signal like other LRR-RLPs (Bergmann and Sack, 2007; Dievart and Clark, 2004; Nadeau and Sack, 2002). *TMM* shows complex genetic interaction with three members of the *ERECTA (ER)* family of leucine-rich repeat-receptor-like kinases (LRR-RLKs): *ER*, *ERL1* and *ERL2* (Shpak *et al.*, 2005). It has been proposed that *TMM* heterodimerizes with one of the *ER* proteins which do possess a kinase domain (Fig. 5) (Petricka *et al.*, 2009; Torii *et al.*, 1996). This heterodimerization would provide *TMM* with intracellular signaling activity. But what induces such activity? One small secretory peptide, called *EPIDERMAL PATTERNING FACTOR 1 (EPF1)*, has been shown to play a role in stomatal patterning (Hara *et al.*, 2007). *EPF1* activity is dependent on *TMM* and *ER*-family kinases, suggesting that *EPF1* may provide the signal that induces signaling via these receptors (Hara *et al.*, 2007). However, its direct interaction has not yet been shown. Evidence for a direct interaction between *EPF1* and *TMM* or an *ER*-family member would make this hypothesis more plausible. Before the identification of *EPF1* as an upstream regulator of *TMM*, another factor upstream of *TMM* had been found. The serine protease *STOMATAL DENSITY DISTRIBUTION1 (SDD1)* was implicated to be involved in the orientation and number of asymmetric cell divisions in the development of stomata (Berger and Altmann, 2000). *SDD1* was shown to be in the same signaling pathway as *TMM* and required *TMM* for its functioning as overexpression of *SDD1* in a *tmm* mutant had no additional effects compared to a *tmm* mutation on itself (Von Groll *et al.*, 2002). At first, according to a simple model, it seemed plausible that *SDD1* would serve to cleave and activate the *EPF1* peptide. However, *SDD1* and *EPF1* both act upstream of *TMM*, but do so in an independent manner (Hara *et al.*, 2007).

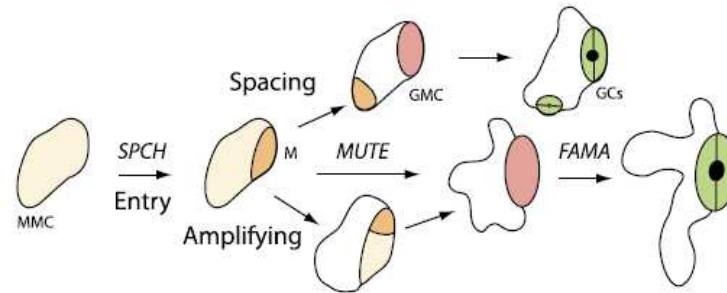


Figure 4 | Development of stomata in *Arabidopsis*

The initial asymmetric division of the meristemoid mother cell (MMC) is mediated by the bHLH transcription factor *SPEECHLESS* (*SPCH*). The smaller meristemoid daughter (M) can then either differentiate into a guard mother cell (GMC), mediated by the bHLH transcription factor *MUTE*, or asymmetrically divide again. Eventually the GMC symmetrically divides into two guard cells (GCs) (under influence of the bHLH protein *FAMA*) that form the stomata. Figure taken from Abrash and Bergmann, 2009

Although no certain activating signal for *TMM* signaling has yet been found, there are data on the downstream effectors of this signaling cascade. Bergmann *et al.* (2004) showed that *YODA* acts downstream of *TMM* in the regulation of asymmetric cell divisions in stomatal development. Normal *YDA* levels are required for the balance between proliferation of MMCs and differentiation (into GMCs) and *YDA* levels must be down-regulated for cells to enter the stomatal lineage (Bergmann *et al.*, 2004). In addition, two MAP kinases, *MPK3* and *MPK6*, and their upstream MAPK kinases *MKK4* and *MKK5* were identified to act downstream of *YDA*, negatively regulating stomatal lineage initiation and differentiation of meristemoids into GMCs (Fig. 5) (Wang *et al.*, 2007). How does this signaling cascade affect asymmetric cell divisions? What are the downstream targets? One finding that gives a partial answer to this question is the identification of *SPEECHLESS* (*SPCH*) as a downstream target of the *YDA-MKK4/5-MPK3/6* cascade that is necessary for the initiation of the stomatal lineage (Fig. 5) (MacAlister *et al.*, 2007; Lampard *et al.*, 2008). *SPCH* is a basic helix-loop-helix (bHLH) transcription factor that can be phosphorylated both by *MPK3* and *MPK6* *in vitro* (Lampard *et al.*, 2008). The *YDA* MAP kinase pathway negatively regulates *SPCH* protein levels (Lampard *et al.*, 2008). This fits

with the finding that indicates that *SPCH* promotes stomatal lineage initiation (MacAlister *et al.*, 2007). However, the precise mechanism is still unclear. Phosphorylation on Serine 193 is required for *SPCH* activity, but other phosphorylations seem to have an inhibiting effect (Lampard *et al.*, 2008).

Two other bHLH transcription factors that act in sequential steps of stomatal development are *MUTE* and *FAMA* (Ohashi-Ito and Bergmann, 2006; Pillitteri *et al.*, 2007). *MUTE* is required for the transition from MMC to GMC (Fig. 5) (Pillitteri *et al.*, 2007; Pillitteri *et al.*, 2008). *FAMA* is required to promote differentiation of the guard cells and inhibits proliferation in their immediate meristemoid precursors (Fig. 5) (Ohashi-Ito and Bergmann, 2006).

Together these data on activating ligands, receptors, downstream MAP kinase signaling cascades and subsequent regulation of transcription factors can be integrated into a model (Fig. 5). The secreted peptide *EPF1* is likely to function as an extracellular signal that activates a transmembrane receptor formed from heterodimerization of *TMM* with a member of the *ER*-family. Downstream of this receptor is a MAP kinase cascade consisting of the MAPKKK *YDA*, the MAPKKs *MKK4* and *MKK5* and the MAPKs *MPK3* and *MPK6* that regulate bHLH transcription factors, possibly regulating the expression of cell fate determinants.

The expression pattern of *YDA* in the one-cell stage has not yet been investigated. This, together with the identification of target proteins would provide new insights in how *YDA* may act in controlling asymmetric divisions. This information still doesn't explain how the asymmetric divisions in stomatal development are regulated. Recently, the first asymmetrically localized proteins involved in plant asymmetric cell divisions were reported (Cartwright *et al.*, 2009; Dong *et al.*, 2009). Dong *et al.* showed that the protein *BASL* localizes to the nucleus and the periphery of premitotic MMCs in a polarized manner. Both daughters of the asymmetrically divided MMC display nuclear *BASL* localization. However, in the larger SLGC *BASL* is also localized to the membrane opposite of the division plane (Dong *et al.*, 2009). Cells that lose nuclear *BASL* but maintain it in the periphery do not divide, but develop into pavement cells. All stomatal-lineage cells with the capacity to divide express nuclear *BASL*. Only with *BASL* expressed in the periphery, these cells divide asymmetrically. GMCs with *BASL* solely in the nucleus divide symmetrically to form a pair of terminally differentiated guard cells.

In the absence of *BASL*, the asymmetry of divisions is disrupted and division planes can be misoriented (Dong *et al.*, 2009). Thus, *BASL* clearly has a role in the asymmetry of stomatal lineage divisions. However, how it acts remains to be determined. Experiments identifying interacting partners may shed some light on the functioning of *BASL*.

Where *BASL* may provide a first indication of a role for intrinsic factors in asymmetric divisions, another protein, *PAN1*, was found to be involved in the transmission of an extrinsic signal required for asymmetry (Cartwright *et al.*, 2009). In maize, stomatal complex formation involves the asymmetric division of a subsidiary mother cell (SMC). This division appears to be influenced by an extrinsic signal coming from an adjacent guard mother cell (GMC). Cartwright *et al.* (2009) found that *PAN1* promotes the polarization of SMCs before the asymmetric cell division. In SMCs, an actin patch is formed at the site of contact with the GMC followed by migration of the nucleus to this side of the SMC.

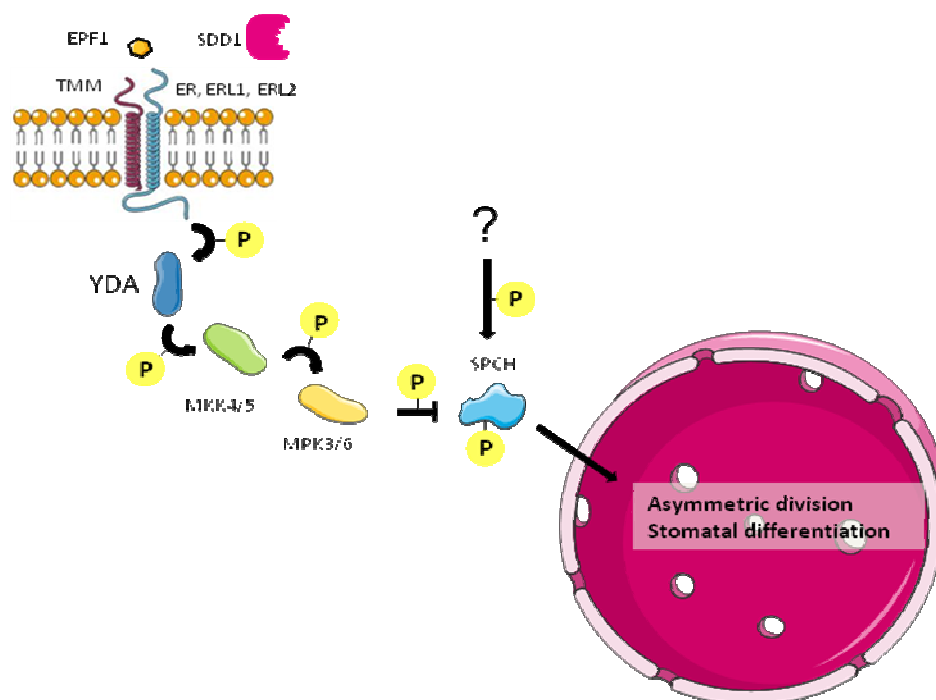


Figure 5 | YDA MAP kinase signaling pathway in stomatal development

EPF1 is a candidate ligand for the TMM receptor upstream of the YDA MAP kinase cascade. TMM heterodimerizes with members of the LRR-RLKs (ER, ERL1 and ERL2) to form a functionally active receptor. The YDA cascade inhibits *SPEECHLESS* via phosphorylation, which in an active state promotes the initiation of stomatal development. There other phosphorylations sites on *SPCH* with activating properties. The factors that mediate this phosphorylation are still unknown, hence the question mark.

PAN1 localizes with this actin patch and segregates with one of the daughters. This suggests a role for PAN1 as a possible polarity determinant. However, the number of SMCs forming actin patches is not significantly affected in *pan1* mutants, but the accumulation of actin in the patches is often reduced and nuclei often fail to localize with the patches (Cartwright *et al.*, 2009; Gallagher and Smith, 2000). This partial effect of *pan1* mutants suggests that PAN1 may act with other proteins with redundant functions. What these proteins are has yet to be uncovered.

BASL and PAN1 are the initial factors providing clues for the presence of intrinsic and extrinsic signals influencing cellular polarity in plants. Identification of additional polarity factors will contribute to understanding the process of polarity establishment in plant cells. If, and how, these two factors can be incorporated into the model comprising the YDA MAP kinase signaling pathway has yet to be investigated. If a link between these systems can be found, it may provide a more complete view on how external signals from surrounding cells can influence intrinsic polarity and asymmetric cell division that create the specific pattern observed in stomatal development.

Polar cell elongation: axons versus pollen tubes

The polarization of the zygote that leads to asymmetric cell division is a crucial process in development. Internal rearrangement of the cytoskeleton results in a guided distribution of cell fate determinants. In *C. elegans*, this process is started by an external cue delivered by a sperm cell (Cowan and Hyman, 2004; Goldstein and Hird, 1996; Jenkins *et al.*, 2006). As mentioned, in plants there is also an indication that sperm may provide a cue for polarization of the embryo (Bayer *et al.*, 2009; Hable and Kropf, 2000).

In most flowering plants this fertilization is preceded by elongation of the pollen tube that delivers the sperm to the egg. Just like the first zygotic division, the guided growth of the pollen tube is a highly polarized process itself. A process in animals that resembles

pollen tube elongation to a great extent is the outgrowth of axons in neuronal development (Fig. 6). Both processes show movement of one side of the cell (the growing tip) resulting in cellular elongation. Here I will make a comparison of how these two polarization processes are regulated.

Polarized axon growth

The development of the nervous system requires proper connectivity between neurons. Disruption of this process can result in a wide variety of neurologic disorders. Understanding of neuronal development may provide insights into the origins of such neurological disorders.

One of the important steps in the development of the nervous system is the polar outgrowth of axons. Useful experimental tools in this field of axonal growth are *C. elegans* and *Drosophila*, because of their relatively simple neuronal systems (*C. elegans* has 302 and *Drosophila* 100,000 neurons). Combining experimental data derived from these models has resulted in the formation of models that help us understand how cells are able to form extended polarized structures.

The initial step in the formation of polarized axons is the specification of one neurite (short process from the neuron during its initial developmental stage) into an axon, whereas the other neurites become dendrites (Fig. 6). Initiation of the axon is accompanied by changes in the cytoskeleton, but it is not yet clear how these changes are induced (reviewed in Tahirovic and Bradke, 2009). A study in cultured rat neurons showed that after the last round of mitosis in neurons, the centrosome moves to the pole where later the axon is formed (de Anda *et al.*, 2005). With the centrosome functioning as a MTOC, this observation indicates that microtubules play a role in axon initiation. However, axons of *Drosophila* that lack any functional centrosomes develop normally (Basto *et al.*, 2006). Other data show that in zebrafish, axons are not formed from the site where the centrosome is located (Zolessi *et al.*, 2006). Together, these observations make the role of centrosomes in axon initiation uncertain.

When one of the neurites has taken the identity of the future axon, it has to elongate in the right direction. This process requires a complex integration of external cues resulting in dynamic reorganization of the internal components in the growing axon. These external signals can attract or repel axons, depending on the receptors of the growing tip (see Killeen and Sybingco, 2008 for a review).

Guidance Cues

In *C. elegans* and *Drosophila*, a group of four axon-guidance cues and their receptors has been described (reviewed in Killeen and Sybingco, 2008). A distinction can be made between guidance molecules that regulate the dorso-ventral (DV) migration and those that mediate anterior-posterior (AP) migration. The group of Netrins (UNC-6 in *C. elegans*) and Slits are involved in the DV-guidance of axons. UNC-6/Netrin, secreted by neurons of the ventral nerve cord (Hedgecock *et al.*, 1990; Wadsworth *et al.*, 1996), interacts with UNC-40 and UNC-5 receptors. However, the effect of each interaction is different. Interaction of UNC-6/Netrin with UNC-40 has an attractive effect (Adler *et al.*, 2006; Chan *et al.*, 1996; Hedgecock *et al.*, 1990; Ishii *et al.*, 1992), whereas the interaction of UNC-6/Netrin with the receptor UNC-5 has a repelling effect (Keleman and Dickson, 2001; Leung-Hagesteijn *et al.*, 1992).

Thus, the combination of an extracellular signaling molecule with a specific receptor determines the orientation of a growing axon; it influences its polarity. The question now is how does the receptor translate the received signal into polarized growth? In response to guidance cues, asymmetric accumulation of F-actin and microtubules occurs in the growth cone, resulting in growth in the direction of this accumulation ((Zhou and Cohan, 2004)). Asymmetry in processes such as vesicular trafficking (Tojima *et al.*, 2007), calcium signaling (Gomez and Zheng, 2006) and protein synthesis (Leung *et al.*, 2006) is also observed in growing axons in response to guidance cues. This makes it seem very likely that signaling modules initiated by the external cues modulate the orientation of these internal processes. The signaling module

downstream of UNC-6 and its receptor UNC-40 has been studied in *C. elegans*.

UNC-40

Adler *et al.* (2006) showed that UNC-6 and its receptor UNC-40 play a role in the generation of a growth cone and in determining the location of axon outgrowth in the symmetric neuron. The growth cone is the area at the tip of the growing axon, the leading edge. In the presence of UNC-6, MIG-10 and UNC-40 are localized to the leading edge of growing axons.

The spatial restriction of MIG-10 to the leading edge requires UNC-40 and asymmetric lipid signaling from PI-3 kinase (PI3K) and PTEN (Adler *et al.*, 2006). PI3K (AGE-1 in *C. elegans*) mediates the production of phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) and PTEN (DAF-18 in *C. elegans*) degrades it. PIP₂ is used for recruitment of Lamellipodin, the mammalian homolog of MIG-10, to the plasma membrane via the PH domain (Krause *et al.*, 2004). Chang *et al.* (2006) showed that AGE-1 may act as a regulator of MIG-10 in neuron development in *C. elegans*. It seems likely that PIP₂ in *C. elegans* also recruits MIG-10 to the plasma membrane, but this direct interaction has not been shown yet. In addition, CED-10, the *C. elegans* homolog of the Rac1 GTPase, was shown to directly interact with MIG-10 and regulate its asymmetric localization (Quinn *et al.*, 2008). This asymmetric MIG-10 distribution is associated with enrichment of the cytoskeleton (both F-actin and microtubules) (Quinn *et al.*, 2008). In mammals, Lamellipodin can bind Ena/VASP which has actin regulatory properties (Krause *et al.*, 2004). Chang *et al.* (2006) showed an interaction between MIG-10 and UNC-34 (the *C. elegans* homolog of Ena/VASP) *in vitro*. It would be interesting to see whether this interaction also occurs *in vivo*.

Actin dynamics are very important in the creation of growth cones and thus the guidance and elongation of axons. A variety of experimental data links UNC-40 to actin dynamics. UNC-40 has two conserved cytoplasmic domains, P1 and P2. The P1

domain acts with the UNC-34 pathway, whereas the P2 domain acts in a pathway with the GTPase CED-10 and the putative actin binding protein UNC-115 (Gitai *et al.*, 2003). This implies that UNC-40 may act as a scaffold to regulate actin dynamics in developing axons via independent pathways as both UNC-34 and UNC-115 may be actin binding proteins.

Further data suggest that, in addition to UNC-34 and UNC-115, a third actin modulating pathway is active during axon guidance. Shakir *et al.* (2008) show a role for the Arp2/3 complex and its activators WVE-1 and WSP-1 in controlling actin dynamics in axon growth cones in *C. elegans* (Shakir *et al.*, 2008). They suggest that WVE-1 may act in the CED-10 pathway and that WSP-1 may function in parallel in the MIG-2 pathway (Shakir *et al.*, 2008). MIG-2 is a Rac-like GTPase that functions redundantly with CED-10 in axon guidance (Lundquist *et al.*, 2001; Zipkin *et al.*, 1997).

These data show that downstream of the guidance cue UNC-6/Netrin, there are three parallel pathways that modulate actin dynamics. Activation of the receptor in response to the extrinsic signal results in localization of a variety of signaling proteins that together coordinate the polarization of actin dynamics that is required for axon outgrowth. We see employment of the same signaling mechanisms that we saw in polarization of the *C. elegans* zygote – GTPases, kinases, phospholipid signaling and direct actin modulation.

Pollen tube elongation

In plants, male gamete development results in a pollen grain containing a tube cell and a generative cell. In most flowering plants, the generative cell divides to form a pair of sperm cells. For fertilization to occur, these sperm cells must reach the ovule. When a pollen grain encounters a stigma it can germinate and the tube cell elongates (pollen tube) into the stigma and the underlying style towards the egg cell in the ovule (Fig. 6) (Leyser and Day, 2006).

The process of pollen tube elongation closely resembles the polar outgrowth of developing

axons in animals. The tip of a single cell grows toward a target while the cell size increases. Do plants make use of the same molecular mechanisms to reach a seemingly similar goal? Intensive research of pollen tube growth in different plant models (mainly *Arabidopsis*, *Lilium longiflorum* and *Nicotiana tabacum*) has provided insights into this crucial process of polarized cell elongation.

Guidance cues

In animals, the presence of attracting and repelling signals is of great importance for proper axon growth. Such guidance cues in plants should be provided by the female tissues through which the pollen tube is directed. Different experiments in lily show that adhesion of the pollen tube tip to the stylar epidermis is important for pollen tube guidance (reviewed in Kim *et al.*, 2004). In *Arabidopsis*, among other plant species, an attracting role for the ovule has been shown (Higashiyama *et al.*, 2003). However, the identity of the different signals has not completely been elucidated. One player that has been identified in pollen tube guidance in *Arabidopsis* is gamma-aminobutyric acid (GABA), produced by so called diploid integument cells from the ovule (Palanivelu *et al.*, 2003). GABA shows a concentration gradient in the style. However, GABA overproduction induced by a non-functional POP2 transaminase that degrades GABA, did not alter guidance of wild type pollen in mature pistils (Palanivelu *et al.*, 2003), suggesting that other guidance cues may control pollen tube guidance in mature pistils. In animals, GABA receptors can signal via G-protein coupled receptors. Whether this is also the case in plants remains to be determined (Yu and Sun, 2007). No homologs of GABA-receptors have been found in plants. However, a family of 20 genes sharing structural homology with mammalian glutamate-receptors has been identified in *Arabidopsis* (AtGLRs) (Lacombe *et al.*, 2001). Different experiments show that these AtGLRs are likely to be involved in Ca²⁺ transport, which has an important role in the polar growth of pollen tubes (reviewed in Bouche *et al.*, 2003). Pollen tubes show a Ca²⁺-gradient towards the tip. Cell wall assembly consumes

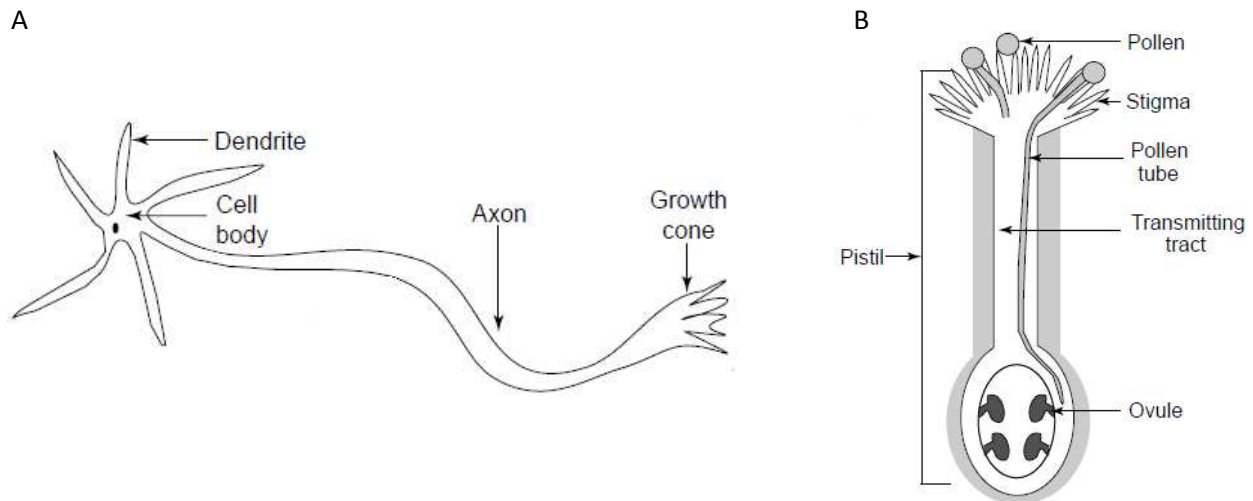


Figure 6 | Axon and pollen tube growth

(A) Axons are the long outgrowths of neurons. One of the neurites forms the axon whereas the others develop into dendrites. (B) Pollen tubes grow from pollen located at the stigma down to the ovule through the pistil, guided by signals from the maternal tissues. Figures taken from Palanivelu and Preus, 2000

a large amount of Ca^{2+} and a lot of Ca^{2+} -dependent signaling proteins are expressed in pollen tubes (reviewed in Cheung and Wu, 2008). Initiation and maintenance of this Ca^{2+} gradient are likely to be regulated by the polar distribution of Ca^{2+} transporters. For example, Ca^{2+} gradients were found to be regulated by exogenous GABA in tobacco pollen tubes (Yu *et al.*, 2006). How GABA and its receptors regulate Ca^{2+} gradients is yet unknown. Different sources of data indicate that the Ca^{2+} gradient is achieved by Ca^{2+} influx at the tip of pollen tubes (Holdaway-Clarke and Hepler, 2003). This influx may be localized to the tip due to stretch-activated Ca^{2+} channels that are activated at the growing tip rather than in the lagging region (Dutta and Robinson, 2004). The Ca^{2+} gradient may contribute to tip growth via its role in a variety of processes.

Some of the processes influenced by Ca^{2+} that are important in tip growth are vesicle trafficking (Camacho and Malho, 2003), actin dynamics (Staiger and Blanchoin, 2006) and signaling via Ca^{2+} dependent protein kinases (e.g. the Ca^{2+} dependent kinases (CDPKs) in *Petunia* (Yoon *et al.*, 2006).

Actin filaments

The role of actin filaments in pollen tubes has been shown in a variety of ways. Visualization of the actin cytoskeleton with various markers

shows that long actin cables extend along the tube up to the apex where a network of shorter actin filaments is visible (Cheung and Wu, 2008). Additional experiments with inhibitors of actin dynamics show disruption of pollen tube growth, indicative of a critical role for the actin cytoskeleton in polar pollen tube growth (reviewed in Hepler *et al.*, 2001). The dynamics of the actin cytoskeleton are regulated by a number of actin binding proteins (ABPs), similar to other systems (Ren and Xiang, 2007). Experiments in which levels of ABPs such as profilin and actin depolymerizing factor (ADF) are changed show disruption of actin organization and pollen tube growth (Chen *et al.*, 2002; Vidali and Hepler, 2001). Profilin enhances actin polymerization and is distributed throughout the pollen tube, but its activity in maize is dependent on Ca^{2+} (Kovar *et al.*, 2000). The Ca^{2+} gradient towards the tip can localize the activity of profilin. The depolymerizing activity of ADF in pollen tubes is under strict regulation. Its actin binding activity is inhibited by phosphorylation, under control of a RhoGTPase signaling cascade (Cheung and Wu, 2008). Also, the ADF activity may be stimulated in the more subapical region under control of a H^+ gradient that results in an acidic tip and a more alkaline subapical region (Cheung and Wu, 2008). In addition, PIP_2 is apically enriched and was shown to inhibit

ADF activity *in vitro* (Nagaoka *et al.*, 1995). Together these mechanisms provide a strict regulation of actin dynamics in the growing pollen tube.

In addition to the proteins mentioned, a number of other regulators of actin dynamics have been reported to have a role in pollen tube elongation (reviewed in Cheung and Wu, 2008). Functionally, the actin network is required for proper vesicle trafficking, as vesicle behavior changes drastically upon treatment with actin dynamics inhibitors in lily (Parton *et al.*, 2001).

Vesicle trafficking

The growth of pollen tubes depends on continuous vesicle trafficking to deliver and recycle plasma membrane components. Visualization experiments making use of GFP-labeled membrane components show the formation of a cytoplasmic stream in the form of a reverse fountain. In the center of the tube the stream moves towards the tip and it goes in the opposite direction close to the membrane (Cheung *et al.*, 2002). Exocytosis is believed to occur mostly at the apex, whereas endocytosis is more abundant at the subapical region (Camacho and Malho, 2003). A family of proteins that is involved in polar vesicle trafficking is that of the ADP-ribosylation factors (ARFs) (Xu and Scheres, 2005a). In polarly outgrowing root hairs, ARF1 accumulates in the tip (Xu and Scheres, 2005b). Genetic and localization studies show that ARF1 possibly regulates the GTPase ROP2 (Rho-of-plants 2) (Xu and Scheres, 2005b). ROPs are homologs of the RHO-family GTPases in yeast and animals where they are known to regulate cell polarity through modulation of actin dynamics (reviewed in Etienne-Manneville and Hall, 2002).

When we put axon and pollen tube development next to each other, we see the same themes coming forward. In both systems there is an important role for guidance cues. Similar to what is seen in asymmetrically dividing zygotes, axons and pollen tubes need a cue to induce polarity. For developing axons it is clear that this is an interplay between attracting and repelling cues (Killeen and Sybingco, 2008). Together the net effect of

these signals determines the direction of growth. Processing of the guidance cues, starts at the receptors on the membranes of axons and pollen tubes. Interpretation of the signals and translation into guided activity is carried out by complex signaling cascades that encompass molecules with different properties. There is a prominent role in both systems for GTPases.

When going through the numerous, but still far from complete, sources of information on developing axons and pollen tubes, it seems that there is a difference in how both processes are examined. In animal experiments analyzing axon growth, the emphasis is put more on genetic interactions rather than mechanistic, structural processes. A lot of genes have been identified to be involved in polar axon initiation and growth, but there seems to be a lack of studies visualizing mechanical processes such as actin dynamics, vesicle trafficking and oscillating cytoplasmic flows. In plant models, these processes have been visualized to some extent. However, the information on genetic interactions of genes involved in pollen tube growth appears relatively small compared to the information on axon growth extracted from animal models such as *C. elegans* and *Drosophila*. This is likely due to the fact that the animal models are used in this field of research for over a longer period of time. However, continuous comparisons of findings in both systems will very likely contribute to a more complete understanding of how polarized growth of single cells is regulated and how it contributes to the development of multicellular organisms.

Conclusion

It is remarkable to see that organisms like *C. elegans*, *Drosophila* and *Arabidopsis*, that differ so much at first sight and have evolved multicellularity independently share cell polarization processes that appear very similar. When we look at the molecular systems behind the establishment and maintenance of polarity, we see that in a number of processes the same principles are used in these evolutionary distant organisms.

Apparently, these systems work well and have survived throughout evolution, although the precise details do differ. Therefore, the use of evolutionary distant organisms and their comparison provides a broader view on cellular processes involved in polarization and is thus of great importance.

The zygotic division is being intensively investigated as it is easily manipulated and analyzed. This resulted in the identification of several factors that are involved in the polarization of a cell. Analysis of these proteins has led to a, yet incomplete, understanding of the working mechanisms of these polarity factors. The main group of polarity factors, the PAR proteins, showed to be conserved throughout the animal kingdom. Research of *Drosophila* neuroblasts shows a role for the same proteins in different setting, indicating its importance. In plants, the zygote is highly inaccessible which has severely hampered experimental manipulation of the process. However, molecular genetic analyses have revealed some insights into zygote polarization. Perhaps surprisingly, no homologs of the PARs could be found in plants. This would mean that plants use other proteins that are able to establish and maintain polarity. One mechanism that contributes to the asymmetry of the zygotic division in *Arabidopsis* is that of the YDA MAP kinase signalling pathway. Mutations in members of this pathway result in disruption of the normal division pattern, comparable to what is observed for *par* mutants in *C. elegans*. For the PAR proteins it has been shown that they actively participate in the maintenance of polarity. They modulate actin dynamics and physically interact with other polarity factors and cell fate determinants. For the YDA signalling cascade, it has thus far only been shown that it regulates expression of target genes via the bHLH transcription factor SPCH (Lampard *et al.*, 2008). It should be investigated how the YDA signalling cascade influences asymmetric cell divisions. Are the downstream targets genes involved in setting up asymmetry? Or are there other function for the members of the YDA signalling cascade that have a more direct effect on asymmetry? For both the *C. elegans* and *Arabidopsis* zygotes, there seems to be an extrinsic cue

that initiates the establishment of polarity. In *C. elegans*, the site of sperm entry marks the posterior side of the embryo. A model of how sperm contributes involves the RhoGAP CYK-4 which is enriched in sperm (Jenkins *et al.*, 2006). CYK-4 was shown, together with the GEF ECT-2, to regulate activity of the GTPase RHO-1 (Jantsch-Plunger *et al.*, 2000; Morita *et al.*, 2005). Localization of CYK-4 at the site of sperm entry may result in posterior restriction of RHO-1 activity, leading to asymmetric contractions of the actomyosin network (Schonegg and Hyman, 2006). In *Arabidopsis*, a factor contributed by sperm was found to act upstream of YDA (Bayer *et al.*, 2009). Transcripts of the gene *short suspensor* (SSP) are delivered by the sperm. SSP may act in the activation of the YDA signalling cascade (Bayer *et al.*, 2009). How SSP translation is activated and how it regulates YDA-signaling has yet to be investigated. But SSP may be an indication of paternal contribution to zygotic polarity.

Another example used to provide insights in the mechanisms underlying cellular polarity is that of axons in animals and pollen tubes in plants. Both cells manifest the polarized outgrowth of a single cell into a specific direction. For axons it was shown external guidance cues regulate the directionality of axon growth (Killeen and Sybingco, 2008). A number of downstream factors have been identified. Important for translation to polarity are the actin modifiers that were found to be regulated downstream of guidance cues. For pollen tube growth, there are some external signals that are suggested to regulate the directionality of growth. However, unravelling of the identity of these factors is still limited. One factor involved in pollen tube guidance that has been identified is GABA (Palanivelu *et al.*, 2003). Via which receptors and signalling mechanisms GABA regulates polarized growth remains to be shown. It has been proposed that glutamate receptors (AtGLRs) may be involved. These receptors are likely to be involved in the transport of Ca^{2+} , which is important in polarization of pollen tubes (reviewed by Bouche *et al.*, 2003). Findings like these are very important in unravelling the mechanisms underlying polarized cellular growth. General mechanisms like cytoskeleton

dynamics and vesicle trafficking have been investigated quite intensively in pollen tubes. Comparison of this work with the behaviour of the cytoskeleton in other cells, for example the *C. elegans* zygote, beautifully shows the versatility of this machinery reaching the same goal in different ways.

Critical analysis of plant and animal systems contributes to a wider understanding of cellular mechanisms that contribute to development. Findings in one system may lead to ideas for experiments in other systems, accelerating the identification of new players involved in these processes. Seeing similarities and differences in these evolutionary distant organisms also keeps us wondering, poised to investigate these elegant processes.

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